Agricultural and urban delivered nitrate pollution input to Mediterranean temporary freshwaters

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Abstract

Nitrate dual stable isotopes ($\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$) have proven to be a powerful technique to trace nitrate sources and transformations in freshwater systems worldwide. However, most studies have focused on perennial systems, and less is known about intermittent ones. The impacts of intensive agricultural practices and wastewaters in Doñana (SW Spain), an iconic Mediterranean temporary wetland protected as a UNESCO World Heritage Site, were quantified using stable isotope mixing models in a Bayesian framework under different denitrification scenarios. We aimed to identify the main nitrate sources and transformation processes in surface waters of interconnected temporary streams, ponds and marshes, and link them with the main human pressures in the watershed (e.g. intensive fruticulture, urban wastewaters). We measured nitrate (NO_3^-) concentrations and stable isotopes ($\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$) in water samples collected during different periods over two years (2015-2016). Most sites showed coupled increases of nitrate isotopic values ($\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$), which were higher than reference values of any possible sources (e.g. synthetic/organic fertilizers and wastewaters), indicating fractionations typical of denitrification processes. The main nitrate sources to the watershed were linked to agricultural practices and the use of synthetic fertilizers, but further investigations in other transformation processes that occur simultaneously should be evaluated. These results highlight an important nitrate removal capacity (i.e. denitrification) of the system, which may positively contribute to natural resilience against eutrophication. However, given the high intra and interannual hydrological fluctuations of Mediterranean aquatic systems, future studies on the relative contribution of nitrate sources and processes should increase spatio-temporal resolution of water sampling, and include measurements of groundwater and interstitial water as well as surface water.

Key words

Nitrate source; stable isotopes; fertilizers; wastewater; denitrification

Highlights

- Dual nitrate isotope approach was used in temporary freshwater systems
- $\delta^{15}N$ and $\delta^{18}O$ reflected different sources, transformations and mixing processes
- Nitrate isotopes confirmed anthropogenic nitrate pollution in the Doñana watershed
- Coupled increase of $\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$ was linked to denitrification processes
- Agricultural land uses contributed largely to the nitrate pollution in the watershed

1. Introduction

Anthropogenic nitrate pollution is a worldwide issue causing negative impacts in surface and groundwater systems, particularly in watersheds with intensive use of agricultural fertilizers (Carpenter et al. 1998; Erisman et al. 2013; Mekonnen et al. 2015). Despite agriculture being one of the major causes of anthropogenic nitrate pollution in aquatic systems, other diffuse and point sources are involved such as domestic or industrial wastewaters, atmospheric deposition and animal farming wastes. Excessive nitrate export into aquatic systems causes eutrophication, with subsequent loss of aquatic organisms and biodiversity reduction (Smith 2003). Nitrate pollution can also lead to toxic effects in both aquatic organisms and human health, mainly related to inhibition of oxygen-carrying capacity of certain pigments (e.g. hemoglobin) and endocrine disruption (Camargo and Alonso, 2006; Poulsen et al., 2018). Improving knowledge about nitrate sources and transformation processes at the watershed scale is critical for a precise understanding of nitrate impacts and management in aquatic systems under anthropogenic pressure (Causse et al., 2015).

Multiple actions have been taken worldwide to reduce and prevent negative impacts of nitrate pollution to humans and the environment. For example, according to the European Nitrate Directive 91/676/EEC (EEC, 1991), each member State should define nitrate vulnerable zones and apply adequate agricultural practices to reduce the impact of fertilizers in surface and groundwaters. Moreover, the Water Framework Directive 2000/60/EC (EC, 2000) requires that nitrate levels in any surface waters within the European Union should not exceed 50 mg L^{-1} NO_3^-. However, despite these and other relevant Directives (EEC, 1991b; EC, 1998; EC, 2006), nitrate still remains a significant pollutant in European freshwater bodies (Mekonnen et al. 2015; EEA, 2018).

This is the case of Doñana World Heritage Site (SW Spain), an iconic Mediterranean wetland, which is currently under threat due to different human pressures in the watershed (Camacho-Muñoz et al., 2013; Green et al., 2017, 2018). According to the Nitrate Directive (EEC, 1991) and its corresponding transposition into the Spanish legislation (Royal Decree 261/1996), part of the surface and groundwaters of the Doñana wetland were designated as "nitrate vulnerable zones" by the Andalusian Government (Decree 36/2008), with the aim of reducing the impact of the ongoing nitrate pollution due to the intensification of agriculture in the watershed (Rodríguez and Stefano 2012, WWF 2016). Nitrate pollution is a major threat to surface and groundwater of the Doñana wetland related to the excessive use of fertilizers in agriculture and the discharge of poorly treated wastewaters into streams (Serrano et al., 2006; Paredes et al., 2019). In several streams, high concentrations of nitrites and ammonia are toxic to many organisms and are incompatible with nature conservation (Paredes et al., unpublished results). Intensive groundwater pumping for irrigation has resulted in a decrease of natural water discharge into streams, enhancing flow intermittency and limiting the dilution capacity of surface waters (Guardiola et al. 2011, Manzano et al. 2013). The strong temporal variability in precipitation and the prolonged arid period in summer, typical of the Mediterranean region, result in a highly irregular frequency of nitrate inputs into the streams entering Doñana. This intermittent and irregular nitrate loading into the aquatic system complicates the monitoring of nitrate inputs and in-stream biogeochemistry.

Stable isotope techniques can be used to trace nitrate pollution sources and nitrogen cycling in aquatic ecosystems (Mayer et al. 2002; Nestler et al. 2011; Kaushal et al., 2011; Soto et al. 2019). Ratios of stable N isotopes ($^{15}N/^{14}N$, expressed as $\delta^{15}N$ in ‰) vary among different nitrate sources. Nitrate derived from human wastewaters or manure are usually more enriched in $\delta^{15}N_{NO3}$ (+10 to +20 ‰) than nitrate from most synthetic fertilizers (-3 to +3 ‰), atmospheric deposition (-15 to +7 ‰) or natural soils (-6 to +9 ‰) (Kendall 1998; Bateman and Kelly, 2007). However, distinguishing between nitrate sources with wide and overlapping $\delta^{15}N_{NO3}$ ranges (e.g. synthetic fertilizers vs. atmospheric deposition), or identifying the influence of different transformation processes is not always possible if only $\delta^{15}N_{NO3}$ is used.

A simultaneous dual nitrate isotope approach ($\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$) offers the advantage of a more precise distinction between sources and processes, since $\delta^{18}O_{NO3}$ (i.e. the ratio $^{18}O/^{16}O$)

shows a greater resolution for the origin of certain sources that overlap for $\delta^{15}N_{NO3}$ (Craine et al., 2015). For example, while synthetic fertilizers and nitrate atmospheric deposition show overlapping $\delta^{15}N_{NO3}$, the $\delta^{18}O_{NO3}$ values of synthetic fertilizers (around +23 ‰) (Michalski et al., 2015) are considerably higher than those of atmospheric deposition (ranging from +60 ‰ to +98 ‰) (Kendall et al., 2008). Furthermore, overlapping of $\delta^{15}N_{NO3}$ values may also occur when there are changes in $\delta^{15}N_{NO3}$ for one of the sources due to transformation processes (e.g. nitrification, denitrification, mineralization, ammonia volatilization or assimilation) (Kendall et al., 2008). Hence, nitrate removal by denitrification or assimilation may produce $^{15}N_{NO3}$ enrichment in the residual nitrate of an originally $^{15}N_{NO3}$-depleted source (e.g. synthetic fertilizers), which can make it undistinguishable from another $^{15}N_{NO3}$ enriched, untransformed source (e.g. human wastewaters) (Kendall, 1998). Such fractionating processes also produce $^{18}O_{NO3}$ enrichment, resulting in comparatively higher $\delta^{18}O_{NO3}$ values in the residual nitrate than in the $^{15}N_{NO3}$ enriched, untransformed sources (Mariotti et al., 1988; Granger et al., 2004; Søvik and Mørkved, 2008). Thus, nitrate isotopic composition in most aquatic systems is the result of simultaneous transformations and nitrate source mixing which are often undistinguishable from each other without the application of multi-isotopic approaches such as the dual nitrate isotope approach (Kendall et al., 2008, Otero et al., 2009, Yue et al., 2017). The latter has been used to study nitrate transport and transformations in numerous watersheds worldwide. However, there is a lack of isotope studies in arid and semiarid areas subjected to warm temperatures, strong rainfall variation and water scarcity, such as the Doñana wetland (Custodio et al., 2009; Tortosa et al., 2011, Wong et al., 2018). Given climate change and the ongoing nutrient inputs from anthropogenic activities, vulnerability to eutrophication is expected to continue increasing in Mediterranean wetlands (Green et al. 2017). In this context, it is critical to improve our understanding of natural nitrate removal processes (e.g. denitrification) which can reduce eutrophication.

In this study we aimed to identify (and quantify) the main anthropogenic nitrate sources and specific transformation processes in the Doñana watershed using the dual nitrate stable isotope approach ($\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$). In combination with nitrate concentrations in surface waters and land use data, we expect to gain information on the relationships between agricultural practices and the nitrate input into these Mediterranean streams. Given results for $\delta^{15}N$ in emergent aquatic vegetation in our study area (Paredes et al. 2019), we hypothesized that sampling sites affected by upstream wastewater treatment plant discharges would show higher inputs from urban sources than sites mainly affected by agricultural practices. Moreover, we hypothesized that biogeochemical processes occurring either in the water column, sediments, riparian zone or groundwater would partly explain nitrate isotopic variability transport and transformation in our study area. Finally, mixing of surface waters with different nitrate isotopic compositions may explain the remaining variability of the nitrate isotopic values.

2. Materials and methods

2.1. Study area

Doñana (SW Spain, Fig. 1) is one of the most important wetland complexes in Europe and in the Mediterranean region, and includes an extensive seasonal marsh partly protected within a UNESCO World Heritage Site (WHS) (Green et al., 2017, 2018). The marsh is flooded by direct precipitation and by a series of temporary entry streams whose flow is determined by strong seasonal and interannual rainfall variations typical of a sub-humid Mediterranean climate, as well as anthropogenic pressure such as groundwater abstraction for agriculture (Green et al. 2017). Water quality in the entry streams is poor due to the influence of agricultural inputs and urban wastewaters (Paredes et al. 2019). In this region, mean annual precipitation is 550 mm, ranging from 170 to 1000 mm (Díaz-Delgado et al., 2016).

We studied the most important streams ("La Rocina", "El Partido" and "Los Sotos") feeding the Doñana marsh in the north-west corner, which drain highly anthropized watersheds, affected to a varying degree by intensive agriculture and urban areas. It is also likely that untreated wastewater from agricultural workers enters all three catchments. We also studied two ponds: (1) the "Laguna de los Mimbrales" (PDmim) is located in Los Sotos catchment within the Doñana National Park (area = 3ha; max. depth = 0.6m; trophic status = eutrophic). It is an artificial, temporary pond fed by both surface and groundwater. It was constructed in 2002 to retain agricultural-derived sediments and pollutants from surface water before it enters the Doñana marsh (Urdiales, 1998; MMA, 2001); (2) the "Laguna Primera de Palos" (PDpal) is located 35 km away to the north-west of Doñana (area = 17 ha; max. depth = 3m; trophic status = mesotrophic to eutrophic). This is the only permanent system in this study, fed by groundwater and intermittent surface water supplies. We used this pond as a reference site because its entire catchment is dedicated to the same land use (i.e. greenhouse berry crops). Finally, we studied the point where both the Rocina and Partido streams reach the marsh at the north-west area.

2.2. Sample collection

We collected 29 surface water samples using acid-washed plastic containers of 1L each at nine different locations (six streams, two ponds and one marsh) (Fig.1) across the Doñana watershed between February and June during 2015 and 2016. We took unequal number of samples from each site. At the end of each sampling day, we transported the samples to the laboratory under refrigerated conditions and immediately filtered them through FILTER-LAB MFV5047 glass-fiber filters (0.45µm pore size) using a low-pressure vacuum pump. We stored all filtered samples in the freezer (-20°C) prior to isotopic analyses ($\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$) and NO_3^- concentration measurements.

Although nitrate sources were generally uncertain, three sampling sites (PDpal, WWTP_roc and WWTP_alm) were assumed to receive nitrate predominantly from one specific source, this being the criterion we used to consider them as "reference sites" (Fig. 1). PDpal receives surface and groundwater affected by chemical fertilizers used in the surrounding intensive greenhouse strawberry production, especially between October and June when only chemical fertilizers are applied (mainly ammonium nitrate, potassium nitrate, mono ammonium phosphate and calcium nitrate). The other two sites (WWTP_roc and WWTP_alm) are directly affected by the discharge of urban wastewater treatment plants (WWTP). Firstly, we collected water at the outflow of El Rocío's WWTP (WWTP_roc). El Rocío's WWTP treats the urban wastewaters of El Rocío village, site of a major religious pilgrimage, with 1,371 habitants (IECA, 2018) but many more people visit on the weekends and particularly during the annual pilgrimage (held 50 days after Easter) when approximately one million people visit the village over a week. Secondly, we collected a sample immediately downstream of Almonte's WWTP (WWTP_alm) where the treated wastewaters were already mixed with the Partido stream water. Almonte's WWTP is the largest in our study area, treating the wastewaters of Almonte and Rociana del Condado towns, with 19,017 and 7,594 inhabitants, respectively (Junta de Andalucía, 2017). Upstream of Almonte's WWTP, El Partido stream also receives the urban treated wastewaters from Bollullos Par del Condado WWTP (14,030 hab). The other six sampling sites were located within the north western area of the Doñana watershed (Fig. 1). Nitrate sources are uncertain at these sites since different anthropogenic point and diffuse nitrate inputs are contributing simultaneously to their surface waters. All sites are influenced by both surface and groundwaters since they are located on a sandy permeable terrain connected to the underlying unconfined aquifer, except for "El Rocío" marsh (MRSro) which is located over silty-clay impermeable deposits where the aquifer is confined below (Serrano et al. 2006).

Besides nitrate isotopes and concentrations, we also determined chlorophyll-*a* concentrations from surface water using acetone extraction (UNESCO, 1966), and recorded dissolved oxygen (DO) and water temperature at 5-20 cm below the surface at each site with a WTW (Weilheim, Germany) Multi-340i handheld meter.

2.3. Stable isotope and nitrate concentration analyses

We measured the nitrate concentration (NO_3^-) using standard colorimetric methods (ISO 13395:1996). We also measured NO_2^- (ISO 13395:1996) and NH_4^+ (ISO 11732:2005) concentrations. All dissolved inorganic N measurements were carried out on a multi-channel SEAL Analytical AA3 AutoAnalyzer (Norderstedt, Germany), at the Laboratory of Aquatic Ecology of EBD-CSIC (Seville, Spain). Limits of detection for the analytical methods were 0.004 µmol L^{-1} for N-NO_3^- and N-NO_2^- and 0.040 µmol L^{-1} for N-NH_4^+.

We measured $\delta^{15}N$ and $\delta^{18}O$ values of dissolved nitrate using the Cd reduction method proposed by McIlvin et al. (2005). This method (ISO 9001: 2008 certification) is based on the reduction of nitrate to N_2O (g) by Cd and its subsequent pre-concentration by means of a gas purification system connected to an IRMS, to perform, once concentrated, the measurement of the isotopic ratio of the $\delta^{18}O_{N2O}$ and $\delta^{15}N_{N2O}$. The N_2O was analyzed using a Pre-Con coupled to a Finnigan MAT 253 Isotope Ratio Mass Spectrometer (IRMS) (Thermo Scientific). In the case of the presence of nitrite, sulfamic acid was added to the water samples to remove NO_2^-, in order to avoid any interference in the measurement of the nitrogen and oxygen isotopic composition of dissolved nitrate (Granger and Sigman, 2008). Following Coplen (2011), several international and laboratory (CCiT) standards were interspersed among samples to normalize the results. For the $\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$ analysis the employed standards were USGS-32, USGS-34, USGS-35 and results were referenced to the international scale (AIR for $\delta^{15}N$ and V-SMOW for $\delta^{18}O$). The reproducibility (1σ) of the samples, calculated from the standards systematically interspersed in the analytical batches, was ±1.0 ‰ for $\delta^{15}N_{NO3}$ and ±1.5 ‰ for $\delta^{18}O_{NO3}$. Samples for the isotopic analyses were processed at the "MAIMA" Research group laboratory and analyzed at the "Centres Científics i Tecnològics" of the "Universitat de Barcelona" (UB).

2.4. Identification of nitrate sources

To identify nitrate sources we plotted all the measured $\delta^{15}N_{NO3}$ vs. $\delta^{18}O_{NO3}$ values together with reference isotope values from major potential watershed sources: chemical fertilizers (Vitòria et al., 2004), soil nitrate from nitrification and wastewaters and/or organic fertilizers from Widory et al. (2004) (Table SP1, Sup. Mat.). According to Kendall et al. (2008) during nitrification there is a large fractionation in the ^{15}N during the transformation of NH_4^+ to NO_2^-, ($\varepsilon_{NH4/NO2}$= -38 to -14 ‰) and negligible ^{15}N fractionation in the transformation of NO_2^-, to NO_3^-, but in N-limited systems, since the transformation is complete, the final NO_2^-, and therefore NO_3^-, will show a small ^{15}N isotopic effect. We have assumed a complete nitrification, and therefore the average $\delta^{15}N$ considered for ammonium derived fertilizers ranges between -5 and +5 ‰. Regarding oxygen, nitrification can incorporate two atoms of O from water and one atom of O from O_2 in some cases, and all oxygen atoms from water in others (Snider et al 2010, Venkiteswaran et al., 2019). Additionally, the $\delta^{18}O_{O2}$ values vary depending on the productivity of the system (Wassenaar et al 2010, Venkiteswaran et al 2015), and its analysis (see Wassenaar and Koehler 1999 for details) may be of great relevance for a better interpretation of nitrate isotope results. In our case, for simplicity, the expected $\delta^{18}O_{NO3}$ derived from nitrification of NH_4^+ (either from soil, manure or fertilizer) was calculated following *Eq. 1* (Mayer et al. 2001), and using the range of $\delta^{18}O_{H2O}$ values of the studied samples (Table SP2, Sup. Mat.) and a $\delta^{18}O_{O2}$ of +23.5‰ (Kroopnick and Craig, 1972).

$$\delta^{18}O_{NO3}=\frac{1}{3}\cdot\delta^{18}O_{O2}+\frac{2}{3}\delta^{18}O_{H2O} \qquad \textit{Eq. 1}$$

Atmospheric deposition (dry/wet) is also a likely pathway of nitrate inputs (Kendall et al., 2008), we did therefore include it as a reference source; however: (1) we expected a low contribution to the streams compared to nitrate derived from intensive human activities in the watershed (agriculture and urban areas) and (2) wet deposition would be limited due to generally low precipitation in the region (annual average= 550 mm).

We estimated proportional contributions of these nitrate sources from the watershed into the dissolved riverine nitrate of each sampling location by using dual isotope values introduced into Bayesian isotope mixing model approach (MixSIAR; Stock and Semmens 2016, Moore and Semmens 2008). Potential sources and their expected isotope values are described in Table SP1. We combined the isotopic composition of primary sources of soil and fertilizer NH_4^+ due to their overlap in isotope values and their subsequent lack of source discrimination. The variable 'site' was included as fixed effect into the models of three chains of 100,000 iterations, a burn-in of 50,000 and a thinning of 50. Using this modelling approach, three scenarios were evaluated because denitrification processes seem to be a main driver of isotopic variation in these temporary systems (see Results and Discussion). For this reason, fractionation factors (and SD) associated to denitrification processes at a level of 25%, 50% and 75% (± 10%) of the fraction denitrified according to the model below (see Section 2.5.) were used in each scenario.

2.5. Denitrification processes

Denitrification typically produces a coupled increase in $\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$, with a slope ranging from 0.5 to 1 (Böttcher et al., 1990; Wunderlich et al., 2013). Since the initial isotopic composition can be different depending on nitrate origin, we roughly estimated how this process could shift the isotopic values of the main potential sources in our system by representing two shaded areas in the $\delta^{15}N_{NO3}$ vs. $\delta^{18}O_{NO3}$ graph (Fig.2). Each shaded area corresponded to the theoretical values of samples that have undergone denitrification with (1) inorganic fertilizer/soil nitrate origin (green) and (2) sewage/manure origin (light purple). Since denitrification slopes may differ, these two theoretical areas partially overlap.

Denitrification processes can be modelled using a Rayleigh distillation model (*Eq. 2*), following Mariotti et al. (1981). The equation can be simplified and expressed as:

$$\varepsilon_{P/S} = ln\frac{\delta_s - \delta_{s,0}}{\ln f} \qquad Eq.\ 2,$$

where $\varepsilon_{P/S}$ is the isotopic fractionation, δ_s and $\delta_{s,0}$ are the isotopic composition of the residual (s) and initial (s,0) nitrate, and f is the remaining nitrate fraction. Both $\varepsilon^{18}N_{NO3}$ and $\varepsilon^{18}O_{NO3}$ can be modeled.

Denitrification percentages were estimated using an average isotopic fractionation of $\varepsilon^{15}N_{NO3/N2}$ = -15‰ (Böttcher et al., 1990) and a $\varepsilon^{18}O_{NO3/N2}$ /$\varepsilon^{15}N_{NO3/N2}$ ratio of 0.7. Different denitrification models were calculated based on the initial isotope values ($\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$) of the original nitrate source:

(1) For water samples affected by nitrate inputs from synthetic fertilizers: since the original NO_3^- could be a mixing of sources such as nitrified NH_4^+ fertilizers and NO_3^- fertilizers, we performed two different models using the same initial $\delta^{15}N_{NO3}$ value (+4‰) but two different $\delta^{18}O_{NO3}$ values (+6‰ and +11‰, respectively). The latter initial values ($\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$) represented different proportions of NO_3^- fertilizers vs. nitrified NH_4^+ fertilizers (Fig. 4) based on values reported in an area with greenhouse cultivation, a predominant use of synthetic fertilizers and no denitrification, and that had an average $\delta^{15}N_{NO3}$ of +4‰ and $\delta^{18}O_{NO3}$ values up to +11‰ (Vitòria et al., 2004).

(2) For water samples affected by nitrate inputs derived from wastewaters: since some of these samples also followed a denitrification trend (WWTP_alm, WWTP_roc), two denitrification models were calculated using different initial $\delta^{15}N_{NO3}$ (+7‰ and +16‰, representing the bibliographic range for wastewater) and a $\delta^{18}O_{NO3}$ of +6‰ (representing the

upper value of nitrification of ammonium in the study area, in order to avoid overestimating the denitrification percentage).

2.6. Effects of seasonal changes in $\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$

To observe whether there was any temporal trend in the nitrate isotopic composition and concentrations over our study period (February to June), we pooled the data for all sites and both years (2015 and 2016) by month (Fig. 3). Additionally, we plotted the isotopic values ($\delta^{15}N_{NO3,}$ $\delta^{18}O_{NO3}$) of each site collected in 2016 together with the precipitation and temperature data. We only represented the isotopic data of those sampling sites with two or more samples collected during 2016 (Table 2). Meteorological data was collected from the Almonte Meteorological Station (37º 08' 53" N, 06º 28' 35" W, near El Rocío town).

3. Results and discussion

3.1. Isotopic values and nitrate concentrations

Isotopic values ($\delta^{15}N_{NO3,}$ $\delta^{18}O_{NO3}$) and nitrate concentrations measured between February and June (2015 and 2016) in surface waters of the Doñana watershed were highly variable (Table 2). Nitrate concentrations varied between 7.3 and 19.2 mg $_{NO3}$ L^{-1} with a median of 14.4 mg $_{NO3}$ L^{-1}, being generally higher in winter than in spring-summer. Isotopic values for $\delta^{15}N_{NO3}$ were higher during spring, ranging between +11.3 ‰ and +27.9 ‰ with a median of +19.1 ‰. Isotopic values for $\delta^{18}O_{NO3}$ ranged between +1.9 ‰ and +29.3 ‰ with a median of +11.4 ‰. However, no clear temporal trend was observed for $\delta^{18}O_{NO3}$ during the study period (Fig. 3).

Nitrite concentrations varied between 0.1 and 3.4 mg $_{NO2}$ L^{-1} with a median of 0.8 mg $_{NO2}$ L^{-1}. Ammonium concentrations varied between 0.001 and 8.6 mg $_{NH4}$ L^{-1} with a median of 2.2 mg $_{NH4}$ L^{-1} (Table SP4 Sup. Mat.).

3.2. Nitrate sources and transformations

To identify the predominant nitrate sources in the study area, we compared our results with reference data and assessed whether different land uses, biological transformations and mixing may have driven the composition of the isotopic values in the samples (Fig. 2, Table SP1, Sup. Mat.).

3.2.1. Measured vs. reference nitrate isotopic values

The two ponds (PDpal, PDmim) and "La Cañada" stream (STca) are located in catchments dedicated mainly to intensive greenhouse production, with no known large urban inputs, therefore we expected a strong influence of nitrate inputs from fertilizers, predominantly of synthetic origin (Fig. SP1, Sup. Mat.). However, $\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$ results not only showed values above the reference values for synthetic fertilizers but they also showed the highest values among all sites (Fig.2). Moreover, we also expected most samples collected downstream of the Almonte WWTP (in the three sites along the Partido stream: WWTP_alm, STpar1, STpar2), El Rocío WWTP (in the two sites along the Marín stream: WWTP_roc and STmar) and in MRSro to show nitrate isotopic values similar to the reference values for urban wastewaters (Widory et al., 2004). However, most of these samples (except for WWTP_alm) also showed higher $\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$ values than expected (Fig. 2). Thus, these results suggest that one or more fractionating processes, in addition to mixing, may have shifted isotopic data to higher values (Lamb et al., 2012; Viana and Bode, 2013).

3.2.2. *Coupled $^{15}N_{NO3}$ and $^{18}O_{NO3}$ enrichment: denitrification vs. assimilation*

We suggest that the coupled increase of $\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$ values observed in the two ponds (PDpal, PDmim), La Cañada stream (STca) and some samples influenced by the Almonte WWTP (WWTP_alm, STpar1, STpar2) and the El Rocío WWTP (WWTP_roc and STmar) may be strongly linked to common biological processes, such as denitrification and/or assimilation, which produce coupled increase in the isotopic values of the original nitrate source due to discrimination of heavier isotopes (^{15}N, ^{18}O) over lighter ones (^{14}N, ^{16}O) (Granger et al., 2004, 2008). Moreover, the correlation slopes in our data (slope $_{\text{PDpal, PDmim, STca, STmar}}$ =0.7 and slope $_{\text{STpar2}}$ = 0.73; Fig. 2) matched the enrichment slopes reported during both these processes, ranging between 0.5 and 1 for denitrification (Böttcher et al., 1990; Wunderlich et al., 2013) and closer to 1 for assimilation (Granger et al., 2004). However, we suggest that denitrification has a stronger effect on nitrate isotopic fractionation than assimilation in some of these sites since large increases of $\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$ values are most likely the result of high isotopic fractionation (ε), typically occurring during denitrification (ε= -5 to -40‰) but not during assimilation (ε= -4 to -10‰) (Kendall and Aravena, 2000; Nikolenko et al., 2018). In the particular case of the two ponds (PDpal, PDmim), a combination of both (denitrification and assimilation) can explain their high coupled isotope enrichments, but the levels of algal production ($\text{chla}_{\text{PDpal}}$= 22.7 ± 11.4 µg L^{-1}; $\text{chla}_{\text{PDmim}}$= 3.4 ± 2 µg L^{-1}) and the presence of a oxygenated water column (DO_{PDpal}= 9.7 ± 1.2 mg L^{-1}; DO_{PDmim}= 11.8 mg L^{-1}) indicated that any denitrification process probably mainly occurred before entering the pond. The high connection between the aquifer and surface waters can result in the discharge of groundwater into these ponds (with potential high levels of denitrified nitrate pool – see below) and the assimilation of remaining nitrate in their standing waters.

3.2.3. Denitrification

Denitrification was likely to cause nitrate isotopic increase in the study sites, so we estimated percentages of denitrification using an average isotopic fractionation value $\varepsilon^{15}N_{NO3/N2}$ = -15‰ (Böttcher et al., 1990). Although this should be considered only as a rough estimate, both ponds (PDpal and PDmim) showed the highest estimates of denitrification percentages among all sites (40% to 80%), especially in samples collected during spring/summer (Fig. 4). Estimated denitrification percentages for the rest of the sampling sites (STca, WWTP_alm, STpar1, STpar2, WWTP_roc and STmar) showed generally lower values than for the ponds (10-60%). Although our results showed that all nitrate concentrations kept below the maximum recommended for surface waters (50 mg L^{-1} NO_3^-), we could expect that real nitrate inputs may be considerably higher since a large proportion is removed by denitrification, according to the estimated denitrification percentages. Thus, water quality monitoring programs measuring only nitrate concentrations in surface water are most likely underestimating the real amount of nitrate exported from the watershed into the streams and ponds. Although we did not study where the nitrate reduction processes take place within our study sites, we assume that it simultaneously occurs: (1) in the water column and sediments of streams and ponds (Tortosa et al. 2011), (2) in the riparian groundwater zone prior to reaching the surface waters (Sebilo et al., 2003; Griffiths et al., 2016), and/or (3) in the deeper groundwater system affected by nitrate leaching from the intensive use of fertilizers (Kim et al., 2015; Otero et al., 2009). In addition, denitrification may often occur within WWTPs as observed in the sample collected at WWTP_roc (directly from the effluent pipe) where both $\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$ values were increased compared to the reference values for WWTP nitrate sources (Fig. 2). Overall, the degree to which denitrification may take part in each compartment would depend on the particular site characteristics and the environmental conditions. Thus, we further evaluate the relative contribution of each source into the surface waters by including different scenarios of denitrification in these Mediterranean wetlands and streams. This information may be particularly relevant for management of surface waters with similar characteristics to the study area, since denitrification constitutes a key process in the attenuation of nitrate.

3.2.4. Source mixture tracking and quantification

Mixing of multiple nitrate sources (e.g. fertilizers, wastewaters, precipitation), is a common process in watersheds and has a direct effect on nitrate isotopic composition (Kendall et al. 2008). Mixing can also occur between transformed and untransformed nitrate from either the same source or different sources. Although denitrification seems to be the most important process explaining nitrate isotopic variation within our sites, we cannot rule out the possibility that mixing also played a key role in the observed nitrate isotopic values. For example, isotopic values of samples collected in STmar fell completely within the denitrification overlapping area (Fig. 2), which could suggest that the nitrate source could be either organic or inorganic, or a mix of the two. Indeed, this site not only receives water from El Rocío WWTP effluent but also from the upstream drainage area where a high percentage of land is dedicated to intensive greenhouse crops (Fig. SP1, Sup. Mat.). Moreover, areas affected by one main nitrate source, such as synthetic fertilizers from agricultural practices in La Rocina, Los Sotos and Laguna de Palos watersheds (without any WWTP influence), could exhibit mixing of waters of the same nitrate source but with different level of isotopic fractionation (e.g. non-denitrified surface waters mixing with denitrified groundwaters). Therefore, we followed a Bayesian approach in order to quantify the relative contribution of the nitrate sources (i.e. soil and fertilizer NH_4^+, nitrate-based fertilizers, organic fertilizer and wastewaters) at a given site under different denitrification scenarios. Some of the samples fell outside the mixing polygon formed by the potential nitrate sources in the first scenario that considers a 25% level of denitrification (Figure SP3), which indicates that the other two scenarios at 50% and 75% level are more likely.

Agricultural practices are important sources for nitrate contamination when considering scenarios of 50 to 75% level of denitrification (Table 3). The nitrate derived from soil and NH_4^+ fertilizers contributed from 37 to 89% at the level of 75% denitrified nitrate, from 4 to 33 % at the level of 50%, and from 0 to 3% at the level of 25%. In addition, nitrate based fertilizers contributed to the mixture around 3-50%, 1-6% and ~1%, respectively, when we consider there is no extensive recycling by bacteria in the soil. Under certain conditions of high microbial activity and sufficient residence time in the unsaturated area, these nitrate-based fertilizers can also be recycled in the soil in a process abbreviated as MIT (Mineralization - Immobilization – Turnover; Mengis et al., 2001). During this process, the isotopic composition of the N is approximately constant, but the $\delta^{18}O_{NO3}^-$ loses its characteristic isotopic signal of + 23 ‰ and will have the same $\delta^{18}O_{NO3}^-$ that nitrified ammonium-based fertilizers. Unfortunately, we do not have direct measurements of microbial activity from soils in the sampled region, and we kept both type of synthetic fertilizers separately in the model. Our estimations of source partitioning are based on nitrate-based fertilizers not recycled in the soil from now on in the text. In contrast, the wastewater sources took a predominant role under the scenarios of lower level of denitrification processes, except for STpar1 that kept its importance in all cases. As expected, the contribution of the nitrate from atmospheric deposition was relatively low.

Furthermore, direct relationships between the proportional contributions of each sources and land use cover were evaluated (Fig.5). The contribution of soil and fertilizer ammonium positively correlated with the percent cover of agricultural crops (without including greenhouses) at a 75% level of denitrification ($R^2=0.37$), but not significantly ($p>0.05$). Greenhouse cover percentage was also correlated with nitrate-based fertilizer contributions in all scenarios ($R^2=0.46-0.53$, $p<0.05$). There is clearly a direct link between the agricultural practices in the watershed and their contamination inputs to the riverine nitrate. For the other sources, we also found a relationship with land uses or with the drainage area for all scenarios (all cases were significant, $p<0.05$). There was a negative correlation between the drainage area and the contribution of atmospheric deposition, which indicates a potential dilution effect with groundwater water sources in the watershed. At last, but not least, a positive relation of wastewater sources and urban cover suggested a direct link with the WWTPs from the area. Said that, these connections with land use practices were evaluated at the same level of denitrification, which is likely not the case.

Overall, our results indicate that at least between 50-75% of the nitrate that inputs this aquatic ecosystem is denitrified before entering the surface waters, and that there is a direct link between nitrate sources and watershed land uses. Thus, we suggest that future research in Mediterranean temporary streams needs to consider the confounding effect of both denitrification and mixing for source tracking and quantification across time and space, so as to facilitate more effective nitrate pollution management of these surface waters. Future studies of nitrate pollution in Mediterranean systems should implement tools such as Bayesian stable isotope mixing models (e.g. Soto et al., 2019, Yi et al., 2017), but while considering important processes such as denitrification to estimate the relative contribution of the main nitrate sources.

3.2.5. Other processes affecting nitrate isotopic composition

Besides denitrification, assimilation or mixing, other processes affecting the isotopic composition of nitrate could potentially be present in the area as follows:

(1) Ammonia volatilization commonly results from application of urea and manure during agricultural practices within the watershed (Bouwman et al., 2002), causing strong enrichment of ^{15}N in the residual NH_4^+ (ε= -25‰) which oxidizes to ^{15}N-enriched nitrate without causing variation in the δ^{18}O values (Nikolenko et al., 2018). Several samples from WWTP_alm and STpar1 showed variability in the $\delta^{15}N_{NO3}$ that was not coupled with shifts in the $\delta^{18}O_{NO3}$, suggesting that these samples were influenced by ammonia volatilization processes prior to nitrification.

(2) Seasonal variations in climatic conditions (temperature and precipitation) and anthropogenic activities (e.g. agricultural cycles) can strongly influence nitrate inputs into watersheds. Although our sampling was not systematic, we assume that seasonality is an important factor affecting nitrate transformation and mixing processes in our study area. Our results revealed that most sites showed increasing coupled $\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$ values from winter to spring (Fig. SP2 Sup. Mat.). Similarly, when combining all sites from the watershed, $\delta^{15}N_{NO3}$ isotopic values increased from winter to late spring (February to June) whereas nitrate concentrations decreased (Fig. 3). These results could be partly related to higher denitrifying microbial activity due to gradual temperature increase (Chen et al. 2009). Furthermore, in the Mediterranean area precipitations are often concentrated within a short period of time. Intense precipitations over a short period can cause considerable watershed runoff and rapid transport of nitrate from the agricultural areas, or inputs of atmospherically-derived nitrate, into the receiving streams or ponds, which may result in abrupt changes of $\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$ values (Divers et al., 2014; Soto et al., 2019). In the Doñana watershed the amount of nitrate loading into the stream and the predominant nitrate sources after a particular heavy rain event would be linked to the ongoing specific agricultural activities at that moment, for example, fertigation of berries from December until June or application of manure for agricultural land conditioning during summer months. In contrast, during prolonged periods of scarce precipitations (late spring, summer and early fall) base-flow in streams remains low, or even ceases completely in some cases, whereas WWTP effluents are continuous throughout the year, thus we expect that nitrate isotopic composition downstream of WWTPs would mainly reflect urban wastewaters (Lin et al. 2019). During dry periods the relative contribution of groundwater to the streams may also be important (Custodio et al., 2009). Denitrification may occur in groundwaters due to the infiltration of nitrates from the agricultural practices (Rodríguez and Stefano, 2012), hence stream water would probably reflect a high proportion of $^{15}N_{NO3}$ and $^{18}O_{NO3}$ enriched groundwater. Dry periods are predicted to increase in the Mediterranean region during coming decades lowering the water table (Guardiola et al. 2011; Cramer et al., 2018), and nitrate

removing processes such as denitrification could be negatively affected (Manis et al., 2014) increasing the sensitivity of the system to eutrophication.

4. Conclusions

The dual nitrate isotope approach can trace nitrate pollution in temporary freshwater systems in the Mediterranean region. This technique sheds new light on the main nitrate sources and processes within the Doñana watershed. The isotopic variability in the samples reflected a complex combination of transformations, mixing processes and human activities that can vary over space and time. We suggest that denitrification was a predominant process given that the majority of the sites showed high coupled increased nitrate isotopic values ($\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$), particularly the ponds exposed to nitrate inputs from synthetic fertilizers. To what extent denitrification occurs in the sediment, riparian zone, WWTP and/or groundwaters was not determined in this study. The nitrate inputs into the system may actually be higher than those indicated by spot sampling of nitrate concentrations, since some nitrate has previously been removed by denitrification. Differences among and within sites shown in this study underline the need to measure nitrate isotopic composition at higher spatio-temporal resolution and include not only measurements of surface waters but also groundwater and interstitial water to enable a more accurate distinction between nitrate sources and processes at a watershed scale (Li et al., 2019). Agricultural practices were important sources of N pollution into this watershed and could be estimated and quantified in a Bayesian framework after considering fractionation associated to denitrification processes. A direct link between the use of synthetic fertilizers in agricultural crops can be established, but investigations in other transformation processes that occur predominantly in the study area (either in water and soil) should be further evaluated. Particularly, in Mediterranean areas affected by strong interannual variability in climatic conditions and increasing human activities, long-term studies with the use of multiple proxies (e.g. $^{15}N_{NO3}$, $^{18}O_{NO3}$ and ^{11}B, biological indicators) are recommended to aid development of management and conservation strategies against anthropogenic nitrogen pollution and eutrophication.

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Table 1. Land use percentage (%) by category for the watershed area of each sampling site. Percentages were calculated from the land use map (Fig. SP1, sup. Mat.) using ArcGIS and Excel softwares.

Watershed	**Sampling point**	**Drainage area (km^2)**	**Agricultural (%)**		**Forested (%)**	**Grassland (%)**	**Urban† (%)**	**Water (%)**
			Greenhouses	**Other crops**				
Primera de Palos	**PDpal***	2.8	85.20	0.20	0.44	0.22	3.39	0.0
El Partido	**WWTP_roc****	15.9	50.9	25.9	2.9	11.7	2.8	1.3
	STmar	18.8	49.9	27.6	2.5	10.0	3.0	1.5
	WWTP_alm***	185.5	4.4	64.5	14.9	4.7	5.7	1.2
	STpar1	267.1	3.2	61.1	18.9	6.4	5.2	1.3
	STpar2	274.9	3.5	60.9	18.7	6.4	5.1	1.5
La Rocina	**STca**	77.6	16.8	7.5	41.2	29.5	1.4	0.8
	MRSro	386.3	8.7	7	66	14.1	1.6	1.0
Los Sotos	**PDmim**	35.4	21.1	21.3	24.1	29.1	1	0.3

* Reference site for nitrate pollution derived from synthetic fertilizers.

** Reference site for nitrate pollution derived from urban wastewaters.

*** Reference site for nitrate pollution derived from mixed sources (urban wastewaters and organic/inorganic fertilizers).

† Includes urban areas and infrastructures (e.g. roads).

Table 3. $\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$ values and NO_3^- concentrations measured in surface water samples collected at nine different sampling sites between February and June in 2015 and 2016. Sampling points are located in the Doñana marsh catchment area except for PDpal. Reference sites (†) are those where the main N source was known.

Site	n	Main N source	Date	$\delta^{15}N_{NO3}$ (‰)	$\delta^{18}O_{NO3}$ (‰)	NO_3^- (mg_{NO3} L^{-1})
WWTP_roc†	1	Urban wastewaters	06/05/2015	+26.7	+16.5	12.1
WWTP_alm†	4	Urban wastewaters*	18/02/2016	+17.5	+5.9	18.3
			20/04/2016	+11.6	+2.4	14.4
			24/05/2016	+15.5	+5.8	15.8
			22/06/2016	+24.6	+10.4	7.3
PDpal†	4	Chemical fertilizers	15/03/2016	+11.3	+17.6	16.9
			20/04/2016	+23.6	+25.6	15.7
			24/05/2016	+22.0	+29.3	11.9
			22/06/2016	+25.3	+26.7	13.2
PDmim	3	Uncertain	11/05/2015	+27.9	+28.0	9.0
			18/02/2016	+17.5	+20.5	14.8
			26/04/2016	+25.2	+24.0	9.0
STca	2	Uncertain	06/05/2015	+13.4	+15.0	13.3
			25/02/2016	+13.7	+18.1	19.2
STmar	4	Uncertain	06/05/2015	+20.5	+17.5	13.2
			16/03/2016	+23.0	+17.4	15.6
			10/04/2016	+17.7	+13.5	17.3
			25/05/2016	+20.7	+17.5	12.9
STpar2	5	Uncertain	06/05/2015	+16.1	+9.6	10.2
			25/02/2016	+14.3	+5.3	15.3
			16/03/2016	+12.5	+5.9	14.8
			10/04/2016	+14.8	+7.8	17.3
			25/05/2016	+16.7	+7.7	15.6
STpar1	5	Uncertain	06/05/2015	+20.5	+1.9	10.3
			10/04/2016	+23.7	+8.7	18.1
			21/04/2016	+14.4	+10.7	14.3
			25/05/2016	+19.2	+7.5	17.3
			21/06/2016	+26.3	+4.8	12.8
MRSro	1	Uncertain	24/05/2016	+19.1	+11.4	8.1

*Water samples at WWTP_alm were collected several meters downstream the WWTP of Almonte so we assume that there is a high influence of nitrate derived from the WWTP outflow though part of the nitrate inputs are expected to be sourced from agricultural fertilizers.

Table 3. Proportional contributions of primary sources (soil and fertilizer NH$_4^+$, fertilizer NO$_3^-$, wastewater, and atmospheric deposition) to dissolved nitrate in the Doñana marsh catchment area estimated using a dual isotope Bayesian mixing model. Median (and SD) contribution values are shown for each source.

25% denitrified

Site	n	Soil and fert NH4+	SD	NO3 fert	SD	Wastewater	SD	Atm Dep	SD
WWTP_roc†	1	0.024	0.037	0.015	0.028	0.801	0.061	0.14	0.048
WWTP_alm†	4	0.008	0.036	0.005	0.015	0.948	0.044	0.026	0.02
PDpal†	4	0.019	0.046	0.014	0.05	0.514	0.06	0.427	0.055
PDmim	3	0.019	0.045	0.013	0.051	0.571	0.066	0.369	0.063
STca	2	0.031	0.104	0.02	0.097	0.612	0.106	0.281	0.079
STmar	4	0.019	0.05	0.013	0.046	0.728	0.06	0.213	0.048
STpar2	5	0.019	0.079	0.009	0.028	0.892	0.081	0.054	0.029
STpar1	5	0.006	0.022	0.003	0.01	0.967	0.029	0.016	0.014
MRSro	1	0.016	0.073	0.01	0.039	0.848	0.096	0.088	0.056

50% denitrified

Site	n	Soil and fert NH4+	SD	NO3 fert	SD	Wastewater	SD	Atm Dep	SD
WWTP_roc†	1	0.15	0.11	0.04	0.05	0.7	0.104	0.08	0.04
WWTP_alm†	4	0.09	0.13	0.01	0.03	0.86	0.131	0.02	0.02
PDpal†	4	0.11	0.11	0.04	0.15	0.44	0.105	0.35	0.1
PDmim	3	0.09	0.11	0.04	0.12	0.53	0.105	0.29	0.09
STca	2	0.3	0.22	0.06	0.15	0.37	0.164	0.17	0.09
STmar	4	0.14	0.15	0.04	0.09	0.63	0.128	0.14	0.07
STpar2	5	0.33	0.21	0.02	0.04	0.61	0.192	0.03	0.02
STpar1	5	0.04	0.06	0.01	0.01	0.93	0.066	0.01	0.01
MRSro	1	0.12	0.18	0.03	0.06	0.74	0.176	0.05	0.05

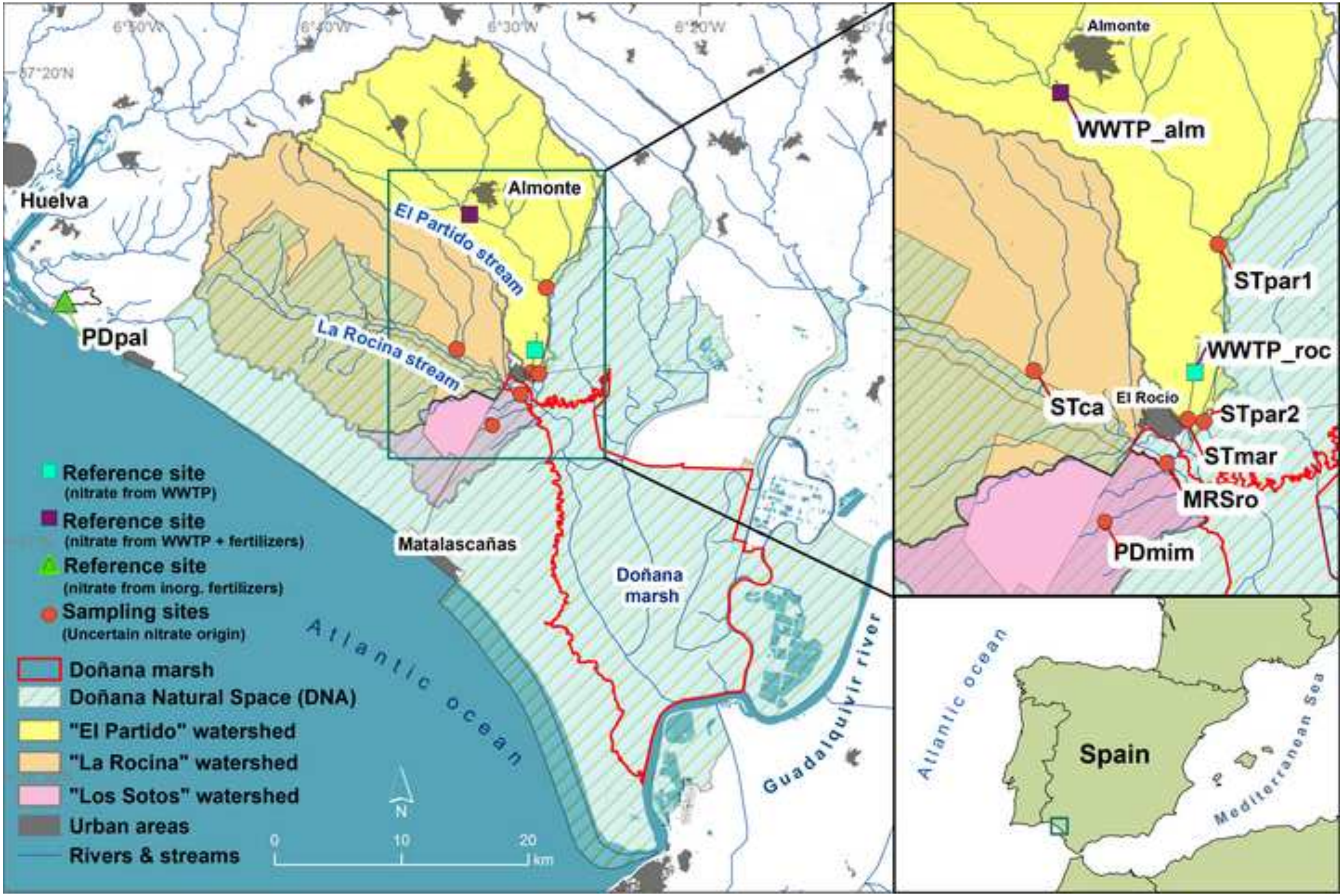
75% denitrified

Site	n	Soil and fert NH4+	SD	NO3 fert	SD	Wastewater	SD	Atm Dep	SD
WWTP_roc†	1	0.7	0.165	0.129	0.085	0.124	0.126	0.02	0.033
WWTP_alm†	4	0.843	0.214	0.035	0.045	0.089	0.193	0.007	0.017
PDpal†	4	0.371	0.183	0.504	0.235	0.068	0.071	0.026	0.118
PDmim	3	0.534	0.216	0.298	0.191	0.084	0.1	0.024	0.089
STca	2	0.507	0.226	0.349	0.223	0.058	0.08	0.02	0.087
STmar	4	0.774	0.186	0.112	0.114	0.059	0.105	0.014	0.041
STpar2	5	0.891	0.13	0.039	0.058	0.04	0.098	0.007	0.02
STpar1	5	0.594	0.271	0.033	0.04	0.35	0.255	0.007	0.015
MRSro	1	0.772	0.217	0.074	0.109	0.084	0.161	0.012	0.039

Caption Figure 1: Study area

Figure 1. Location of the sampling points selected for this study. Red dots represent locations where the contribution and type of nitrate sources are uncertain. Purple squares indicate reference sites for nitrate related to urban wastewaters (WWTP_alm and WWTP_roc) and the green triangle a reference site for nitrate related to inorganic fertilizers used in strawberry production under plastic (PDpal). PD indicates a pond, ST a stream and MR a marsh.

Figure 1


6°50'W
6°40'W
6°30'W
6°20'W
37°20'N
Huelva
Almonte
El Partido stream
La Rocina stream
PDpal
Matalascañas
Doñana marsh
Atlantic ocean
Guadalquivir river
🟩 Reference site (nitrate from WWTP)
■ Reference site (nitrate from WWTP + fertilizers)
▲ Reference site (nitrate from inorg. fertilizers)
● Sampling sites (Uncertain nitrate origin)
▭ Doñana marsh
▨ Doñana Natural Space (DNA)
"El Partido" watershed
"La Rocina" watershed
"Los Sotos" watershed
Urban areas
Rivers & streams
0
10
20
km
N
Almonte
WWTP_alm
STpar1
WWTP_roc
STca
El Rocio
STpar2
STmar
MRSro
PDmim
Atlantic ocean
Spain
Mediterranean Sea

Caption Figure 2: Isotopic data

Figure 2. δ^{15}N vs. δ^{18}O values of the samples together with published reference data (in boxes, Table SP3, Sup. Mat.) from chemical fertilizers (Vitòria et al., 2004), wastewaters and/or organic fertilizers (Widory et al., 2004). Shaded areas between slopes 0.5 and 1 represent theoretical "denitrification" values when the sources are: chemical fertilizers and soil NO_3^- or organic fertilizers and wastewaters. Samples falling within the "overlapping area" may be linked to any of the reference sources. Green dots are sampling sites (PDpal, PDmim and STca) where chemical fertilizers represent the main potential nitrate source and do not receive any WWTP outflow. Blue/black squares (WWTP_alm, STpar1, STpar2), blue triangles (WWTP_roc, STmar) and a yellow dot (MRSro) are sampling sites affected by both agricultural fertilizers and upstream WWTP discharges. Nitrate inputs from agricultural sources may occur either through watershed runoff or groundwater discharges. Symbol size indicates the nitrate concentrations for each sample.

Figure 2

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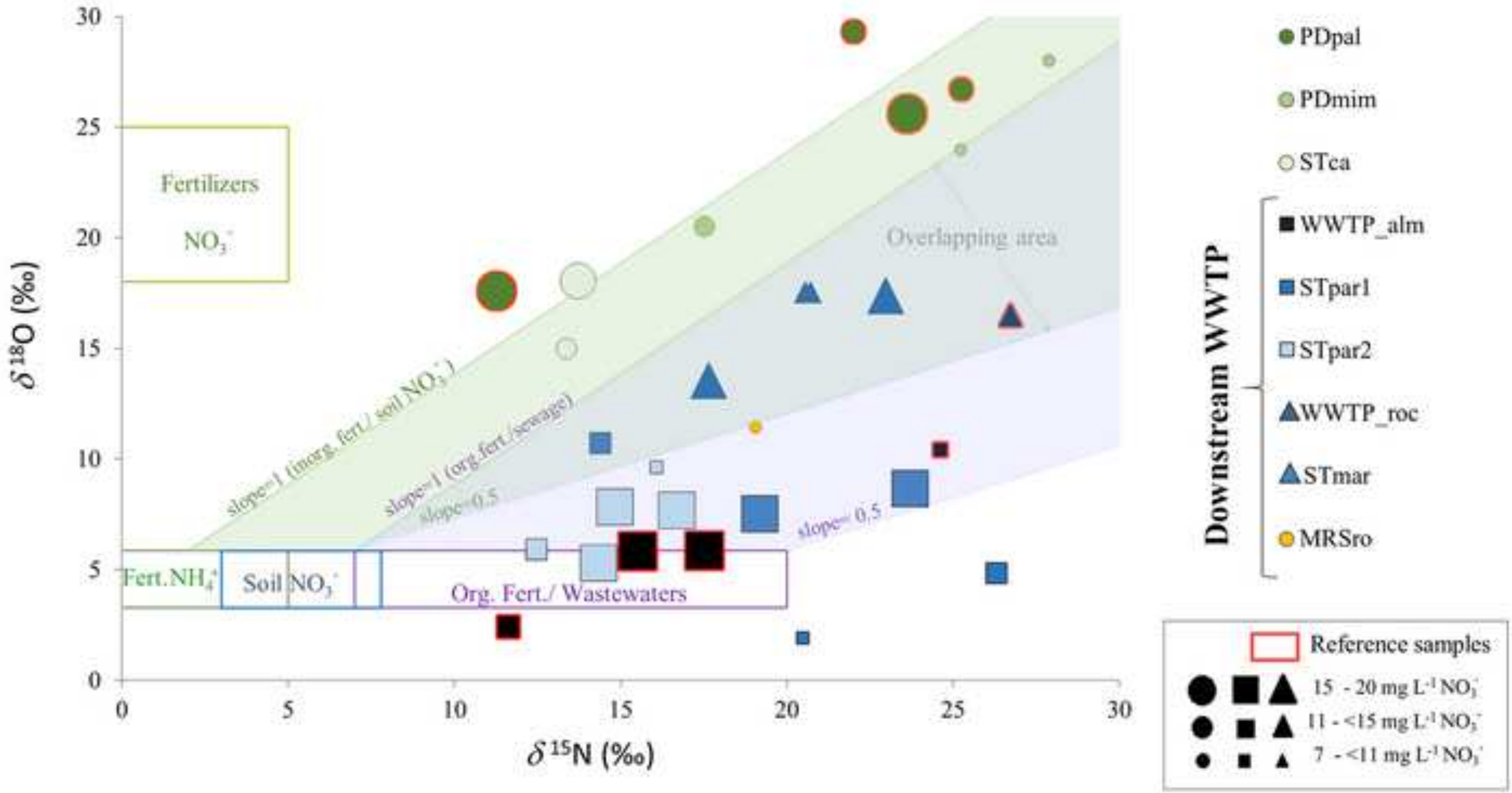
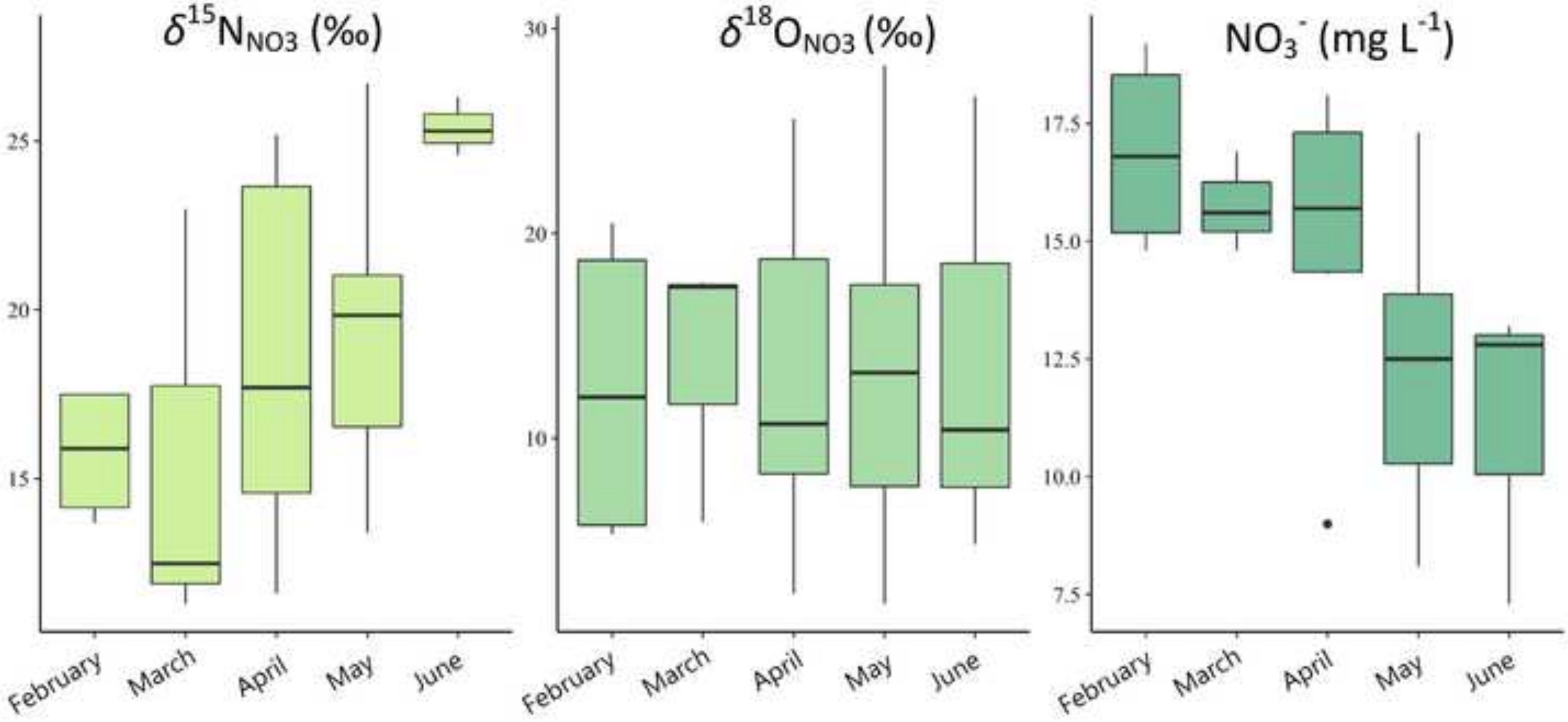


Caption Figure 3: Temporal variation

Figure 3. Temporal variation of isotopic values (δ^{15}N and δ^{18}O) and NO_3^- concentrations. Each boxplot contains pooled data for all sites and both years (2015 and 2016) by month (February to June).

Figure 3



$\delta^{15}N_{NO3}$ (‰)
25
20
15
$\delta^{18}O_{NO3}$ (‰)
30
20
10
NO_3^- (mg L^{-1})
17.5
15.0
12.5
10.0
7.5
February
March
April
May
June

Figure 4. δ^{15}N vs. δ^{18}O values of the samples together with the modelled denitrification trends.

The shadowed areas represent the initial values used in the models.

Figure 4

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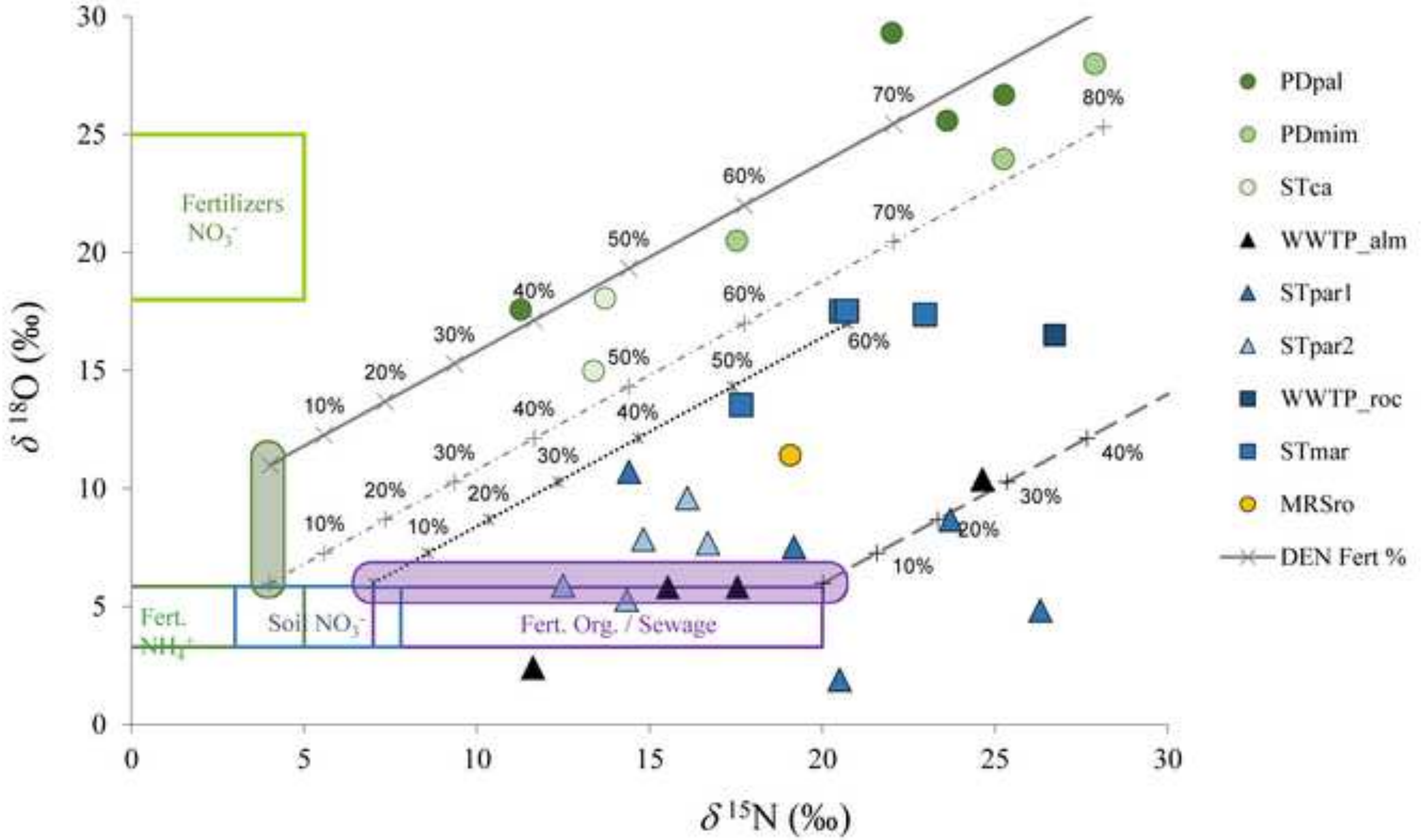

Figure 5. Relationships between the estimated proportional contributions of each source at 25% (black triangles), 50% (white circles), and 75% (grey circles) level of denitrification and potential land use parameters (from Table 1).

Figure 5

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