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## Nitrite isotope characteristics and associated soil N transformations

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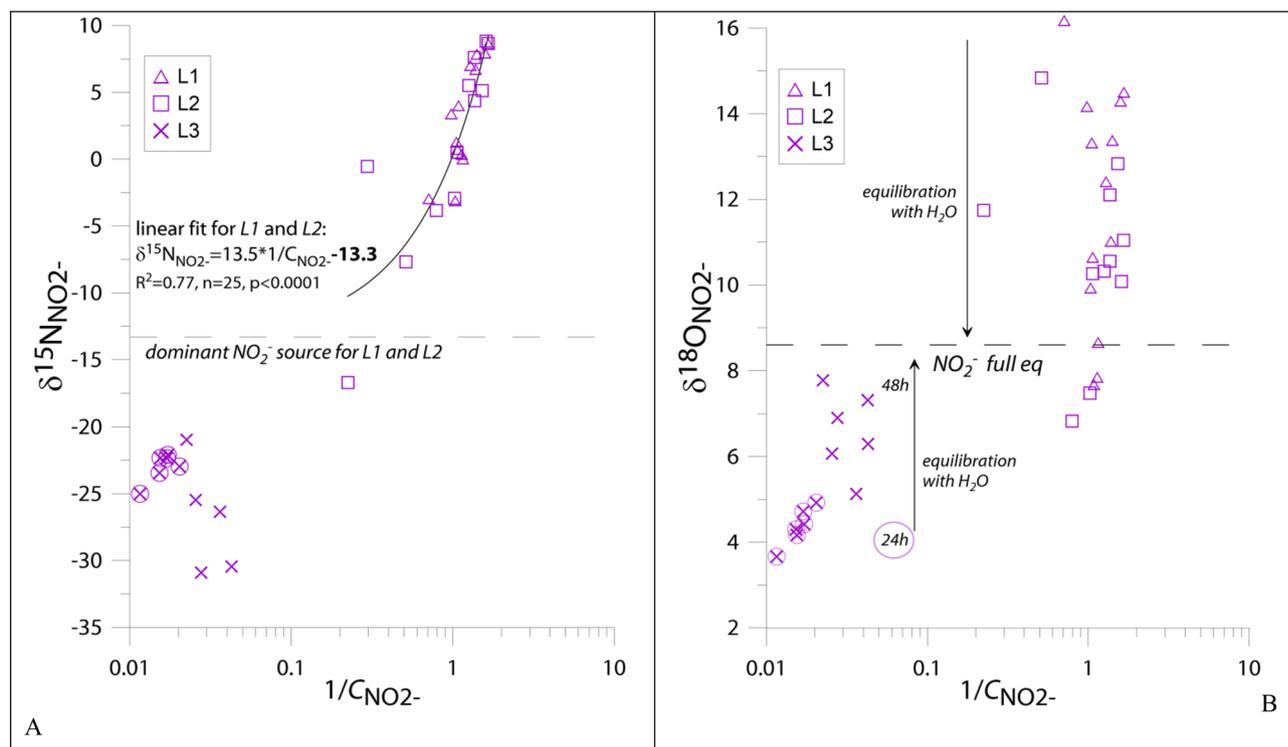
Nitrite ( $\text{NO}_2^-$ ) is a crucial compound in the N soil cycle. As an intermediate of nearly all N transformations, its isotopic signature may provide precious information on the active pathways and processes.  $\text{NO}_2^-$  analyses have already been applied in  $^{15}\text{N}$  tracing studies, increasing their interpretation perspectives. Natural abundance  $\text{NO}_2^-$  isotope studies in soils were so far not applied and this study aims at testing if such analyses are useful in tracing the soil N cycle. We conducted laboratory soil incubations with parallel natural abundance and  $^{15}\text{N}$  treatments, accompanied by isotopic analyses of soil N compounds ( $\text{NO}_3^-$ ,  $\text{NO}_2^-$ ,  $\text{NH}_4^+$ ). The double  $^{15}\text{N}$  tracing method was used as a reference method for estimations of N transformation processes based on natural abundance nitrite dynamics. We obtained a very good agreement between the results from nitrite isotope model proposed here and the  $^{15}\text{N}$  tracing approach. Natural abundance nitrite isotope studies are a promising tool to our understanding of soil N cycling.

Nitrite ( $\text{NO}_2^-$ ), as an intermediate of nearly all N transformations, is a crucial compound to understand the complexity of the N soil cycle with its many contributing pathways. Moreover, as a very reactive compound it usually occurs at very low concentrations, hence conveying information on currently active N transformations. The *Ntrace* model used for interpretation of  $^{15}\text{N}$  labelled soil studies has been recently expanded with the  $\text{NO}_2^-$  content and isotopic analyses, which vastly increased its interpretation perspectives<sup>1</sup>. Thanks to incorporation of  $\text{NO}_2^-$  dynamics in this model it appeared possible to distinguish and quantify three  $\text{NO}_2^-$  and  $\text{N}_2\text{O}$  production pathways: denitrification, autotrophic nitrification and heterotrophic nitrification. Although  $^{15}\text{N}$  tracing studies can precisely identify various soil N transformations<sup>1,2</sup>, they require addition of  $^{15}\text{N}$ -labelled substances, which is associated with additional fertilization, soil disturbance, and potential problems with label distribution homogeneity<sup>3,4</sup>. Moreover, due to high costs and fast consumption of the  $^{15}\text{N}$  label,  $^{15}\text{N}$  tracing approach can be applied mostly for short-term and micro-plot studies<sup>5</sup>. Development of reliable methods for identifying N transformations based on natural abundance stable isotopes can overcome these problems and provide an approach allowing studies in undisturbed soil conditions ensuring original N transformation rates that can be traced in larger time and space scale.

Natural abundance  $\text{NO}_2^-$  isotope studies are so far mostly applied in aquatic studies<sup>6–9</sup> and appeared particularly informative for the oceanic oxygen deficient zones, where  $\text{NO}_2^-$  can be accumulated<sup>7,9</sup>. However, for soil studies the natural abundance  $\text{NO}_2^-$  analyses are so far lacking. Also in soils  $\text{NO}_2^-$  accumulation may happen and the monitoring of  $\text{NO}_2^-$  content in soils can provide important information to understand the N cycle<sup>10–12</sup>. In particular,  $\text{NO}_2^-$  plays a central role for  $\text{N}_2\text{O}$  formation<sup>12,13</sup>. However, even in situations when  $\text{NO}_2^-$  accumulation is not observed, and the interpretation of, typically very low, soil  $\text{NO}_2^-$ -contents is ambiguous, the N transformations can potentially be followed by the stable isotopic signature of  $\text{NO}_2^-$ , which has neither been tested nor applied so far.

Nitrite can be formed during nitrate reduction (NAR) in the course of denitrification, ammonium oxidation (AOX) in the course of autotrophic nitrification and organic N oxidation (ORG) associated with heterotrophic nitrification, and consumed during nitrite reduction (NIR) to NO or  $\text{N}_2\text{O}$ , and nitrite oxidation (NIOX) to  $\text{NO}_3^-$ <sup>1,7</sup>. Each of these sources and sinks are characterised by specific isotopic fractionation<sup>7,9,14</sup>, which makes

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**Figure 1.** Relationship between  $\text{NO}_2^-$  isotopic signature  $\delta^{15}\text{N}_{\text{NO}_2^-}$  (A) or  $\delta^{18}\text{O}_{\text{NO}_2^-}$  (B) and reciprocal  $\text{NO}_2^-$  content (Keeling plot analysis—see “Methods” section). For  $\delta^{18}\text{O}_{\text{NO}_2^-}$  (B) the dashed line indicates the  $\delta$  value of  $\text{NO}_2^-$  in full equilibrium with ambient water in 20 °C of 8.6‰<sup>23</sup> and the arrows indicate the direction of change in  $\delta$  values of  $\text{NO}_2^-$  in course of equilibration with water (points ‘move’ towards full equilibrium). For L3 the first samples taken after 24 h are marked with circles and the second samples taken after 48 h are shown with crosses only. Note the logarithmic scale of the X-axis.

it possible to trace them back to their origins and sinks of  $\text{NO}_2^-$ , and consequently, for a better understanding of the N cycling<sup>7</sup>.

This study presents the first attempt to interpret the  $\text{NO}_2^-$  isotopic signatures ( $\delta^{15}\text{N}_{\text{NO}_2^-}$  and  $\delta^{18}\text{O}_{\text{NO}_2^-}$ ) in agricultural soil to decipher soil transformation processes. Three laboratory incubations were performed: under oxic conditions with lower water content (L1), under oxic conditions with higher water content (L2) and under anoxic conditions (L3), to monitor the differences when various N transformation processes are enhanced. The incubations at natural abundance level (NA treatment) and under  $^{15}\text{N}$  enrichment ( $^{15}\text{NO}_3^-$  treatment and  $^{15}\text{NH}_4^+$  treatment) were performed simultaneously. Based on the  $^{15}\text{N}$  treatments the *Ntrace* model<sup>1</sup> was applied to determine  $\text{NO}_2^-$  sources and sinks. The results of NA treatment were used to construct the soil  $\text{NO}_2^-$  model, which is based on the model used for oceanic studies<sup>7</sup>, including the processes that have contributed to production and consumption of  $\text{NO}_2^-$  in soils. This study provides the first attempt to validate the results of  $\text{NO}_2^-$  isotope modelling with an independent  $^{15}\text{N}$  tracing approach.

## Results

**Soil  $\text{NO}_2^-$  characteristics.** The oxic experiment was performed in two moisture treatments: L1 (drier conditions) and L2 (wetter conditions), with water addition in the middle of experiment which increased the soil moisture from 61 to 68% water-filled pores space (WFPS) for L1a and L1b and from 72 to 81% WFPS for L2a and L2b, respectively. The detailed experimental conditions and information on general soil properties can be found in<sup>15</sup> and in the supplement.  $\text{NO}_2^-$  content varied from 0.6 to 1.4  $\mu\text{mol N kg}^{-1}$  soil for L1 and from 0.1 to 4.7 for L2, whereas the  $\text{NO}_3^-$  content was three orders higher and quite stable ranging from 1300 to 1700  $\mu\text{mol N kg}^{-1}$  soil. The  $\delta^{15}\text{N}_{\text{NO}_2^-}$  was similar for L1 and L2 with a mean of  $3.2 \pm 4.2\text{‰}$  and  $3.9 \pm 4.2\text{‰}$ , respectively, whereas  $\delta^{15}\text{N}_{\text{NO}_3^-}$  was very stable with a mean of  $4.5 \pm 0.4\text{‰}$  and  $4.7 \pm 0.6\text{‰}$ , respectively. There was a negative correlation between  $\delta^{15}\text{N}_{\text{NO}_2^-}$  and the  $\text{NO}_2^-$ -content (Fig. 1A). Similar values for  $\delta^{18}\text{O}_{\text{NO}_2^-}$  were found for both L1 and L2 with a mean of  $11.8 \pm 2.8\text{‰}$  and  $12.5 \pm 5.0\text{‰}$ , respectively.

The anoxic experiment L3 was performed to favour denitrification.  $\text{NO}_2^-$  content was much higher when compared to oxic conditions (L1 and L2) reaching  $63.9 \pm 12.5 \mu\text{mol N kg}^{-1}$  soil and  $32.4 \pm 8.9 \mu\text{mol N kg}^{-1}$  soil after 24 h and 48 h of incubation, respectively. The average  $\delta^{15}\text{N}_{\text{NO}_2^-}$  was  $-24.8 \pm 3.3\text{‰}$  without significant differences between the two samplings, whereas  $\delta^{18}\text{O}_{\text{NO}_2^-}$  showed significantly lower values of  $4.4 \pm 0.4\text{‰}$  after 24 h compared to  $6.6 \pm 1.0\text{‰}$  after 48 h (Fig. 1B).

In both  $^{15}\text{N}$  treatments ( $^{15}\text{NH}_4^+$  and  $^{15}\text{NO}_3^-$ ) in L1 and L2, we observed a sudden drop in  $^{15}\text{N}$  abundance in  $\text{NO}_2^-$  ( $a^{15}\text{N}_{\text{NO}_2^-}$ ) from 12.7 to 5.1 at.% after water addition to the soil, whereas  $^{15}\text{N}$  abundance in  $\text{NO}_3^-$  ( $a^{15}\text{N}_{\text{NO}_3^-}$ )

Experiment	$^{15}\eta_{\text{NO}_2^-/\text{NO}_3^-}$	$^{15}\eta_{\text{N}_2\text{O}-\text{NO}_3^-}$	$^{15}\eta_{\text{N}_2\text{O}-\text{NO}_2^-}$
L1a	$-5.0 \pm 1.9$	$-23.5 \pm 1.9$	$-18.5 \pm 1.8$
L1b	$1.8 \pm 1.8$	$-15.0 \pm 9.3$	$-16.6 \pm 7.4$
L2a	$-3.8 \pm 5.2$	$-22.7 \pm 5.9$	$-18.1 \pm 11.2$
L2b	$1.4 \pm 2.8$	$-41.6 \pm 2.1$	$-42.8 \pm 0.3$
L3	$-31.4 \pm 4.3$	$-53.1 \pm 2.7$	$-21.3 \pm 2.9$

**Table 1.** Apparent isotopic fractionation factors for  $\delta^{15}\text{N}$  of  $\text{NO}_3^-$ ,  $\text{NO}_2^-$  and  $\text{N}_2\text{O}$ .

showed only slight decrease from 13.2 to 12.1 at.% (means for all treatments, individual values in Fig. S1 and Table S1). This indicates an incorporation of another source of unlabelled  $\text{NO}_2^-$  for the wet part of these experiments. In natural abundance isotopes this change was also reflected in a higher apparent isotope effect  $^{15}\eta_{\text{NO}_2^-/\text{NO}_3^-}$ . For the wet part it was even positive with an average + 1.6‰, whereas for the dry part it was lower with - 4.4‰ (Table 1). Interestingly, this significant isotopic change in  $\text{NO}_2^-$  was not reflected in the  $\text{N}_2\text{O}$   $^{15}\text{N}$  abundance ( $\delta^{15}\text{N}_{\text{N}_2\text{O}}$ ) in the  $^{15}\text{N}$  treatments (Fig. S1, Table S1).

**Isotope effects between  $\text{NO}_3^-$ ,  $\text{NO}_2^-$  and  $\text{N}_2\text{O}$ .** With the NA dataset for  $\text{NO}_2^-$  presented in this paper and the dataset for  $\text{N}_2\text{O}$  presented in a previous paper for L1 and L2<sup>15</sup>, and here for L3 (Table S1), we can investigate the relation between the isotopic characteristics of both N compounds and determine the apparent isotope effects between  $\text{NO}_2^-$  and  $\text{N}_2\text{O}$ , and comparing them with isotope effects between  $\text{NO}_3^-$  and  $\text{N}_2\text{O}$ . Therefore, we need the isotopic signatures of the produced  $\text{N}_2\text{O}$  prior to isotopic fractionation due to  $\text{N}_2\text{O}$  reduction. For L3 the incubations were partially conducted with  $\text{N}_2\text{O}$  reduction inhibition (acetylated treatments) and we only report here the  $\delta_{\text{N}_2\text{O}}$  values of the inhibited treatment (Table S1) which represent the produced  $\text{N}_2\text{O}$  isotopic signatures. For L1 and L2 a detailed study of  $\text{N}_2\text{O}$  reduction was performed<sup>15</sup> where the  $\text{N}_2\text{O}$  reduced fraction ( $r_{\text{N}_2\text{O}}$ ) was determined with  $^{15}\text{N}$  treatment, and the produced  $\text{N}_2\text{O}$  ( $\delta_{\text{N}_2\text{O}_p}$ ) can be calculated according to the equation:

$$\delta_{\text{N}_2\text{O}_p} = \delta_{\text{N}_2\text{O}_m} - \ln r_{\text{N}_2\text{O}} * \epsilon_{\text{red}}$$

based on the measured  $\text{N}_2\text{O}$  ( $\delta_{\text{N}_2\text{O}_m}$ ) and the isotopic fractionation associated with  $\text{N}_2\text{O}$  reduction ( $\epsilon_{\text{red}}$ )<sup>15</sup>. The determined apparent N isotope effects ( $^{15}\eta$ ) was calculated as:

$$^{15}\eta_{\text{product-substrate}} = \delta^{15}\text{N}_{\text{product}} - \delta^{15}\text{N}_{\text{substrate}}$$

**Determination of  $\text{NO}_2^-$  dominant source.** Keeling plots were applied to identify the  $\text{NO}_2^-$  dominant source (see Methods Section for methodical explanation)<sup>15-18</sup>. For the oxic experiment, a significant linear fit between  $\delta^{15}\text{N}_{\text{NO}_2^-}$  and reciprocal  $\text{NO}_2^-$  content was found, where a linear equation intercept of - 13.3‰ indicated the isotopic signature of the dominant  $\text{NO}_2^-$  source. It must be denitrification since the applied conditions of quite high soil moisture and nitrate amendment should have favoured denitrification. In a previous study, denitrification was identified as the dominant source for  $\text{N}_2\text{O}$ <sup>15</sup> and also the applied *Ntrace* model indicated the dominance of denitrification nitrate reduction (*NAR*) in the  $\text{NO}_2^-$ -sources ( $f_{\text{NAR}}$  of 0.53 and 0.55 for L1 and L2, respectively, Table 2). Hence, based on the value found from the Keeling plot (Fig. 1A) we can determine the nitrogen isotopic fractionation for denitrification ( $^{15}\epsilon_{\text{NAR}}$ ) between  $\delta^{15}\text{N}_{\text{NO}_3^-}$  (mean measured value) and  $\delta^{15}\text{N}_{\text{NO}_2^-}$  (Keeling plot intercept) for this incubation experiment:

$$^{15}\epsilon_{\text{NAR}}(\text{NO}_2^-/\text{NO}_3^-) = -13.3\text{‰} - (+4.5\text{‰}) = -17.8\text{‰}$$

This value fits quite well in the literature range<sup>7,14</sup> and is further used in the  $\text{NO}_2^-$  isotope model as  $^{15}\epsilon_{\text{NAR}}$ .

Under anoxic conditions, denitrification should be the only source of  $\text{NO}_2^-$ , hence a typical Keeling correlation is not expected. We rather observed the opposite trend in L3 than under oxic conditions, i.e. lower  $\delta^{15}\text{N}_{\text{NO}_2^-}$  values with lower  $\text{NO}_2^-$  contents (Fig. 1A). Most probably this reflects the variability of apparent isotope effects, which are typically larger for lower reaction rates<sup>19,20</sup>.

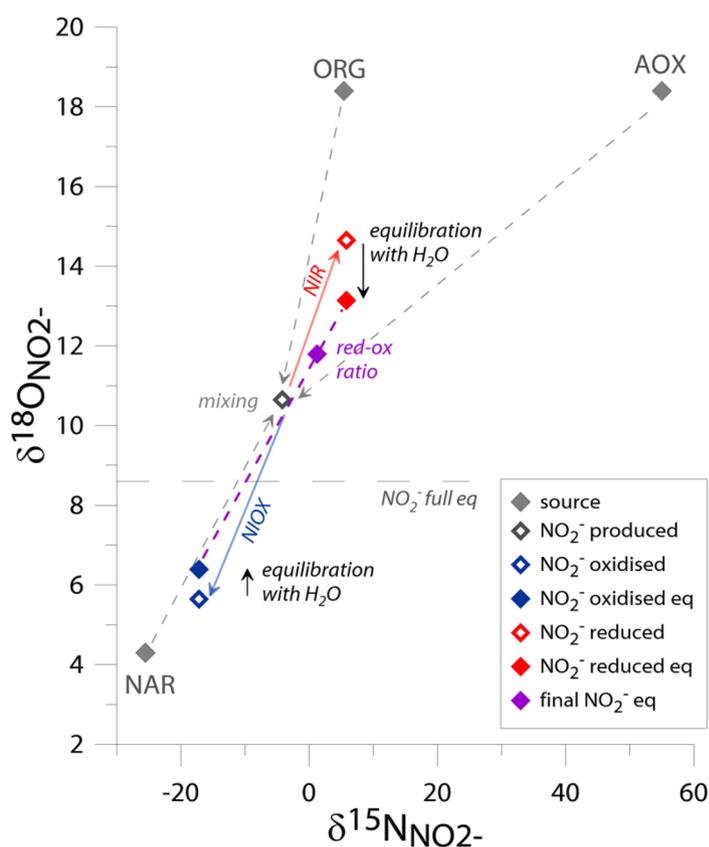
For  $\delta^{18}\text{O}_{\text{NO}_2^-}$  values, beside sources mixing, we also deal with isotope exchange of O-atoms between  $\text{NO}_2^-$  and ambient water, hence the Keeling plot method cannot be applied. We observed that  $\delta^{18}\text{O}_{\text{NO}_2^-}$  values were modified by the O exchange process, especially under anoxic conditions (L3), where lower  $\text{NO}_2^-$  content and incubation progress shifted  $\delta^{18}\text{O}_{\text{NO}_2^-}$  values towards equilibrium with water ( $\text{NO}_2^-$  full eq, Fig. 1B).  $\text{NO}_2^-$  samples taken after 48 h showed more equilibrated  $\delta^{18}\text{O}_{\text{NO}_2^-}$  values and lower  $\text{NO}_2^-$  content.

**$\text{NO}_2^-$  isotope model.** The model is constructed based on the  $\text{NO}_2^-$  isotope model proposed for oceanic studies<sup>7</sup> and adapted for typical soil N pathways after the *Ntrace* model, designed for  $^{15}\text{N}$  labelled soil studies applying  $\text{NO}_2^-$  as a key intermediate in soil N transformations<sup>1</sup>, assuming steady state conditions. It takes into account three main  $\text{NO}_2^-$  sources (*NAR*, *AOX* and *ORG*) and two main  $\text{NO}_2^-$  sinks (*NIR* and *NIOX*), as well as  $\delta^{18}\text{O}_{\text{NO}_2^-}$  equilibration with ambient water (Fig. 2), according to the following equations:

$$\delta^{15}\text{N}_{\text{NO}_2^-} = \delta^{15}\text{N}_{\text{NAR}} * f_{\text{NAR}} + \delta^{15}\text{N}_{\text{AOX}} * f_{\text{AOX}} + \delta^{15}\text{O}_{\text{ORG}} * f_{\text{ORG}} - ^{15}\epsilon_{\text{NIR}} * f_{\text{NIR}} - ^{15}\epsilon_{\text{NIOX}} * f_{\text{NIOX}} \quad (1)$$

Source	Substrate		Source fractionation		Produced NO <sub>2</sub> <sup>-</sup>		f <sub>mix</sub>	Mixed NO <sub>2</sub> <sup>-</sup>		Sink	Sink fractionation		Residual NO <sub>2</sub> <sup>-</sup>		NO <sub>2</sub> <sup>-</sup> eq	f <sub>red-ox</sub>	Final modeled		True measured		f <sub>mix</sub>	
	δ <sup>18</sup> O	δ <sup>15</sup> N	<sup>18</sup> ε	<sup>15</sup> ε	δ <sup>18</sup> O	δ <sup>15</sup> N	N <sub>trace</sub>	δ <sup>18</sup> O	δ <sup>15</sup> N		<sup>18</sup> ε	<sup>15</sup> ε	δ <sup>18</sup> O	δ <sup>15</sup> N	δ <sup>18</sup> O	N <sub>trace</sub>	δ <sup>18</sup> O	δ <sup>15</sup> N	δ <sup>18</sup> O	δ <sup>15</sup> N	fitted	
L1	NAR	4.3	4.5	0	-17.8	4.3	-13.3	<b>0.53</b>	10.9	0.3	NIR	-4.0	-10.0	14.9	10.3	13.3	0.86	12.4	7.1	11.8	3.2	<b>0.55</b>
	AOX	23.5; -6.4	93.9	20	-25.0	18.4	68.9	<b>0.08</b>			NIOX	5.0	13.0	5.9	-12.7	6.6	0.14					<b>0.02</b>
	ORG	23.5	7.4	0	-2.0	18.4	5.4	<b>0.39</b>														<b>0.43</b>
L2	NAR	4.7	4.7	0	-17.8	4.7	-13.1	<b>0.55</b>	10.9	3.2	NIR	-4.0	-10.0	14.9	13.2	13.3	0.70	11.3	6.3	12.5	3.9	<b>0.58</b>
	AOX	23.5; -6.4	65.5	-20	-25.0	18.4	40.5	<b>0.23</b>			NIOX	5.0	13.0	5.9	-9.8	6.6	0.30					<b>0.18</b>
	ORG	23.5	7.4	0	-2.0	18.4	5.4	<b>0.23</b>														<b>0.25</b>
L3	NAR	15.3	7.0	-10	-30	5.3	-23.0	<b>1</b>	5.3	-23.0	NIR	-4.0	-10.0	9.3	-13.0	9.1		9.1	-13.0	5.5	-24.8	
											no fract			5.3	-23.0	6.1		6.1	-23.0			

**Table 2.** Nitrite stable isotope model to determine sources mixing proportions. In the first step, nitrite isotopic signature (δ<sup>18</sup>O, δ<sup>15</sup>N) is modelled based on: (i) the nitrite sources taking into account measured substrate isotopic signatures (NO<sub>3</sub><sup>-</sup> for NAR, NH<sub>4</sub><sup>+</sup> for AOX and organic N for ORG), fractionation factors (<sup>18</sup>ε, <sup>15</sup>ε), and sources mixing proportions according to the results of the N<sub>trace</sub> model (f<sub>mix</sub> N<sub>trace</sub>); (ii) nitrite sinks with their characteristic isotopic fractionation factors (<sup>18</sup>ε, <sup>15</sup>ε) including the nitrite reduction–oxidation ratio after results of the N<sub>trace</sub> model; and (iii) nitrite equilibration with water (NO<sub>2</sub><sup>-</sup> eq) including measured extent of O-exchange of 0.25, δ<sup>18</sup>O of -5‰ and <sup>18</sup>ε<sub>eq</sub> for 20 °C. In the second step, modelled nitrite isotopic signature were fitted to the measured values by adjusting the sources mixing proportions (f<sub>mix</sub> fitted) to find the ideal fit of modelled vs. measured δ<sup>15</sup>N values.



**Figure 2.** General scheme of the NO<sub>2</sub><sup>-</sup> stable isotope model. The isotopic signatures of NO<sub>2</sub><sup>-</sup> sources shown are based on the measured mean isotopic signatures of substrates for L1 and L2 and the isotopic fractionation associated with NAR, AOX and ORG (Table 1). Dashed gray arrows illustrate the mixing of 3 NO<sub>2</sub><sup>-</sup> sources with mean mixing proportions found in N<sub>trace</sub> study (f<sub>NAR</sub> = 0.55, f<sub>AOX</sub> = 0.15, f<sub>ORG</sub> = 0.30, Table 1) resulting in the produced δ<sub>NO<sub>2</sub><sup>-</sup></sub> (grey open point). This δ value can be modified by NIR (red arrow) and NIOX (blue arrow). The δ<sup>18</sup>O<sub>NO<sub>2</sub><sup>-</sup></sub> after reduction or oxidation (red and blue open point, respectively) is further modified by equilibration with ambient water with the extent of 0.25 of the equilibrated oxygen atoms (red and blue filled point, respectively). The ratio of NO<sub>2</sub><sup>-</sup> reduction and oxidation processes (red-ox ratio, here 4:1, as mean from N<sub>trace</sub> study, Table 1) determines the final δ<sub>NO<sub>2</sub><sup>-</sup></sub> (purple point).

	<i>k</i>	<i>C</i> <sub>NO<sub>2</sub><sup>-</sup></sub>	<i>F</i> <sub>eq</sub>	<i>F</i> <sub>B</sub>	<i>F</i> <sub>Ntrace</sub>	<i>T</i> <sub>B</sub>	<i>T</i> <sub>eq</sub>
	[d <sup>-1</sup> ]	[μmol kg <sup>-1</sup> ]	[μmol kg <sup>-1</sup> d <sup>-1</sup> ]	[μmol kg <sup>-1</sup> d <sup>-1</sup> ]	[μmol kg <sup>-1</sup> d <sup>-1</sup> ]	[h]	[h]
L1	17.7	0.9	15.5	16.3	28.0	1.3	1.4
L2	17.7	1.4	24.3	39.6	26.4	0.8	1.4
L3	17.7	48.1	851.8	639.7		1.8	1.4

**Table 3.** Nitrite transformation fluxes (due to equilibration ( $F_{eq}$ ) and biological turnover ( $F_B$ )) and residence time due to biological turnover ( $T_B$ ) or abiotic equilibration ( $T_{eq}$ ) determined with  $\delta^{18}O_{NO_2^-}$  values compared to nitrite turnover rate determined with *Ntrace* model ( $F_{Ntrace}$ ).

$$\delta^{18}O_{NO_2^-} = (\delta^{18}O_{NAR} * f_{NAR} + \delta^{18}O_{AOX} * f_{AOX} + \delta^{18}O_{ORG} * f_{ORG} - \epsilon_{NIR} * f_{NIR} - \epsilon_{NIOX} * f_{NIOX}) * (1 - x) + \delta^{18}O_{eq} * x \quad (2)$$

where  $\delta_{NO_2^-}$  is the measured residual  $NO_2^-$  isotopic signature,  $\delta_{NAR/AOX/ORG}$  are the isotopic signatures of source  $NO_2^-$  calculated with the measured stable isotope values for  $NO_2^-$  substrates ( $NO_3^-$ ,  $NH_4^+$ ,  $N_{org}$ , respectively for three sources, Table 2) and the characteristic isotopic fractionation associated with each  $NO_2^-$  formation pathway ( $\epsilon_{NAR/AOX/ORG}$ , Table 2).  $\epsilon_{NIR/NIOX}$  are the isotopic fractionation factors associated with  $NO_2^-$  sinks ( $\epsilon_{NIR/NIOX}$ , Table 2). See also Methods Section for detailed description of isotope effects for particular processes; final values used in the model are shown in Table 2. The  $\delta^{18}O_{eq}$  stands for O isotopic signature of  $NO_2^-$  in complete equilibrium with water, which equals 8.6‰ for the incubation temperature of 20 °C<sup>7</sup> and  $\delta^{18}O_{H_2O}$  of -5‰.  $x$  is the extent of oxygen atom exchange between nitrate and ambient water determined with the <sup>17</sup>O approach<sup>21</sup> for  $N_2O$  originating from denitrification processes under anoxic conditions (L3) and is equal 0.25 (see Methods Section). The exchange for  $NO_2^-$  cannot be higher than the value determined for  $N_2O$ . Since most of the exchange observed for  $N_2O$  is associated with the  $NO_2^-$ - $H_2O$  isotope exchange<sup>21</sup> this value was incorporated in the model calculations.

For NAR we were able to determine the isotope effect ( $\epsilon_{NAR} = -17.8$ ) based on the Keeling plot (Fig. 1A) for L1 and L2. This value of -17.8‰ is on the lower range of previously determined values<sup>22-29</sup> (see summary in the Methods Section) and was used in the model for L1 and L2. For anoxic experiment L3 the fractionation was larger (Fig. 1A) and we included the typical  $\epsilon_{NAR}$  value of -30‰ in the model. For  $\epsilon_{NAR}$  the fractionation must be very low to obtain the observed range of  $\delta^{18}O_{NO_2^-}$ : -10‰ for L3 and no fractionation for L1 and L2. Similar ranges of  $\epsilon_{NAR}$  values were modelled previously with indication of lower values for smaller reaction rates<sup>21</sup>. This is in accordance with our observations indicating much lower N transformation rates for the oxic experiments L1 and L2 than for the anoxic L3. Also the isotopic fractionation for NIR appears to be lower under anoxic conditions, where  $NO_2^-$  is accumulating. We obtained best fit between modelled and measured values for L3 when no fractionation associated with NIR was assumed (Table 2). This can be due to observed accumulation of  $NO_2^-$ , indicating that the steady-state model assumption is not valid. In case of  $NO_2^-$  accumulation the isotopic fractionation associated with its reduction has a very low impact on the final isotopic signature of the residual  $NO_2^-$ .

**$NO_2^-$  turnover.** When  $\delta^{18}O_{NO_2^-}$  values are not completely equilibrated with soil water, measured  $\delta^{18}O_{NO_2^-}$  values can be used to estimate the rates of biological  $NO_2^-$  turnover relative to abiotic exchange<sup>7</sup>. This estimation is based on the abiotic equilibration rate as a function of temperature and pH<sup>7</sup>. Furthermore, we can determine the flux of  $NO_2^-$  oxygen atoms abiotic exchange as  $F_{eq} = k * C_{NO_2^-}$ . The  $NO_2^-$  flux of biological production (or consumption) can be determined from the  $\delta^{18}O_{NO_2^-}$  isotope mass balance following the method proposed for oceanic studies<sup>7</sup> adapted to soil  $NO_2^-$  transformations:

$$F_B = \frac{F_{eq}(\delta^{18}O_{NO_2^-} - \delta^{18}O_{eq})}{\delta^{18}O_{NAR} * f_{NAR} + \delta^{18}O_{AOX} * f_{AOX} + \delta^{18}O_{ORG} * f_{ORG} - \epsilon_{NIR} * f_{NIR} - \epsilon_{NIOX} * f_{NIOX} - \delta^{18}O_{NO_2^-}} \quad (3)$$

where  $\delta^{18}O_{NO_2^-}$  is the measured  $NO_2^-$ ,  $\delta^{18}O_{NAR/AOX/ORG}$  are the calculated  $NO_2^-$  sources NAR, AOX and ORG,  $\epsilon_{NIR/NIOX}$  are the isotopic fractionation associated with  $NO_2^-$  sinks NIR and NIOX,  $f$  are the respective contributions of  $NO_2^-$  sources determined by *Ntrace* model (Table 2), and  $\delta^{18}O_{eq}$  is the value for  $NO_2^-$  in complete equilibrium with ambient water (of 8.6‰ for this case study). In turnover rate calculations (Table 3) we have neglected NIOX because due to the inverse fractionation of this process for some cases the isotope mass balance did not work due to unrealistic discrepancies between calculated and measured  $\delta^{18}O_{NO_2^-}$  values. Since  $\epsilon_{NIOX}$  can be very low<sup>30</sup> and the NIOX contribution is low for our case study (up to 30%, Table 2), this process has most probably little impact on the final  $\delta^{18}O_{NO_2^-}$  values. In the  $NO_2^-$  isotope model, neglecting NIOX would result in higher final modelled  $\delta^{18}O_{NO_2^-}$  values of 13.3‰ for both treatments, which would fit to the measured values equally well as when the NIOX fractionation is included (Table 2).

Our results indicate that the  $NO_2^-$  flux in L2 is larger than in L1 (Table 3), which is reasonable since L2 was the wetter treatment showing more intensive nitrogen fluxes based  $N_2O$  and  $N_2$  fluxes, which were twice as high in L2 compared to L1<sup>15</sup>. Similar differences can be observed here for calculated FB values (Table 3), however this is not directly confirmed by the *Ntrace* results.

## Discussion

We found very good congruity between *Ntrace* and the NA  $\text{NO}_2^-$  model. The modelled  $\delta^{18}\text{O}_{\text{NO}_2^-}$  and  $\delta^{15}\text{N}_{\text{NO}_2^-}$  values using measured source fractions provided by the *Ntrace* model differed up to 1.2‰ and 4.0‰, respectively, when compared to true measured values (Table 2). When we solely used the NA  $\text{NO}_2^-$  model to assess the fraction of  $\text{NO}_2^-$ -sources contribution based on  $\delta^{15}\text{N}_{\text{NO}_2^-}$ , i.e., fitting modelled  $\delta^{15}\text{N}_{\text{NO}_2^-}$  values to the true measured values by adjusting the fraction of  $\text{NO}_2^-$ -sources contribution, the fitted fractions are in good agreement with fractions provided by the *Ntrace* model (Table 2). Both results show similar dominance of NAR in  $\text{NO}_2^-$  production ( $f_{\text{NAR}}$  of ca. 0.55) but the NA  $\text{NO}_2^-$  model indicates even higher contribution of heterotrophic vs autotrophic nitrification ( $f_{\text{ORG}}$  vs.  $f_{\text{AOX}}$ ).

The *Ntrace* approach, which is able to identify the contribution of ORG, was actually the first one that paid attention to this process in soils<sup>31</sup>. Here, with the NA  $\text{NO}_2^-$  model we get a confirmation of the potentially high ORG relevance in soil N transformations. Without this process the final  $\delta^{15}\text{N}_{\text{NO}_2^-}$  and  $\delta^{18}\text{O}_{\text{NO}_2^-}$  values could not be explained. Namely, if only considering two source processes: NAR and AOX, to meet the measured  $\delta^{15}\text{N}_{\text{NO}_2^-}$  value we would need domination of NAR ( $f_{\text{NAR}} > 0.75$ ) and to meet the measured  $\delta^{18}\text{O}_{\text{NO}_2^-}$  value we would need an unrealistically high contribution of AOX ( $f_{\text{AOX}} > 0.70$ ). Hence, the application of both isotope signatures ( $\delta^{15}\text{N}_{\text{NO}_2^-}$  and  $\delta^{18}\text{O}_{\text{NO}_2^-}$ ) simultaneously allows for a proper identification of  $\text{NO}_2^-$  sources.

The presented  $\text{NO}_2^-$  isotope model may not be very typical, since for our case study we had exceptionally high  $\delta^{15}\text{N}_{\text{NH}_4^+}$  values (from 36 to 100‰), hence this worked partially as a naturally low level  $^{15}\text{N}$  tracing allowing for very clear separation of NAR and AOX with  $\delta^{15}\text{N}_{\text{NO}_2^-}$  values. In case of similar  $\delta$  values for substrate  $\text{NO}_3^-$  and  $\text{NH}_4^+$ , this separation would be very weak, but still, in combination with  $\delta^{18}\text{O}_{\text{NO}_2^-}$  values, may be useful in assessing source contributions. The  $^{15}\text{N}$  enrichment of  $\text{NH}_4^+$  was not purposely induced but was a consequence of the fast ammonium consumption. *Ntrace* analysis revealed that the dominant ammonium sink is immobilisation, responsible for more than 90% of ammonium consumption. This process is associated with pronounced enrichment of residual ammonium in  $^{15}\text{N}$ <sup>32–34</sup>, which we observed in this study. The very fast  $\text{NH}_4^+$  immobilisation and its further release due to Norg oxidation to  $\text{NO}_3^-$  were unexpected in this study and cannot be fully explained. The *Ntrace* model assumes the existence of the labile Norg pool, which is associated with these extremely fast fluxes. In the NA model, the assumed substrate for ORG nitrite production is the measured  $\delta^{15}\text{N}$  of the bulk organic N pool. This is probably the largest uncertainty in the model, since the labile Norg pool may be isotopically different than the bulk Norg. Similar uncertainties may also be associated with the measured bulk  $\delta^{15}\text{N}_{\text{NO}_3^-}$ , since this value may be significantly higher in the intensively denitrifying soil microsites.

The  $\text{NO}_2^-$  isotopic signature time series in the  $^{15}\text{N}$  treatments (Fig. S1), strongly indicates the appearance of new unlabelled  $\text{NO}_2^-$  for L1 and L2 after water addition in the course of the incubation. *Ntrace* clearly indicated an increase in ORG contribution after water addition (from 0.11 to 0.49 and from 0.07 to 0.33 for L1 and L2, respectively, Table S3) and the NA  $\text{NO}_2^-$  model confirms this finding (from 0.11 to 0.52 and from 0.21 to 0.31 for L1 and L2, respectively, Table S3). This suggests that NA  $\text{NO}_2^-$  analyses can be used to trace the dynamic changes in soil N transformations.

The  $^{15}\text{N}$  treatment indicated that  $\text{N}_2\text{O}$   $^{15}\text{N}$  enrichment ( $a^{15}\text{N}_{\text{N}_2\text{O}}$ ) follows rather  $a^{15}\text{N}_{\text{NO}_3^-}$  than  $a^{15}\text{N}_{\text{NO}_2^-}$  (Fig. S1). This indicates that mostly NAR  $\text{NO}_2^-$  is further reduced and emitted as  $\text{N}_2\text{O}$  and suggests that the ORG  $\text{NO}_2^-$  forms an isolated  $\text{NO}_2^-$  pool, as also suggested earlier<sup>1</sup>, which is probably not further reduced to  $\text{N}_2\text{O}$  in significant magnitude. The calculated  $a_{\text{P,N}_2\text{O}}$  value representing the  $^{15}\text{N}$  enrichment of the  $^{15}\text{N}$ -pool derived  $\text{N}_2\text{O}$  is higher than  $a^{15}\text{N}_{\text{N}_2\text{O}}$  due to the contribution of non-labelled  $\text{N}_2\text{O}$  to the total  $\text{N}_2\text{O}$  flux, so that  $a^{15}\text{N}_{\text{N}_2\text{O}} = f_{\text{P,N}_2\text{O}} * a_{\text{P,N}_2\text{O}} + (1 - f_{\text{P,N}_2\text{O}}) * a_{\text{NA}}$ , where  $a_{\text{NA}}$  is the  $^{15}\text{N}$  abundance of the natural abundance samples (0.367 at.‰). However,  $a_{\text{P,N}_2\text{O}}$  values always have a higher  $^{15}\text{N}$  abundance than found for any soil N-pool (Table S1). This indicates that in denitrification soil microsites, where the  $^{15}\text{N}$ -pool derived  $\text{N}_2\text{O}$  is produced, we deal with higher  $a^{15}\text{N}_{\text{NO}_2^-}$  and  $a^{15}\text{N}_{\text{NO}_3^-}$  values than the mean analysed values. This confirms the  $\text{N}_2\text{O}$  emission originating from various isolated soil N-pools. The fraction of the  $^{15}\text{N}$ -pool  $\text{N}_2\text{O}$  is dominating – from 0.7 to 1.0—with higher values for higher soil moisture (Table S1). This is in contrast to  $\text{NO}_2^-$  which gets  $^{15}\text{N}$  depleted after water addition (Fig. S1) and  $f_{\text{NAR}}$  is only around 0.5.

Regarding the NA isotope effects, we can see that for pure denitrification processes under anoxic conditions in L3, we deal with very low  $^{15}\eta_{\text{NO}_2^-/\text{NO}_3^-}$  values (Table 1) indicating a pronounced isotope effect between  $\text{NO}_3^-$  and  $\text{NO}_2^-$ , whereas for L1 and L2, where  $\text{NO}_2^-$  is formed not only due to NAR but also AOX and ORG (Table S2), this effect is much smaller (as indicated by  $^{15}\eta_{\text{NO}_2^-/\text{NO}_3^-}$  closer to 0, Table 1). Interestingly, in the wetter parts of the experiments (L1b, L2b), when increased contribution of ORG  $\text{NO}_2^-$  occurs, even an inverse effect is observed, i.e.  $\text{NO}_2^-$  is  $^{15}\text{N}$  enriched compared to  $\text{NO}_3^-$ . As a result, for L1 and L2 very similar isotope effects for  $\text{N}_2\text{O}$  production with both substrates are observed ( $^{15}\eta_{\text{N}_2\text{O}/\text{NO}_3^-}$  and  $^{15}\eta_{\text{N}_2\text{O}/\text{NO}_2^-}$  are not significantly different). This is in contrast with L3, where  $^{15}\eta_{\text{N}_2\text{O}/\text{NO}_2^-}$  is much lower compared to  $^{15}\eta_{\text{N}_2\text{O}/\text{NO}_3^-}$ . For oxic conditions, clearly the highest isotope effect for  $\text{N}_2\text{O}$  production with both substrates is noted for L2b, for which the  $^{15}\eta_{\text{N}_2\text{O}/\text{NO}_3^-}$  is nearest to the values typical for denitrification, as found in L3. Indeed, for L2b the denitrification pool derived fraction ( $f_{\text{P,N}_2\text{O}}$ , Table S1) is the highest. Also the previous study<sup>15</sup> indicated that the  $\text{N}_2\text{O}$  fraction produced due to denitrification, including both bacterial and fungal denitrification, is near 1 for this part of the experiment (L2b)<sup>15</sup>. However, the  $^{15}\eta_{\text{N}_2\text{O}/\text{NO}_2^-}$  values are much lower for L2b than for L3 which is probably caused by admixture of other nitrite sources present for L2b but absent for L3.

Despite the fact that  $\text{NO}_2^-$  turnover rates determined with  $\delta^{18}\text{O}_{\text{NO}_2^-}$  ( $F_B$ ) differed somewhat from the  $F_{\text{Ntrace}}$  results (Table 3), the congruence can be considered to be adequate because very plausible ranges for  $\text{NO}_2^-$  turnover rates were observed. Both methods provide a similar range of values, however, the *Ntrace* model does not reflect the significant difference in turnover rates between L1 and L2 (Table 3). This may be due to lower sensitivity of the *Ntrace* approach in precise  $\text{NO}_2^-$  fluxes determination, since these are determined as a result of complex modelling of all N pools and the final result is an average of the best fit fluxes for both treatments ( $^{15}\text{NH}_4^+$  and

$^{15}\text{NO}_3^-$ ). This turnover rate estimation provides a unique opportunity to predicting process rates based on natural abundance isotopic measurements.

Summing up, the natural abundance signatures of  $\text{NO}_2^-$  can be applied for identification of  $\text{NO}_2^-$  sources by applying the NA isotope model, which allows to estimate the contribution of the main pathways: NAR, AOX and ORG. This study showed that these are in a very good agreement with the results provided by the *Ntrace* model. Moreover, analysis of  $\delta^{18}\text{O}_{\text{NO}_2^-}$  values allows for estimation of  $\text{NO}_2^-$  turnover rates. The natural abundance signatures of  $\text{NO}_2^-$  may potentially be used in linking the soil N transformations with gaseous emissions in the form of  $\text{N}_2\text{O}$ . However, this connection still cannot be fully understood and needs further studies.

## Methods

**Laboratory incubations.** *Oxic incubations: L1 and L2 experiment.* Silt loam soil *Albic Luvisol* from arable cropland of Merklingsen experimental station located near Soest (North Rhine-Westphalia, Germany,  $51^\circ 34' 15.5'' \text{N}$ ,  $8^\circ 00' 06.8'' \text{E}$ ) was used in the incubations (0.87 silt, 0.11 clay, 0.02 sand). The soil density of intact cores was  $1.3 \text{ g cm}^{-3}$ , pH value 6.8, total C content 0.0130, total N content 0.0016, organic matter content 0.0214, initial  $\text{NO}_3^-$  content  $864 \mu\text{mol N kg}^{-1}$  dry soil and initial  $\text{NH}_4^+$  content  $50 \mu\text{mol N kg}^{-1}$  dry soil. The soil, upper 30 cm soil layer, was collected on the 18.01.2018 and the incubation was conducted from 19.02.2018 to 05.03.2018. The soil was air dried and sieved at 4 mm mesh size. Afterwards, the soil was rewetted to achieve a water content equivalent to 60% water-filled pore space (WFPS) and fertilised with 20 mg N per kg soil, added as  $\text{NaNO}_3$  (10 mg N) and  $\text{NH}_4\text{Cl}$  (10 mg N). Three treatments were prepared: natural abundance (NA), labelled with  $^{15}\text{N}$  nitrate ( $^{15}\text{NO}_3^-$ ) and labelled with  $^{15}\text{N}$  ammonium ( $^{15}\text{NH}_4^+$ ). For the  $^{15}\text{NO}_3^-$  treatment,  $\text{NaNO}_3$  solution with 72 atom %  $^{15}\text{N}$  was added and for the  $^{15}\text{NH}_4^+$  treatment,  $\text{NH}_4\text{Cl}$  solution with 63 atom %  $^{15}\text{N}$  was added. Then soils were thoroughly mixed to obtain homogenous distribution of water and fertilizer and an equivalent of 1.69 kg dry soil was repacked into each incubation column with bulk density of  $1.3 \text{ g cm}^{-3}$ .

For each treatment 14 soil columns were prepared, and half of them received additional water injected on the top of the column (100 mL water added) to prepare two moisture treatments: L1 (61% WFPS) and L2 (72% WFPS). The incubation lasted 12 days. In the meantime, on the 6th day of incubation, water addition on the top of each column was repeated (80 mL water added) to increase the soil moisture in both treatments to ca. 68% WFPS in L1 and ca. 81% WFPS in L2. The strategy of adding water on the top of the column to achieve target water content was necessary to allow mixing and compaction at a suitable (low) water content of the soil and thus to optimise homogeneity of water and fertilizer distribution<sup>3</sup>. The incubation temperature was 20 °C. The columns were continuously flushed with a gas mixture with reduced  $\text{N}_2$  content to increase the measurements sensitivity (2%  $\text{N}_2$  and 21%  $\text{O}_2$  in  $\text{He}^{35}$ ) with a flow of  $9 \text{ mL min}^{-1}$ . Gas samples were collected daily into two 12 mL septum-capped Exetainer vials (Labco Limited, Ceredigion, UK) connected to the vents of the incubation columns. Soil samples were collected 5 times during the incubation by sacrificing one incubation column per sampling event, which was then divided into three subsamples (replicate samples of mixed soil).

*Anoxic incubations: L3 experiment.* The same soil was used for the static incubations performed under an anoxic atmosphere ( $\text{N}_2$ ) in closed, gas-tight vessels, where denitrification products accumulated in the headspace. The incubation was conducted from 13.07.2020 to 15.07.2020. The soil was air dried and sieved at 4 mm mesh size. Afterwards, the soil was rewetted to achieve a water content equivalent to 70% water-filled pore space (WFPS) and fertilised with 100 mg N per kg soil, added as  $\text{NaNO}_3$  using natural *Chile saltpetre* ( $\text{NaNO}_3$ , Chili Borium Plus, Prills-Natural origin, supplied by Yara, Dülmen, Germany,  $\delta^{18}\text{O} = 56\text{‰}$ ,  $\Delta^{17}\text{O} = 21.8\text{‰}$ ) to prepare 12 incubation soil samples of NA treatment and  $\text{Na}^{15}\text{NO}_3$  to prepare 6 incubation soil samples of  $^{15}\text{NO}_3^-$  treatment. The soil was thoroughly mixed to obtain a homogenous distribution of water and fertilizer and an equivalent of 85 g of dry soil was repacked into each incubation jar at bulk densities of  $1.3 \text{ g cm}^{-3}$ . The 0.5  $\text{dm}^3$  Mason jars were used with airtight rubber seals and with two three-way valves installed in their cover to enable sampling and flushing. The jars were flushed with  $\text{N}_2$  at approximately  $500 \text{ cm}^3 \text{ min}^{-1}$  (STP: 273.15 K, 100 kPa) for 10 min to create anoxic conditions. In 6 NA vessels and three  $^{15}\text{NO}_3^-$  vessels 50  $\text{dm}^3$  of headspace  $\text{N}_2$  was replaced with 50  $\text{dm}^3$  of acetylene to inhibit  $\text{N}_2\text{O}$  reduction to  $\text{N}_2$ . Half of the incubation vessels of each treatment was incubated for 45 h and the other half was finished after 21 h for destructive sampling for soil mineral N analyses. The incubation temperature was 20 °C. Four gas samples were collected in 10 to 12 h-intervals by transferring 30  $\text{cm}^3$  of headspace gases into two pre-evacuated 12  $\text{cm}^3$  Exetainer vials (Labco Limited, Ceredigion, UK). The excess 3  $\text{cm}^3$  of headspace gas in each vial ensured that no ambient air entered the vials. The removed sample volume was immediately replaced by pure  $\text{N}_2$  gas.

**Soil analyses.** All soil samples were homogenized. Soil water content was determined by weight loss after 24 h drying at 110 °C. Soil pH was determined in 0.01 mol  $\text{CaCl}_2$  solution (ratio 1:5). Nitrate and ammonium concentrations were determined by extraction in 2 M KCl in 1:4 ratio by 1 h shaking. Nitrite concentration was determined in alkaline extraction solution of 2 M KCl with addition of 2 M KOH (25 mL per L) in 1:1 ratio for 1 min of intensive shaking<sup>36</sup>. The amount of added KOH was adjusted to keep the alkaline conditions in extracts (pH over 8). After shaking, the samples were centrifuged for 5 min and filtered. The extracts for  $\text{NO}_2^-$  measurements were stored at  $-4 \text{ °C}$  and analyzed within 5 days.  $\text{NO}_3^-$ ,  $\text{NH}_4^+$  and  $\text{NO}_2^-$  concentrations were determined colorimetrically with an automated analyser (Skalar Analytical B.V., Breda, the Netherlands).

To determine isotopic signatures of mineral nitrogen in NA treatments, microbial analytical methods were applied. For nitrate, the bacterial denitrification method with *Pseudomonas aureofaciens* was applied<sup>37,38</sup>. For nitrite, the bacterial denitrification method for selective nitrite reduction with *Stenotrophomonas nitritireducens* was applied<sup>6</sup>, also for  $^{15}\text{N}$ -enriched samples from  $^{15}\text{N}$  treatments. For ammonium, a chemical conversion to nitrite with hypobromite oxidation<sup>39</sup> followed by bacterial conversion of nitrite after pH adjustment was applied<sup>40</sup>.

$\delta^{15}\text{N}$  of the organic N was analysed in the flushed and dried soil sample after mineral N extractions by EA combustion coupled to Delta Plus mass spectrometer (Thermo Finnigan, Bremen, Germany).

In  $^{15}\text{N}$  treatments,  $^{15}\text{N}$  abundances of  $\text{NO}_3^-$  ( $a_{\text{NO}_3^-}$ ) and  $\text{NH}_4^+$  ( $a_{\text{NH}_4^+}$ ) were measured as described in Eschenbach, et al.<sup>41</sup>.  $\text{NO}_3^-$  was reduced to NO by Vanadium-III chloride ( $\text{VCl}_3$ ) and  $\text{NH}_4^+$  was oxidized to  $\text{N}_2$  by hypobromite ( $\text{NaOBr}$ ). NO and  $\text{N}_2$  were used as measurement gas. Measurements were performed on isotope ratio mass spectrometer (Delta Plus, Thermo Finnigan, Bremen, Germany).

Soil water was extracted with the method described by Königer, et al.<sup>42</sup> and the  $\delta^{18}\text{O}$  of water samples (with respect to VSMOW) was measured using cavity ringdown spectrometer Picarro L1115-*i* (Picarro Inc., Santa Clara, USA). The measurement repeatability ( $1\sigma$ ) of the internal standards (three calibrated waters with known  $\delta^{18}\text{O}$ :  $-19.67\text{‰}$ ,  $-8.60\text{‰}$ ,  $+1.37\text{‰}$ ) was below  $0.1\text{‰}$ . The overall error associated with the soil water extraction method determined as standard deviation ( $1\sigma$ ) of the 5 samples replicates was below  $0.5\text{‰}$ .

All isotopic values are expressed as ‰ deviation from the  $^{15}\text{N}/^{14}\text{N}$  and  $^{18}\text{O}/^{16}\text{O}$  ratios of the reference materials (i.e. atmospheric  $\text{N}_2$  and Vienna Standard Mean Ocean Water (VSMOW), respectively).

**Gas analyses.** The samples for gas concentration analyses were collected in Exetainer vials (Labco Limited, Ceredigion, UK) and were analysed using an Agilent 7890A gas chromatograph (GC) (Agilent Technologies, Santa Clara, CA, USA) equipped with an electron capture detector (ECD). Measurement repeatability as given by the relative standard deviation ( $1\sigma$ ) of four standard gas mixtures was typically  $1.5\%$ .

The gas samples collected from  $^{15}\text{N}$  treatments were analyzed for  $a^{15}\text{N}_{\text{N}_2\text{O}}$  ( $^{15}\text{N}$  abundance in the emitted  $\text{N}_2\text{O}$ ),  $a_{\text{P-N}_2\text{O}}$  ( $^{15}\text{N}$  abundance in the  $^{15}\text{N}$ -pool derived  $\text{N}_2\text{O}$ ) and  $f_{\text{P-N}_2\text{O}}$  ( $^{15}\text{N}$ -pool derived fraction of  $\text{N}_2\text{O}$ )<sup>15</sup> with a modified GasBench II preparation system coupled to MAT 253 isotope ratio mass spectrometer (Thermo Scientific, Bremen, Germany) according to Lewicka-Szczebak et al.<sup>43</sup>. In this set-up,  $\text{N}_2\text{O}$  is converted to  $\text{N}_2$  during in-line reduction, and stable isotope ratios  $^{29}\text{R}$  ( $^{29}\text{N}_2/^{28}\text{N}_2$ ) and  $^{30}\text{R}$  ( $^{30}\text{N}_2/^{29}\text{N}_2$ ), of  $\text{N}_2$  are determined.

The gas samples of the NA treatment were analysed for  $\text{N}_2\text{O}$  isotopocules ( $\delta^{15}\text{N}_{\text{N}_2\text{O}}$ ,  $\delta^{18}\text{O}_{\text{N}_2\text{O}}$ ,  $\delta^{15}\text{N}^{\text{SP}}_{\text{N}_2\text{O}}$ ) using a Delta V isotope ratio mass spectrometer (Thermo Scientific, Bremen, Germany), coupled to an automatic preparation system with Precon + Trace GC Isolink (Thermo Scientific, Bremen, Germany), where  $\text{N}_2\text{O}$  was pre-concentrated, separated and purified, and  $m/z$  44, 45, and 46 of the intact  $\text{N}_2\text{O}^+$  ions as well as  $m/z$  30 and 31 of  $\text{NO}^+$  fragment ions were determined. The results were evaluated accordingly<sup>44–46</sup> which allows the determination of average  $\delta^{15}\text{N}$ ,  $\delta^{15}\text{N}^{\alpha}$  ( $\delta^{15}\text{N}$  of the central N position of the  $\text{N}_2\text{O}$  molecule), and  $\delta^{18}\text{O}$ .  $\delta^{15}\text{N}^{\beta}$  ( $\delta^{15}\text{N}$  of the peripheral N position of the  $\text{N}_2\text{O}$  molecule) was calculated as  $\delta^{15}\text{N} = (\delta^{15}\text{N}^{\alpha} + \delta^{15}\text{N}^{\beta})/2$  and  $^{15}\text{N}$  site preference ( $\delta^{15}\text{N}^{\text{SP}}$ ) as  $\delta^{15}\text{N}^{\text{SP}} = \delta^{15}\text{N}^{\alpha} - \delta^{15}\text{N}^{\beta}$ .

**Determination of  $\Delta^{17}\text{O}$  excess in  $\text{N}_2\text{O}$  and  $\text{NO}_3^-$  and estimation of O-atoms exchange ( $x$ ).**  $\text{N}_2\text{O}$  samples collected in the L3 NA treatment and  $\text{N}_2\text{O}$  produced from soil  $\text{NO}_3^-$  by the bacterial denitrifier method were analysed for  $\Delta^{17}\text{O}$  after microwave equilibration in a sapphire tube and separation of  $\text{N}_2$  and  $\text{O}_2$  on a mole sieve column<sup>47</sup>. The  $^{17}\text{O}$  excess,  $\Delta^{17}\text{O}$ , is defined as<sup>48</sup>:

$$\Delta^{17}\text{O} = \frac{1 + \delta^{17}\text{O}}{(1 + \delta^{18}\text{O})^{0.5279}} - 1 \quad (4)$$

The measurement repeatability ( $1\sigma$ ) of the international standards (USGS34, USGS35) was typically  $0.5\text{‰}$  for  $\Delta^{17}\text{O}$ .

The extent of isotope exchange ( $x$ ) was determined based on the comparison of  $\Delta^{17}\text{O}$  in soil nitrate and produced  $\text{N}_2\text{O}$ . It requires the application of nitrate characterised by high  $\Delta^{17}\text{O}$ . Therefore, for this determination, soils in L3 were amended with natural  $\text{NaNO}_3$  Chile saltpetre showing high  $\Delta^{17}\text{O}$  (of  $21.8\text{‰}$ ) and the  $\Delta^{17}\text{O}$  of the  $\text{N}_2\text{O}$  product was measured.  $\Delta^{17}\text{O}$  of soil water was assumed to be  $0\text{‰}$ .

The magnitude of oxygen isotope exchange ( $x$ ) was calculated as:

$$x = 1 - \frac{\Delta^{17}\text{O}(\text{N}_2\text{O})}{\Delta^{17}\text{O}(\text{NO}_3^-)} \quad (5)$$

The accuracy of  $x$  determination was better than  $1\%$ .

**Application of the Keeling plot.** The original idea for Keeling plot application applies for mixing of the background low level (atmospheric  $\text{CO}_2$ ) and one dominant source responsible for the significant increase of the  $\text{CO}_2$  concentration<sup>16</sup>. In such a case, plotting the  $\delta$  values against the reciprocal  $\text{CO}_2$  concentration reveals the isotopic signature of the dominant as intercept of the linear fit<sup>16</sup>. Afterwards, the application of Keeling approach to isotopic studies has expanded to the other environments and substances, including nitrates source identification<sup>17,18,49</sup>. In these studies the requirement of only two sources is not necessarily fulfilled, but the occurrence of a clear linear relation between isotopic signature and reciprocal concentration of the studied substance indicates that there is a dominant source which can be isotopically characterised<sup>49</sup>. This is clearly the case for our nitrite samples, where we find a very significant linear relation (Fig. 1A). Nitrite contents in soils are typically very low and only rarely accumulate, mostly as a result of intensified nitrification or denitrification processes<sup>11,12,49–51</sup>. Hence, with Keeling plot we can isotopically identify the dominant  $\text{NO}_2^-$  source and identify the pathway responsible for this accumulation.

**Isotope fractionation factors for the nitrite model.** The isotope fractionation factors are always expressed as:

$$\varepsilon_{\text{product/substrate}} = \delta_{\text{product}} - \delta_{\text{substrate}}$$

Hence, negative  $\varepsilon$  values inform about normal isotope effect resulting in product depletion in heavy isotopes.

**Nitrite sources.** NAR is associated with quite high isotopic fractionation of N and O, resulting in significant depletion in  $^{15}\text{N}$  and  $^{18}\text{O}$  in the product  $\text{NO}_2^-$ . The nitrate reductase enzymatic experiments showed a mean  $^{15}\varepsilon_{\text{NAR}}$  of  $-26.6 \pm 0.2\text{‰}$ , similar to  $^{18}\varepsilon_{\text{DEN}}$  with a mean of  $-24.9 \pm 0.3\text{‰}$ <sup>25</sup>. In pure culture bacterial studies much larger variations of  $^{15}\varepsilon_{\text{DEN}}$  were observed, i.e. ranging from  $-30.5$  to  $-5.4\text{‰}$ <sup>22,24,26</sup> and it has been suggested that the range from  $-15$  to  $-10\text{‰}$  is most representative for typical cellular nitrate reduction rates for bacterial strains<sup>27</sup>. The strongest fractionation was found for pure culture fungal studies with a mean  $^{15}\varepsilon_{\text{NAR}}$  of  $-37.8 \pm 6.6\text{‰}$ <sup>20</sup>. Similar values were found for  $^{18}\varepsilon_{\text{DEN}}$  in pure culture studies: ranging between  $-30$  and  $-25\text{‰}$  for bacterial denitrification<sup>23</sup> and between  $-30$  and  $-10\text{‰}$  for fungal denitrification<sup>20</sup>. In the sediment denitrification experiments  $^{15}\varepsilon_{\text{DEN}}$  ranged from  $-24.4$  to  $-18.9\text{‰}$  and  $^{18}\varepsilon_{\text{DEN}}$  from  $-21.9$  to  $-15.8\text{‰}$ <sup>8,29</sup>. A slightly lower  $^{15}\varepsilon_{\text{DEN}}$  of  $-29.4 \pm 2.4\text{‰}$  was determined for soil studies<sup>28</sup>.

Nitrite produced from AOX is depleted in  $^{15}\text{N}$  compared to its ammonium substrate. Bacterial ammonia oxidation show a mean  $^{15}\varepsilon_{\text{AOX}}$  of  $-25.8 \pm 9.8\text{‰}$ <sup>52</sup>, similar to archaeal ammonia oxidation with a mean  $^{15}\varepsilon_{\text{AOX}}$  of  $-22 \pm 5\text{‰}$ <sup>53</sup>.  $\delta^{18}\text{O}_{\text{NO}_2^-}$  from AOX depends on  $\delta^{18}\text{O}_{\text{O}_2}$  ( $+23.5\text{‰}$ ),  $\delta^{18}\text{O}_{\text{H}_2\text{O}}$  ( $-5\text{‰}$ ) and  $\delta^{18}\text{O}_{\text{N}_2\text{O}_{\text{eq}}}$  ( $8.6\text{‰}$ ) according to the equation<sup>7,54</sup>:

$$\delta^{18}\text{O}_{\text{AOX}} = 0.5 * (\delta^{18}\text{O}_{\text{O}_2} + \delta^{18}\text{O}_{\text{H}_2\text{O}} + 20) * 0.92 + (\delta^{18}\text{O}_{\text{H}_2\text{O}} + \delta^{18}\text{O}_{\text{NO}_2^- \text{eq}}) * 0.08 \quad (6)$$

Nitrite produced from ORG show much lower  $^{15}\text{N}$  enrichment with a mean  $^{15}\varepsilon_{\text{ORG}}$  of about  $-2\text{‰}$  as measured for marine sediments fractionation<sup>55</sup>.  $\delta^{18}\text{O}_{\text{NO}_2^-}$  from ORG was assumed to be the same as for AOX according to Eq. (3) ( $+18.4\text{‰}$ ).

**Nitrite sinks.** Two major nitrite sinks—reduction and oxidation—show opposite isotopic fractionation. Nitrite reduction is associated with normal isotope effect resulting in enrichment in  $^{15}\text{N}$  and  $^{18}\text{O}$  of the nitrite pool, whereas nitrite oxidation is characterised by inverse isotope effect, where heavy isotopes are preferentially transferred to the oxidised product leaving nitrite pool depleted in  $^{15}\text{N}$  and  $^{18}\text{O}$ . For NIR different fractionation may be associated with various nitrite reductases involved, showing a  $^{15}\varepsilon_{\text{NIR}}$  of  $-22 \pm 2\text{‰}$  and an  $^{18}\varepsilon_{\text{NIR}}$  of  $-2 \pm 2\text{‰}$  for Cu-NIR and  $-8 \pm 2\text{‰}$  and  $-6 \pm 2\text{‰}$  respectively for Fe-NIR<sup>56</sup>. In batch experiments with environmental bacterial communities a  $^{15}\varepsilon_{\text{DNIR}}$  ranging from  $-15$  to  $-10\text{‰}$  was observed when nitrite was investigated as an intermediate product but much lower when nitrite was a substrate<sup>29</sup>. Here we probably also observe this for L3—where nitrite is accumulating we get the best fit with the measured values when no fractionation associated with NIR is assumed (Table 2).

For nitrite oxidation the inverse isotope effects with a  $^{15}\varepsilon_{\text{NOX}}$  of  $+12.8\text{‰}$ <sup>57</sup> and an  $^{18}\varepsilon_{\text{NOX}}$  of  $+5\text{‰}$ <sup>30</sup> were found.

**Nitrite equilibration with water.** The oxygen isotope signature of  $\text{NO}_2^-$  is additionally modified by the abiotic equilibrium exchange with ambient water<sup>23</sup>. The magnitude of this exchange is governed by the equilibrium isotope effect between  $\text{NO}_2^-$  and water ( $\varepsilon_{\text{eq}}$ ) which is a function of temperature<sup>7,23</sup> and the extend of O atoms exchange.  $\varepsilon_{\text{eq}}$  for the incubation temperature of  $20\text{ °C}$  equals  $13.63$ ,  $\delta^{18}\text{O}_{\text{H}_2\text{O}}$  is  $-5\text{‰}$ , consequently, the  $\delta^{18}\text{O}$  of nitrite in complete equilibrium with water is  $8.6\text{‰}$ . The extend of O atoms exchange was determined with the  $^{17}\text{O}$  approach<sup>21</sup> for  $\text{N}_2\text{O}$  originating for denitrification processes in anoxic experiment L3 and equalled  $0.25$ .

## Data availability

Original data are available upon request. Material necessary for this study findings is presented in the paper and supplementary materials.

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## Author contributions

D.L.S. and R.W. designed the laboratory experiments and D.L.S. was in charge of carrying them out. D.L.S. and J.D. performed the isotopic analyses and data evaluation. D.L.S. performed the interpretations based of natural abundance isotope studies and constructed the nitrite isotope model. A.J.W. performed the *Ntrace* model with support of C.M. D.L.S. prepared the manuscript with significant contribution of R.W. and C.M.

## Competing interests

The authors declare no competing interests.

## Additional information

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