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ABSTRACT

The isotopic composition of N and O of nitrate (NO_3^-) is usually employed to trace its sources of pollution in groundwater. In agricultural areas, the amount of NO_3^- that reaches the aquifers after fertilization is controlled by different transformation processes that can affect the nitrogen species isotopic composition. Aiming to address the reliability of using isotope tools to trace sources of groundwater NO_3^-, the goal of this study was to check the effect of fertilization on the isotopic composition of N compounds retained and leached from soils. The concentration and isotopic composition ($\delta^{15}N$ and $\delta^{18}O$) of ammonium (NH_4^+), NO_3^- and nitrite (NO_2^-) was characterized after the application of pig slurry in lysimeters containing either soil under fallow (LF) or the same soil continuously cropped and fertilized (LC) during the previous six years. Results showed that the leached NO_3^- isotopic signature did not directly reflect the isotopic composition of the applied pig slurry. Just after fertilization, nitrification led to lower $\delta^{15}N_{NO3}$ values in soil extracts and leachates (e.g. from +5.9±0.9 ‰ to +3.8±3.1 ‰ in soil extracts of LF lysimeters). These values increased after complete nitrification (+11.5±1.3 ‰) towards the $\delta^{15}N_{bulk}$ of pig slurry (+19.6±0.5 ‰). Later on, due to soil organic matter and plant debris mineralization and subsequent nitrification, values decreased towards the initial $\delta^{15}N_{NO3}$ of soil but remained above them (+8.6±1.0 ‰). Both LF and LC experiments showed a similar trend and the latter ones allowed to reinforce that long-term fertilization with pig slurry can increase the soil $\delta^{15}N_{NO3}$. Concerning the $\delta^{18}O$ of NO_3^- from soil extracts and leachates, it mainly depended on the $\delta^{18}O$ of irrigation water and oxygen, after nitrification of NH_4^+ from pig slurry. Therefore, studies aiming to trace groundwater NO_3^- pollution sources in rural areas by using an isotopic approach should consider the fertilization history of each setting. Also, analyzing the $\delta^{15}N_{bulk}$ of soil is recommended, since it could mask the isotopic signature of the N applied through fertilization.

Keywords: groundwater pollution, lysimeters, nitrate, soil fertilization, stable isotopes

1. INTRODUCTION

Groundwater nitrate (NO_3^-) pollution is mainly caused by diffuse (non-point) sources linked to intensive use of synthetic and organic fertilizers in agricultural lands. In synthetic fertilizers, N is commonly found as ammonium (NH_4^+) or NO_3^-, while in organic fertilizers it can also be found as organic N compounds. Organic N is mineralized to NH_4^+, which can be directly uptaken by plants or oxidized to NO_3^- through nitrification. After nitrification, NO_3^- excess that cannot be uptaken by crops is leached to the hydrosphere, leading to a significant loss of available N for plants and to groundwater pollution (Beeckman et al., 2018; Subbarao et al., 2006).

Measures to decrease N inputs into soil and therefore to improve groundwater quality have been applied since 1991 in response to European directives (2000/60/EC, 2000; 2006/118/EC, 2006; 91/676/EEC, 1991). One of the most challenging issues to evaluate its effectiveness, is the determination of NO_3^- pollution sources in aquifers. Isotopic characterization ($\delta^{15}N$ and $\delta^{18}O$) has proven to be a powerful tool since NO_3^- and NH_4^+ in synthetic and organic fertilizers, present well defined and different isotopic compositions (Aravena et al., 1993; Clark and Fritz, 1997; Kendall, 1998; Vitoria et al., 2004; Wassenaar, 1995). However, the residence time of N derived from fertilizers in the soil organic matter pool can amount to several decades (Sebilo et al., 2013; Wang et al., 2013). Consequently, during the delay between fertilizer application and NO_3^- leaching to groundwater, several transformation processes in the non-saturated zone potentially modify the isotopic composition of N compounds that will be leached to the hydrosphere.

The most important N cycling reactions in agricultural lands are nitrification, mineralization-immobilization-turnover (MIT), plant uptake, denitrification, and NH_4^+ volatilization (**Figure 1**) (Craine et al., 2015; Kelley et al., 2013; Mengis et al., 2001). Nitrification involves the oxidation of NH_4^+ to hydroxylamine (NH_2OH), nitrite (NO_2^-) and finally NO_3^- (Heil et al., 2016; Masclaux-Daubresse et al., 2010; Sharma and Ahlert, 1977). MIT refers to the recycling of NO_3^- via immobilization as organic N, subsequent mineralization to NH_4^+ via organic matter degradation and a turnover back to NO_3^- via nitrification (Mengis et al., 2001). Immobilization, together with plant uptake, are two N assimilation pathways, which involve the production of organic N from inorganic compounds such as NO_3^-, NO_2^- or NH_4^+ (Cabello et al., 2004; Emmerton et al., 2001; Lin and Stewart, 1997). On the other hand, denitrification leads to a decrease of available N reactive species by reducing NO_3^- to NO_2^-, nitric oxide (NO), nitrous oxide (N_2O), and finally N_2 (Knowles, 1982; Moreno-vivián et al., 1999). Due to the different reaction rates between the heavy and light isotopes during these reactions, the isotopic composition of the involved N compounds can undergo significant variations (Craine et al., 2015; Denk et al., 2017; Kendall, 1998; Mariotti et al., 1981; Nikolenko et al., 2018).

In the case of the $\delta^{15}N$, immobilization, nitrification, denitrification and volatilization, cause an enrichment in the heavy isotope (^{15}N) of the residual substrate (either NO_3^- or NH_4^+), since the lighter isotope (^{14}N) reacts preferentially (Aravena and Robertson, 1998; Böttcher et al., 1990; Högberg, 1997; Mariotti et al., 1981). During mineralization, the ^{14}N is preferentially released, leading to an enrichment in ^{15}N of the soil organic N pool compared to the soil inorganic N pool (Craine et al., 2015; Dijkstra et al., 2008, 2006). In contrast, changes in the substrate isotopic composition during plant uptake are rather weak, although a slight enrichment in ^{15}N is usually observed for plant tissues compared to the substrate (Craine et al., 2015; Estrada et al., 2017; Spoelstra et al., 2010). With respect to the $\delta^{18}O$ in NO_3^-, the main reactions controlling its

composition are the incorporation of oxygen during nitrification as well as the enrichment in the heavy isotope (^{18}O) caused by denitrification. During chemolithoautotrophic nitrification of NH_4^+ to NO_3^-, two oxygen atoms are derived from water and one from O_2 (Andersson and Hooper, 1983; Hollocher, 1984). This atom ratio is usually employed to determine the $\delta^{18}O$ of nitrified NO_3^-, but some authors have pointed out that this hypothesis is not always valid due to several reasons, such as I) different processes can modify the $\delta^{18}O$ of O_2 and H_2O, II) the isotopic fractionation during the incorporation of oxygen atoms from O_2 and H_2O may not be negligible, III) $\delta^{18}O$ of the intermediate NO_2^- can equilibrate with $\delta^{18}O$ of H_2O, and IV) chemolithoautotrophic and heterotrophic nitrification might present different ratios for the O_2 and H_2O atoms incorporation (Mayer et al., 2001; Snider et al., 2010 and references therein). As a result, we might use cautiously the $\delta^{18}O_{NO3}$, when assessing NO_3^- groundwater pollution origin. Even when employing NO_3^- fertilizers, the $\delta^{18}O_{NO3}$ after the MIT process may no longer reflect the original NO_3^- source as described by Mengis et al., (2001).

The present study aims to gain knowledge on the isotopic composition of NO_3^- retained and leached from agricultural soils fertilized with pig slurry. Therefore, it can be helpful to assess the reliability of using isotope tools to trace NO_3^- sources in polluted groundwater bodies in rural areas where stockbreeding residues are applied to crops, a scenario commonly found across Europe. To achieve the goal, the concentration and isotopic shifts on NO_3^-, NO_2^- and NH_4^+ retained and leached from soil were checked during a complete agronomical year in lysimeter experiments. The influence of long-term fertilization was also checked by comparing a soil left fallow and a soil fertilized during the previous 6 years. The results could question the range of isotopic values currently employed to identify sources of groundwater NO_3^- pollution derived from fertilizers.

2 METHODS

2.1 Experimental setup

A lysimeter experiment was set up in a greenhouse at Torre Marimón (IRTA, Caldes de Montbui, Spain). The lysimeters consisted of polyvinyl chloride tubes (48 height x 20 cm diameter), with a perforated lid at the bottom covered with a 2 mm plastic mesh. The filling material of the lysimeters corresponded to a 2 cm layer of rinsed quartz sand, to allow drainage, and two 20 cm layers of experimental agricultural soil (equivalent to 13.4 kg), simulating A and B horizons.

For A horizon filling (top 20 cm), two types of topsoil were collected from the same field, a calcareous Fluventic haploxerept (Soil Survey Staff, 2014) described in detail in Marks et al. (2016), but from two neighboring areas subjected to different managements during the previous 6 agronomical years (2011-2017). One of the two areas corresponded to 1x1 m mesocosms continuously cropped with barley and fertilized with thermally dried pig slurry at 50 kg N ha^{-1} $year^{-1}$ (C), while the other area corresponded to soil adjacent to the mesocosms kept under fallow and non-fertilized (F). Fallow consisted on a fall tillage between 2011-2015, while between 2015-2017 plots were mowed instead. Horizon B of this fallow area was collected to fill horizon B of all lysimeters. According to the use of topsoil (horizon A), continuously cropped or under fallow, two types of lysimeters were established and designated as LC and LF, respectively (**Figure 2**), with 5 replicates each.

The setup of the lysimeters was performed on March 23rd, 2017, an initial watering was carried out and the lysimeters were left to stabilize for 11 days. On April 3rd, 2017, each lysimeter was sowed with fifteen barley seeds (*Hordeum vulgare*) (later thinned to keep only the most robust plant) and 7.3 g of dried pig slurry were added as fertilizer (100 kg N ha^{-1} $year^{-1}$). Watering was performed by keeping moisture below the maximum water holding capacity (around 20% w/w) from April to July. After harvesting in July 3rd, 2017, in order to simulate Mediterranean climate, a

drought period was simulated. Only three spaced irrigation events were performed during summer and early fall (17th August, 5th September and 11th November) that, coupled to the high temperatures in the greenhouse, led to dry soil conditions. Drought conditions were partly suppressed after a fourth irrigation in late fall (2nd December), that, coupled with the lower temperatures, led to higher soil moisture. Irrigation water (groundwater from the study site) had NO_3^- concentration around 200 mg/L. Temperatures recorded in the greenhouse and moisture measured in the soil throughout the study period are reported in the supporting information (**Table S1** and **Table S2**, respectively).

2.2 Sample collection

During the experiment, at five relevant stages in relation to cropping practices, soil, leachates and gas samples were obtained. The dates and specific samples collected for each stage are described in **Table 1**.

Namely, topsoil samples were collected by using a 5.5 x 7 cm core. For soil samples, KCl extracts were immediately prepared by weighting 20 g of soil and adding them to 100 ml of KCl 2M (1:5 w/v), followed by a 30 min-period of shaking in a vertical agitator (120 rpm) (ISO/TS 14256-1: 2003). In parallel, water extracts were prepared using 40 g of soil in a 1:5 ratio (w/v), followed by a 1 h-period of shaking. These KCl and water extracts were centrifuged (5 min at 8000 rpm) and then filtered in Whatman no. 42 and frozen at -20 °C before analysis. To collect ~200 ml of leachate, each lysimeter was placed on a glass tray (raised 1.3 cm) to allow drainage and the suitable amount of water was added considering the water content at each lysimeter and determined by previous lysimeter weighting. The leachates samples were just filtered and frozen. These soil extracts and leachate samples were analyzed for NO_3^-, NO_2^- and NH_4^+ content and isotopic composition determination. In addition, aliquots of soil samples (dried at room temperature for several days) were finely grounded to determine bulk N content and isotopic composition.

Soil gas emissions were measured using the static chamber-based method described in Collier et al. (2014). For this purpose, vented chambers (21.5 cm high, 21 cm diameter) were placed and sealed on top of the lysimeters to accumulate gaseous emissions during 20 min or 30 min (the latter when the ambient temperature was colder). Gas samples were extracted from the static chambers at three equally spaced time points using a plastic syringe (20 ml) and injected into a 12 ml vial (Exetainers®, Labco Ltd., Ceredigion, UK). In these gas samples, N_2O content was determined.

In addition, plant samples were collected, at the end of their life cycle, when dried in summer (July 3th, 2017), a pig manure sample was also collected at the beginning of the study, and irrigation water samples were collected at the beginning and end of the study. The collected plant shoots and leaves (dried at 60ºC for 48 h) and the pig slurry sample (already dry) were finely grounded to determine bulk N content and isotopic composition. In irrigation water samples, NO_3^-, NO_2^- and NH_4^+ content and its isotopic composition were determined.

2.3 Analytical methods

NH_4^+ was measured using the salicylate method (Willis et al., 1996), in a Spectronic 20 Genesys 4001/4 spectrophotometer. NO_2^- and NO_3^- concentrations were determined by ionic chromatography in an AS4A-SC Dionex anion column in a Dionex DX-100 Ion Chromatograph. N_2O concentration was analysed by a gas chromatograph (Agilent 7890A) coupled to an electron capture detector (ECD). Rates of flux were estimated from the slope of the linear regression between gas concentration and accumulated time within the chamber headspace, then corrected by the air temperature, the atmospheric pressure and the surface-volume ratio of the chamber. Bulk N content of soil and pig slurry samples was determined by elemental analysis (EA) (Carlo Erba). Bulk N content of plant samples was measured by near infrared spectrometry by scanning

the grounded samples from 1100 to 2500 nm using a NIRSystems 5000 scanning monochromator (FOSS, Hilleröd, Denmark) using the calibrations described in Martos et al. (2020).

To measure the isotopic composition of N compounds, dissolved NH_4^+ was initially oxidized to NO_2^- by using a hypobromite solution (Zhang et al., 2007). In parallel, dissolved NO_3^- was reduced to NO_2^- using spongy cadmium (McIlvin and Altabet, 2005; Ryabenko et al., 2009). Then, the generated NO_2^- was reduced to N_2O with a sodium azide solution with acetic acid (McIlvin and Altabet, 2005; Ryabenko et al., 2009; Zhang et al., 2007). The later step was also employed in parallel for NO_2^- containing samples. Simultaneous $\delta^{15}N$ and $\delta^{18}O$ analysis of N_2O produced during the conversion of NH_4^+, NO_3^- and NO_2^- following the cadmium or hypobromite and azide methods, was carried out using a Pre-Con (Thermo Scientific) coupled to a Finnigan MAT-253 Isotope Ratio Mass Spectrometer (IRMS) (Thermo Scientific). The bulk $\delta^{15}N$ of soil, plant and pig slurry samples was determined in a Carbo Erba EA-Finnigan Delta C IRMS. According to Coplen (2011), several international and laboratory (UB) standards were interspersed among samples for the normalisation of the isotope results (USGS-32, USGS-34, USGS-35, UB-IWS_{NO3} ($\delta^{15}N$ = +16.9 ‰, $\delta^{18}O$ = +28.5 ‰) and UB-IWS_{NO2} ($\delta^{15}N$ = -28.5 ‰), for the $\delta^{15}N$ and $\delta^{18}O$ of dissolved NO_3^- and NO_2^-; USGS-25, IAEA-N2 and UB-IWS_{NH4} ($\delta^{15}N$ = -0.8 ‰), for the $\delta^{15}N$ of dissolved NH_4^+; and USGS-40, IAEA-N1, IAEA-N2 and UCGEMA-P, for the $\delta^{15}N_{bulk}$ of solid materials). The standard deviation reproducibility of the samples was ±1.0 ‰ for $\delta^{15}N$ of dissolved NO_3^-, NO_2^- and NH_4^+; ±1.5 ‰ for $\delta^{18}O$ of dissolved NO_3^- and NO_2^-; and ±0.2 ‰ for $\delta^{15}N_{bulk}$ of solid materials. The isotopic notation is expressed in terms of δ per mil relative to international standards Vienna Standard Mean Oceanic Water (V-SMOW) for $\delta^{18}O$ and Atmospheric N_2 (AIR) for $\delta^{15}N$, following **Equation 1**:

$$\delta = \frac{R_{sample} - R_{standard}}{R_{standard}}, \qquad \text{where } R = {}^{18}O/{}^{16}O \text{ and } {}^{15}N/{}^{14}N, \text{ respectively} \qquad \textbf{Equation 1}$$

The concentration analyses were performed at the soil laboratory in CREAF (Cerdanyola del Vallès, Spain). The isotopic analyses were prepared at the MAiMA laboratory (Barcelona, Spain) and analysed at the Centres Científics i Tècnològics of the Universitat de Barcelona (Barcelona, Spain).

3. RESULTS

Complete datasets of the study are reported as supporting information (**Tables S3** to **S4**). These datasets include the results for the soil extracts and leachates N compounds concentration and isotopic composition, which are described along the results section. Datasets involving further information on gas, pig manure, plants, irrigation water and soil samples, which is employed to sustain the discussion, have also been provided.

3.1. Evolution of inorganic N content in LF and LC lysimeters

Focusing on the soil extracts, average NO_3^- concentrations remained constant (0.4±0.2 mmol/Kg) until 8 months after pig slurry application (December 4th), when it increased drastically to 12.9±8.3 and 2.8±1.2 mmol/Kg in the LF and LC lysimeters, respectively (**Figure 3AB**). NH_4^+ concentration in soil extracts showed a peak two days after pig slurry application (April 5th) reaching an average of 6.6±5.1 and 3.7±3.1 mmol/Kg in KCl and water extracts, respectively for the LF lysimeters, and 9.8±4.1 and 2.7±1.7 mmol/Kg, respectively for the LC lysimeters (**Figure 3AB**). Afterwards, NH_4^+ concentration decreased below 0.3 mmol/Kg for both types of extracts and experiments and remained constant until the end of the study. NO_2^- in soil water extracts was below the detection limit except two days after pig slurry application (April 5th), showing a concentration of 0.1±0.04 mmol/Kg for both the LF and LC lysimeters (**Table S3**).

Focusing on leachates from the LF ad LC lysimeters, NO_3^- slightly decreased from initial values of 0.5±0.3 mmol/Kg to 0.3±0.04 mmol/Kg at the harvest campaign, performed 3 months after pig

slurry application (July 5th). Nevertheless, the highest NO_3^- concentration (0.9±0.1 and 0.7±0.4 mmol/Kg for the LF and LC lysimeters, respectively) was detected at the last sampling, performed 8 months after pig slurry application (December 4th) (**Figure 3CD**). NH_4^+ and NO_2^- were below detection limit in all leachate samples.

3.2. Evolution of inorganic N compounds isotopic composition

In the LF lysimeters, the $\delta^{15}N_{NO3}$ values of soil extracts and leachates showed parallel variations along the study (**Figure 4A**). Hence, only $\delta^{15}N_{NO3}$ changes for soil extracts are detailed below. Two days after pig slurry application (April 5th), the $\delta^{15}N_{NO3}$ in soil extracts slightly decreased from an average +5.9±0.9 ‰ to +3.8±3.1 ‰ (**Figure 4A**). Two months after pig slurry application, the $\delta^{15}N_{NO3}$ values increased giving an average of +11.5±1.3 ‰ (**Figure 4A**). The highest $\delta^{15}N_{NO3}$ (average of +14.2±2.7 ‰) was measured the following month (July 5th). Then, the values decreased until reaching an average of +8.6±1.0 ‰ at the end of the year (December 4th) (**Figure 4A**). Concerning the $\delta^{18}O_{NO3}$, no significant differences were observed along time either in the NO_3^- from soil extracts or leachates from the LF lysimeters (**Figure 4B**).

The $\delta^{15}N_{NH4}$ from soil KCl extracts also showed remarkable variations along the LF lysimeters study. Two days after pig slurry application (April 5th), the average $\delta^{15}N_{NH4}$ was +16.8±3.0 ‰, which is slightly lower that the $\delta^{15}N_{bulk}$ of pig slurry (+19.6±0.5 ‰) (**Figure 4A**). Two months after pig slurry application (June 7th), the $\delta^{15}N_{NH4}$ values decreased to an average +8.6±5.4 ‰ (**Figure 4A**). One month later, the average increased to +13.5±5.4 ‰ (July 5th), and then it decreased again until reaching +4.7±1.9 ‰ in the last campaign (December 4th).

Slight differences were observed in the $\delta^{15}N_{NO3}$ of soil extracts between the LF and LC lysimeters (**Figure 5A**). The average $\delta^{15}N_{NO3}$ before fertilization (April 3rd) and five months after the harvest (December 4th) was slightly higher in the LC compared to the LF lysimeters (+7.3±1.5 ‰ and

+5.9±0.9 ‰, respectively, in April 3rd and +10.1±2.1 ‰ and +8.6±1.0 ‰, respectively, in December 4th). This trend was also observed for the plant development and harvest campaigns (June 7th and July 5th), showing averages of +12.8±2.4 ‰ and +14.0±1.8 ‰, respectively in the LC lysimeters and of +11.5±1.3 ‰ and +14.2±1.7 ‰, respectively in the LF lysimeters. In contrast, for the campaign performed two days after fertilization (April 5th), which presented a higher range of $\delta^{15}N_{NO3}$ values, the average for lower for the LC compared to LF lysimeters (+1.8±3.9 ‰ and +3.8±3.1 ‰, respectively). Concerning the $\delta^{18}O_{NO3}$, no significant differences were observed along time between the two types of lysimeters (**Figure 5B**). Also, in a dual isotope plot, the evolution of the $\delta^{15}N$ and $\delta^{18}O_{NO3}$ of NO_3^- from soil extracts, showed again a similar trend in both the LF and LC experiments (**Figure 6**). A comparison on the isotopic composition of NH_4^+ between the two types of lysimeters (LF and LC) is not provided since the focus of the paper was on the effect of pig slurry application on leachable NO_3^-.

4. DISCUSSION

4.1. N cycling effects on concentration and isotopic composition of N compounds

Focusing on LF lysimeters (under fallow for six years), NH_4^+ concentration in soil extracts showed a peak two days after pig slurry application (April 5th) (**Figure 3A**). In this sampling campaign, the concomitant detection of NO_2^- and N_2O (**Table S3**) pointed the beginning of nitrification. Both NH_4^+ nitrification and volatilization provoke a $\delta^{15}N_{NH4}$ enrichment (Högberg, 1997; Mariotti et al., 1981; Robinson, 2001), while mineralization leads to decreased $\delta^{15}N_{NH4}$ values with respect to the $\delta^{15}N_{bulk}$ of soil organic matter (SOM) (Craine et al., 2015; Dijkstra et al., 2008, 2006). In contrast, plant uptake might not exert significant isotopic effect on NH_4^+ (Craine et al., 2015; Estrada et al., 2017; Spoelstra et al., 2010). The average $\delta^{15}N_{NH4}$ (+16.8±3.0 ‰) for April 5th, falling below the

$\delta^{15}N_{bulk}$ of pig slurry (+19.6±0.5 ‰) suggests that volatilization did not play an important role (**Figure 4A** as expected since the employed fertilizer was thermally dried and because it was applied below the soil surface. These results also show that a possible heavy isotope enrichment due to nitrification was cancelled out by the effect of SOM mineralization.

Afterwards, NH_4^+ concentration in soil extracts decreased and remained constant until the end of the study (**Figure 3A**). Two months after pig slurry application (June 7th), the $\delta^{15}N_{NH4}$ average values decreased from +16.8±3.0 ‰ to +8.6±5.4 ‰ (**Figure 4A**) due to the preferential release of ^{14}N from SOM during mineralization and given that the $\delta^{15}N_{bulk}$ of soil before fertilizer application was +5.6±0.1 ‰. One month later (July 5th), the average $\delta^{15}N_{NH4}$ increased to +13.5±5.4 ‰, and then it decreased again until reaching +4.7±1.9 ‰ in the last campaign (December 4th). An increase in $\delta^{15}N_{NH4}$ values due to volatilization during the warmest months (June-July) could not be discarded. During those months, the $\delta^{15}N_{NH4}$ could reflect a mix between NH_4^+ from pig slurry and mineralization, which had suffered nitrification and volatilization. At the end of the year, the influence of the $\delta^{15}N_{bulk}$ of pig slurry on the mineralized NH_4^+ was null, and since ^{14}N continued to be preferentially released from SOM, the $\delta^{15}N_{NH4}$ became even lower than soil $\delta^{15}N_{bulk}$ (**Figure 4A**). The NH_4^+ concentration being below the detection limit in all the leachate samples reinforced the NH_4^+ decrease in soil mainly due to nitrification, but also as a result of plant uptake and microbial assimilation and possibly volatilization.

Regarding NO_3^- in soil extracts of the LF lysimeters, its content was low (0.4±0.2 mmol/Kg) until the end of the study (12.9±8.3 mmol/Kg) (**Figure 3AC**). Two days after pig slurry application (April 5th), the $\delta^{15}N_{NO3}$ in soil extracts slightly decreased from an average +5.9±0.9 ‰ to +3.8±3.1 ‰, due to the beginning of nitrification (**Figure 4A**). The generated NO_3^- through nitrification is depleted in $\delta^{15}N$ with respect to the substrate, especially at the beginning of the reaction (Högberg, 1997; Kendall and Aravena, 2000; Robinson, 2001). The measured $\delta^{15}N_{NO2}$ of -31.7±1.3 ‰ in soil extracts,

two days after fertilizer application, proved the significant isotopic fractionation produced during this process (**Table S3**). After pig slurry nitrification was completed, two months after its application, the $\delta^{15}N_{NO3}$ values increased towards the $\delta^{15}N_{bulk}$ of pig slurry (+19.6±0.5 ‰) and the $\delta^{15}N_{NO3}$ of irrigation water (+14.1±1.8 ‰), giving an average of +11.5±1.3 ‰ (**Figure 4A**). The highest $\delta^{15}N_{NO3}$ (average of +14.2±2.7 ‰) was measured the following month (July 5th) and decreased until reaching an average of +8.6±1.0 ‰ at the end of the year (December 4th) (**Figure 4A**). This decrease was due to the higher MIT rates after the harvest, leading to a higher influence of the $\delta^{15}N_{bulk}$ of soil and plants. The average $\delta^{15}N_{bulk}$ measured in the harvested plants of +10.8±0.7 ‰, was consistent with the $\delta^{15}N_{NO3}$ decrease due to mineralization and nitrification of plant debris (**Figure 4A**). A boost in MIT has been already observed in soil N cycling studies after the addition of organic compounds rich in carbon and nitrogen such as wheat straw and oil-seed rape (Shindo and Nishio, 2005; Watkins and Barraclough, 1996) and after intermittent drought periods (Appel, 1998; Sparling et al., 1995). On the other hand, the low $\delta^{15}N_{NO3}$ values measured in December 4th (+8.6±1.0 ‰) suggested that the influence of the $\delta^{15}N_{NO3}$ of irrigation water (+14.1±1.8 ‰) on the soil extracts $\delta^{15}N_{NO3}$ was low even during irrigation periods.

The $\delta^{15}N_{NO3}$ values variation for NO_3^- in leachates of the LF lysimeters was comparable to that of NO_3^- in soil extracts (**Figure 4A**). The increase in NO_3^- content in leachates at the end of the study (**Figure 3C**) was likely produced because: I) during the five previous months the system was not leached, II) plant uptake was not possible after harvest, and III) mineralization and nitrification rates increased since plant debris provided an important source of organic N, as concluded from the soil extracts isotope results.

Concerning the $\delta^{18}O_{NO3}$ in the NO_3^- from soil extracts and leachates from the LF lysimeters (**Figure 4B**), its value depended on the $\delta^{18}O$ of NO_3^- from irrigation water (+6.9 ‰, SD = 0.4) and on the isotopic effect produced during nitrification, which is in turn influenced by the $\delta^{18}O$ of O_2 (+23.5

‰, (Kroopnick and Craig, 1972)) and that of H_2O. The limited variation of $\delta^{18}O_{NO3}$ values along the study indicated a negligible isotopic effect from plant uptake. Also, it pointed that denitrification was not significant along the experimental period even if organic carbon in pig slurry could have induced this process, especially during the spring-summer season due to the higher temperatures (Dawson and Murphy, 1972; Elefsiniotis and Li, 2006; Margalef-Marti et al., 2019). Hence, it was reinforced that the measured NO_2^- and N_2O during the experiment (**Table S3**) were produced from nitrification rather than denitrification (Beeckman et al., 2018; Caranto and Lancaster, 2017; Morley et al., 2008; Wunderlin et al., 2012). This tight dependence of the soil $\delta^{18}O_{NO3}$ on nitrification process has been previously observed by other authors (Kelley et al., 2013; Mengis et al., 2001).

4.2. NO_3^- isotopic composition differences between LF and LC lysimeters

The NO_3^- peak detected in soil extracts at the end of the year was higher in the LF (under fallow for 6 years) compared to the LC lysimeters (soil continuously cultivated and fertilized), showing averages of 12.9±8.3 and 2.8±1.2 mmol/Kg, respectively (**Figure 3AB**). However, slight differences were observed in the $\delta^{15}N_{NO3}$ of soil extracts between the LF and LC lysimeters (**Figure 5A**), and no significant differences were observed for the $\delta^{18}O_{NO3}$ (**Figure 5B**). A lack of large differences between cultivated and non-cultivated soils has been previously related to a mineralization that provokes a higher influence on soil NO_3^- isotopic composition of the $\delta^{15}N_{bulk}$ of soil compared to that of fertilizers (Ostrom et al., 1998). The average $\delta^{15}N_{NO3}$ in the soil extracts was slightly higher in the LC compared to the LF lysimeters except for the campaign performed two days after fertilization (April 5th), which presented a higher range of $\delta^{15}N_{NO3}$ values due to the high nitrification rates after pig slurry application (**Figure 5A**). The higher $\delta^{15}N_{NO3}$ values measured in LC lysimeters were attributed to the previous fertilization with pig slurry of the soil employed for the

LC lysimeters compared to the soil of the LF lysimeters, which was laid fallow for 6 years before the study.

In a dual isotope plot, the evolution of the $\delta^{15}N$ and $\delta^{18}O_{NO3}$ of NO_3^- from soil extracts, showed again a similar trend in both the LF and LC experiments. The initial values measured for NO_3^- from soil extracts fitted well in the soil theoretical area (**Figure 6**). Therefore, the use of a simplified model to calculate de $\delta^{18}O_{NO3}$ range by considering a ratio 2:1 for the oxygen incorporation from H_2O and O_2, respectively, seemed to be appropriate under our experimental conditions. Two days after fertilizer application, since nitrification of pig slurry was still limited, the $\delta^{15}N_{NO3}$ of soil extracts decreased. When pig manure nitrification was likely completed after two months, the $\delta^{15}N_{NO3}$ increased towards the $\delta^{15}N_{bulk}$ of pig slurry and the $\delta^{15}N_{NO3}$ of irrigation water, in the two types of lysimeters. The $\delta^{18}O_{NO3}$ values were slightly higher than the theoretical values for nitrification, this can be either by the influence of irrigation water or by higher values of $\delta^{18}O_{H2O}$ due to evaporation since the highest temperatures were recorded on June and July. Five months after the harvest, the $\delta^{15}N_{NO3}$ values in the soil extracts decreased towards the initial values of the soil $\delta^{15}N_{bulk}$ likely due to the incorporation of plant debris in the soil organic pool by MIT (**Figure 6**). Despite this decrease of $\delta^{15}N_{NO3}$ towards the initial soil values, a slight but systematic shift towards higher $\delta^{15}N_{NO3}$ values between the initial and final isotopic composition of NO_3^- for both LF and LC experiments was observed (**Figure 6**). This shift was attributed to an effect of fertilization, which is consistent with the higher initial $\delta^{15}N_{NO3}$ values measured in the soil extracts from the LC lysimeters (previously cultivated and fertilized) compared to LF lysimeters (previously laid fallow). This modification of the $\delta^{15}N_{NO3}$ values of soil NO_3^- could lead to a modification of the $\delta^{15}N_{bulk}$ of soil over time if a given agricultural land is periodically fertilized.

4.3. Implications of fertilizer application on the isotopic composition of NO_3^- leached to groundwater

When using isotope approaches to determine groundwater NO_3^- pollution sources, the $\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$ determined in groundwater samples is usually compared in dual isotope plots to the isotopic composition range of potential NO_3^- sources in the study site, such as in **Figure 6.**

The $\delta^{15}N_{NO3}$ range for sources is preferably specifically determined on the studied area or alternatively obtained from studies performed nearby and reported in the literature (Puig et al., 2013; Vitòria et al., 2008; Widory et al., 2005). However, as observed in our study, transformation processes occurring to NO_3^- before being leached to aquifers, can modify its isotopic composition. The accuracy of the information provided by this approach depends on the influence of transformation processes occurring to NO_3^- sources before the contaminant is leached to aquifers. This influence could be even higher at field-scale where increased thickness of the vadose zone compared to our lysimeter experiment could increase the N compounds retention time and N cycling.

If NO_3^- is leached to groundwater shortly after application of NH_4^+ fertilizers, when nitrification is uncompleted, it presents lower $\delta^{15}N_{NO3}$ values than its source. Just when nitrification is complete, the exported NO_3^- presents $\delta^{15}N_{NO3}$ values closer to the $\delta^{15}N$ of the NH_4^+ source. The $\delta^{15}N_{NO3}$ values can also be increased due to plant uptake and immobilization and due to denitrification, although in our study denitrification did not seem to play an important role. Finally, after harvest, the $\delta^{15}N_{bulk}$ of pig slurry can became masked by the $\delta^{15}N_{bulk}$ of SOM and therefore, the $\delta^{15}N_{NO3}$ values of leached NO_3^- can decrease again. In addition, under our experimental conditions, fertilization with pig slurry caused an increase of the $\delta^{15}N_{NO3}$ of NO_3^- retained in soil over time in all the lysimeters. Given that the amount of N from pig slurry applied in the present study was low (100 Kg N ha^{-1} $year^{-1}$) compared to the quantities applied at field-scale (up to 170 Kg N ha^{-1} $year^{-1}$ in

nitrate vulnerable zones (NVZ) and up to 250 Kg N ha^{-1} $year^{-1}$ in non-vulnerable areas), the increase in the soil $\delta^{15}N_{NO3}$ due to the influence of $\delta^{15}N_{bulk}$ of pig slurry in agricultural areas could be even higher. Since a direct comparison of the isotopic composition of the source (in this case pig slurry) and the leached NO_3^- might not be valid, to assess the source of NO_3^- inputs into groundwater, we should consider the enlarged $\delta^{15}N_{NO3}$ range due to past fertilization events to define the SOM boxes. For this reason, in studies aiming to trace groundwater NO_3^- pollution sources in rural areas by using an isotopic approach it can be useful to consider the fertilization history of each setting and to analyze the $\delta^{15}N$ of the soil, since it could mask the influence of the N applied through fertilization.

Concerning the $\delta^{18}O_{NO3}$ data, the range for NH_4^+ sources is usually calculated using the simplified model showing that after nitrification the $\delta^{18}O_{NO3}$ = 2/3 $\delta^{18}O_{H2O}$ and 1/3 $\delta^{18}O_{O2}$, which seemed to be appropriate under our experimental conditions. However, as shown by our results, when using this approach, the presence of NO_3^- in the irrigation water should be considered. Also, the isotopic effects associated to water evaporation, oxygen atoms incorporation during nitrification and $\delta^{18}O_{NO2}$ equilibration with $\delta^{18}O_{H2O}$, should not be neglected systematically. In summary, the results of this study highlighted that, as previously recommended by other authors, as much hydrochemical data as possible, including seasonal variations, land use and livestock activities must be coupled to isotopic data in order to provide an appropriate interpretation of groundwater pollution sources.

4. CONCLUSIONS

After pig slurry was applied as fertilizer in lysimeters seeded with barley, NH_4^+ was nitrified rapidly in all the lysimeters (LF and LC). The generated NO_3^- was uptaken by plants and immobilized, allowing the closure of the MIT process (after SOM mineralization). Besides, few N losses were

produced due to the controlled leaching, while volatilization and denitrification did not seem to play an important role. As a result of the beginning of nitrification of pig slurry, the initial $\delta^{15}N_{NO3}$ of soil extracts and leachates first decreased and after two months started to increase towards the $\delta^{15}N_{bulk}$ of pig slurry and the $\delta^{15}N_{NO3}$ of irrigation water. The $\delta^{15}N_{NO3}$ also increased due to plant uptake and immobilization. Five months after harvest, the $\delta^{15}N_{NO3}$ values in soil extracts decreased again due to a higher rate of SOM and plant debris mineralization and subsequent nitrification. As a result, by the end of the agricultural year the $\delta^{15}N_{bulk}$ of pig slurry was masked by the $\delta^{15}N_{bulk}$ of plant debris and soil. Nevertheless, pig slurry application led to $\delta^{15}N_{NO3}$ values in the soil NO_3^- extracts and leachates slightly above the initial ones. The lysimeters with soil previously under fallow or continuously cropped showed a similar trend. The latter ones were useful to reinforce the occurrence of this $\delta^{15}N_{NO3}$ increase due to fertilization also at field-scale. Modification of the $\delta^{15}N_{bulk}$ of the soil after decades of fertilizer application should be considered when using the $\delta^{15}N_{NO3}$ to trace sources of groundwater NO_3^- pollution. On the other hand, the $\delta^{18}O$ of soil nitrate mainly depended on the $\delta^{18}O$ of NO_3^- from irrigation water, the $\delta^{18}O$ of H_2O and the $\delta^{18}O$ of O_2, due to nitrification of NH_4^+ from pig slurry. Consequently, isotopic studies aiming to trace groundwater NO_3^- pollution must consider the fertilization history of a given setting and the analysis of $\delta^{15}N$ of soil and $\delta^{18}O$ of groundwater, for an improved accuracy of results interpretation.

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Table 1. Experimental schedule. The lysimeters were established on March 23rd, the plants were sown on April 3rd and harvested on July 3rd.

Sampling date	Days after setup	Experimental stage	Samples
03/04/2017	11	Pre-fertilization	Soil + leachate
05/04/2017	13	Post-fertilization	Soil + leachate + gas
07/06/2017	76	Developed plant	Soil + leachate
05/07/2017	104	Harvest	Soil + leachate + gas
04/12/2017	256	Bare soil	Soil + leachate + gas

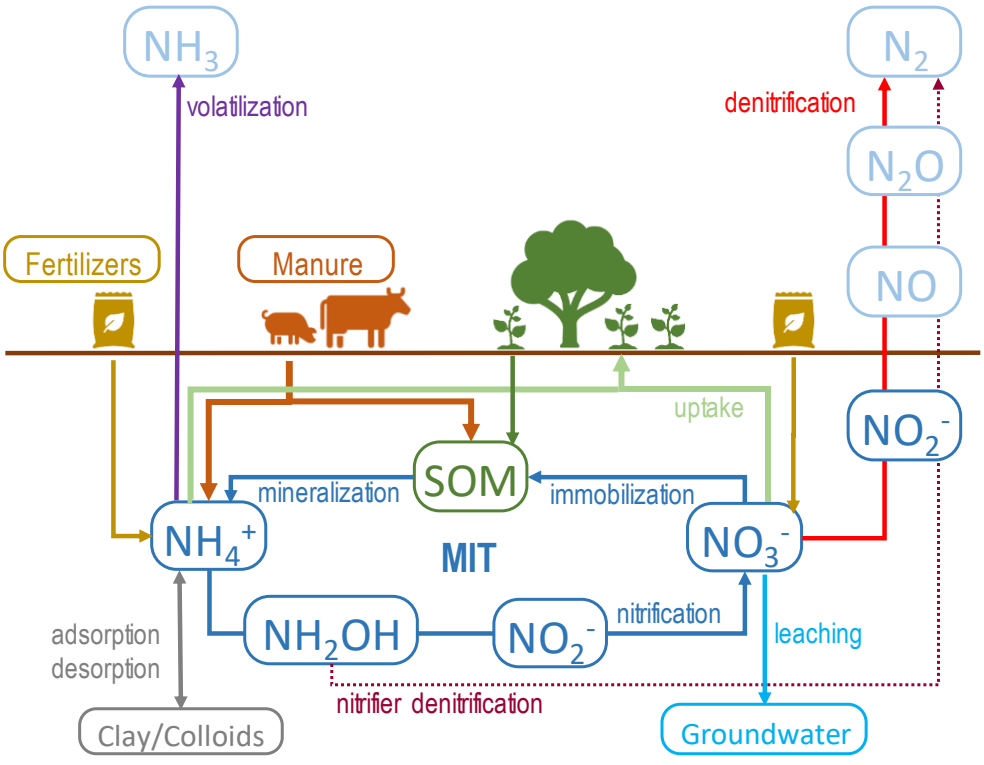

Figure 1. N cycling in non-saturated zones.

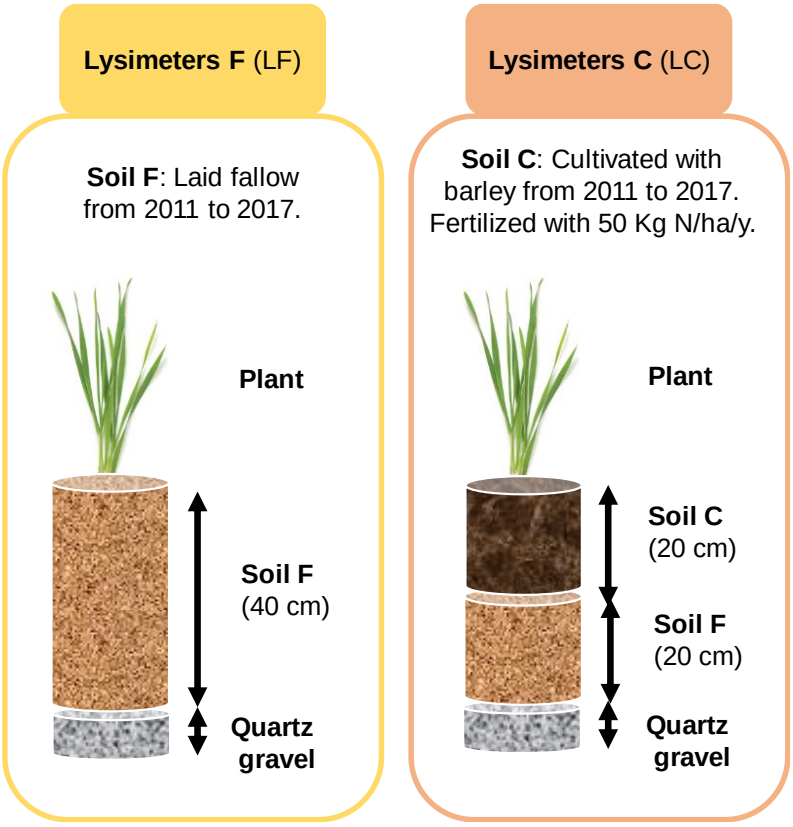

Figure 2. Lysimeter schemes. The experimental setup included five replicates of each of these two types of lysimeters established according to the soil content.

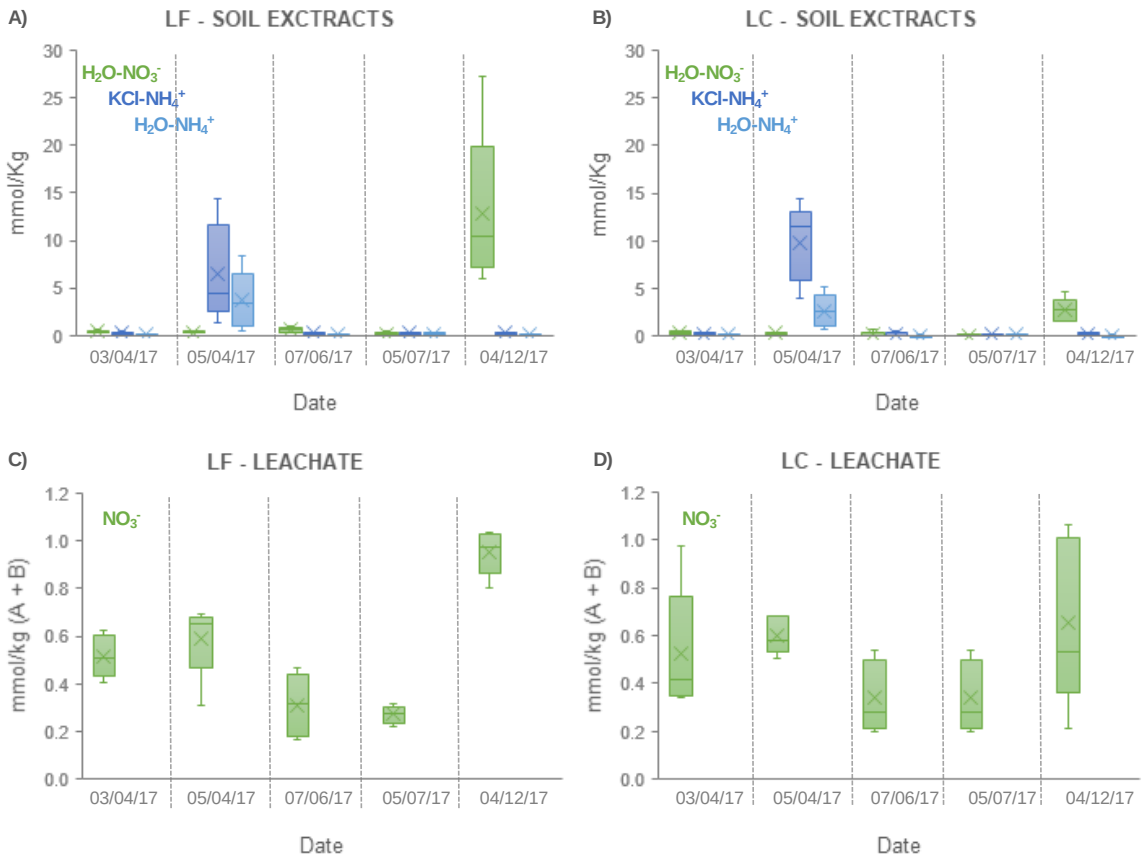

Figure 3. Concentration of NO_3^- and NH_4^+ retained in the soil (A + B) and leached (C + D) over time. Each boxplot involves five replicates of each type of lysimeter, namely LF (A + C) or LC (B + D). In the legend, the type of soil extract is expressed as KCl or H_2O. NH_4^+ was below detection limit in all leachate samples.

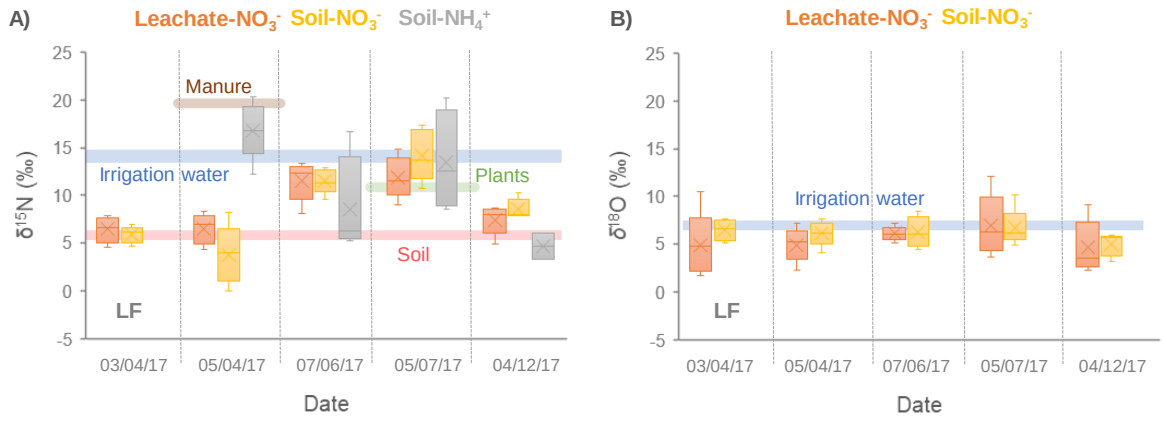

Figure 4. $\delta^{15}N_{NO3}$ (A) and $\delta^{18}O_{NO3}$ (B) in NO_3^- and NH_4^+ retained in the soil and leached during the LF lysimeter experiments. Each boxplot involves five replicates. The average isotopic composition measured for soil (bulk N), manure (bulk N), irrigation water (NO_3^-), and harvested plants (bulk N) is also shown.

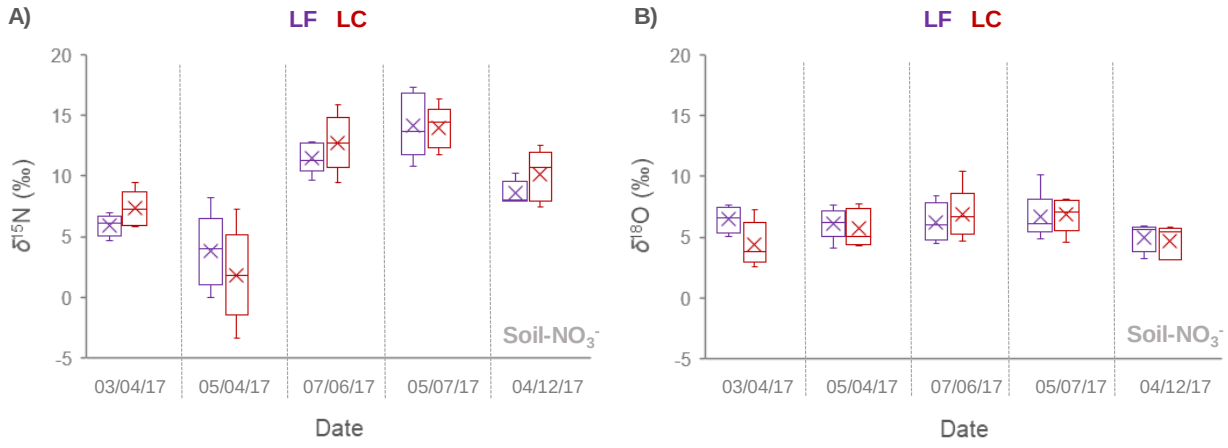

Figure 5. $\delta^{15}N_{NO3}$ (A) and $\delta^{18}O_{NO3}$ (B) of NO_3^- retained in the soil in the LF and LC lysimeters. Each boxplot involves five replicates.

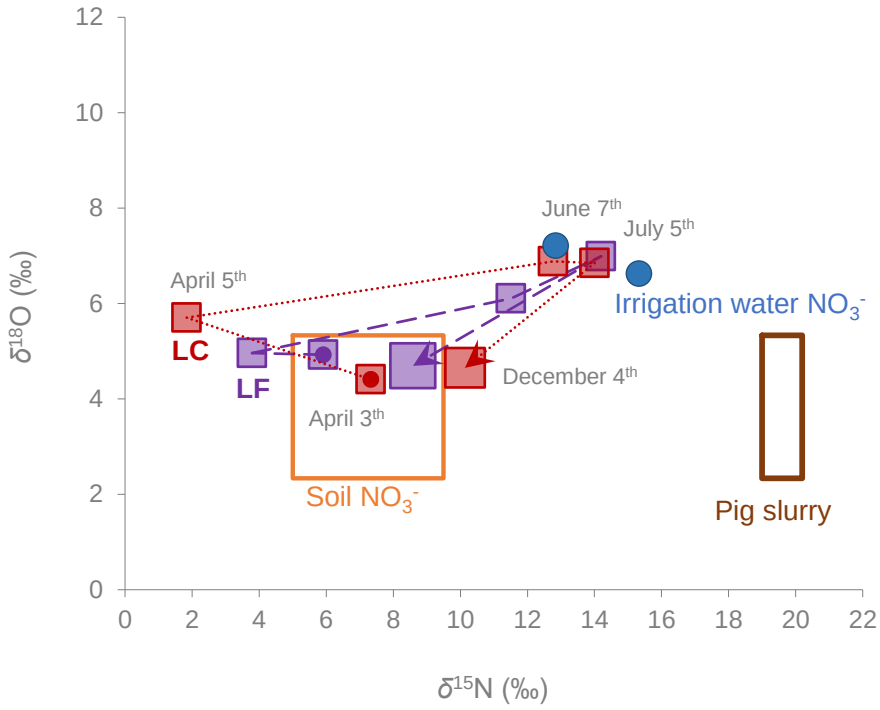

Figure 6. Isotopic composition evolution of NO_3^- from soil extracts in LF and LC lysimeters. The sampling campaigns are joined by a line, where circles and arrows reflect the first and last one, respectively. The isotopic composition of irrigation water, pig slurry and soil are shown. For the soil NO_3^- box, the $\delta^{15}N$ involves the range of measured initial values in both the LF and LC lysimeters, while for pig slurry it corresponds to its measured bulk $\delta^{15}N$ considering the standard deviation. For both, the $\delta^{18}O$ has been calculated following: $\delta^{18}O_{NO3} = 1/3\delta^{18}O_2 + 2/3\delta^{18}O_{H2O}$. The $\delta^{18}O_2$ = +23.5 ‰ (Kroopnick and Craig, 1972) and the $\delta^{18}O_{H2O}$ ranges between -8 and -3.5 ‰ in the study area (Otero et al., 2009; Puig et al., 2017).