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Paredes, Irene; Otero, Neus; Soler, Albert; Green, Andy J.; Soto, David X.  
2020. **Agricultural and urban delivered nitrate pollution input to  
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**The definitive version was published in *Agriculture, Ecosystems and  
Environment*, 294, 106859. <https://doi.org/10.1016/j.agee.2020.106859>**

The definitive version is available at <https://www.elsevier.com/>

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# Agricultural and urban delivered nitrate pollution input to Mediterranean temporary freshwaters

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## Abstract

Nitrate dual stable isotopes ( $\delta^{15}\text{N}_{\text{NO}_3}$  and  $\delta^{18}\text{O}_{\text{NO}_3}$ ) 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 ( $\text{NO}_3^-$ ) concentrations and stable isotopes ( $\delta^{15}\text{N}_{\text{NO}_3}$  and  $\delta^{18}\text{O}_{\text{NO}_3}$ ) in water samples collected during

27 different periods over two years (2015-2016). Most sites showed coupled increases of nitrate  
28 isotopic values ( $\delta^{15}\text{N}_{\text{NO}_3}$  and  $\delta^{18}\text{O}_{\text{NO}_3}$ ), which were higher than reference values of any possible  
29 sources (e.g. synthetic/organic fertilizers and wastewaters), indicating fractionations typical of  
30 denitrification processes. The main nitrate sources to the watershed were linked to agricultural  
31 practices and the use of synthetic fertilizers, but further investigations in other transformation  
32 processes that occur simultaneously should be evaluated. These results highlight an important  
33 nitrate removal capacity (i.e. denitrification) of the system, which may positively contribute to  
34 natural resilience against eutrophication. However, given the high intra and interannual  
35 hydrological fluctuations of Mediterranean aquatic systems, future studies on the relative  
36 contribution of nitrate sources and processes should increase spatio-temporal resolution of water  
37 sampling, and include measurements of groundwater and interstitial water as well as surface  
38 water.

39

## 40 **Key words**

41 Nitrate source; stable isotopes; fertilizers; wastewater; denitrification

42

43

## 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  $50 \text{ mg L}^{-1} \text{ NO}_3^-$ . 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)

70 and its corresponding transposition into the Spanish legislation (Royal Decree 261/1996), part of  
71 the surface and groundwaters of the Doñana wetland were designated as “nitrate vulnerable  
72 zones” by the Andalusian Government (Decree 36/2008), with the aim of reducing the impact of  
73 the ongoing nitrate pollution due to the intensification of agriculture in the watershed (Rodríguez  
74 and Stefano 2012, WWF 2016). Nitrate pollution is a major threat to surface and groundwater of  
75 the Doñana wetland related to the excessive use of fertilizers in agriculture and the discharge of  
76 poorly treated wastewaters into streams (Serrano et al., 2006; Paredes et al., 2019). In several  
77 streams, high concentrations of nitrites and ammonia are toxic to many organisms and are  
78 incompatible with nature conservation (Paredes et al., unpublished results). Intensive  
79 groundwater pumping for irrigation has resulted in a decrease of natural water discharge into  
80 streams, enhancing flow intermittency and limiting the dilution capacity of surface waters  
81 (Guardiola et al. 2011, Manzano et al. 2013). The strong temporal variability in precipitation and  
82 the prolonged arid period in summer, typical of the Mediterranean region, result in a highly  
83 irregular frequency of nitrate inputs into the streams entering Doñana. This intermittent and  
84 irregular nitrate loading into the aquatic system complicates the monitoring of nitrate inputs and  
85 in-stream biogeochemistry.

86 Stable isotope techniques can be used to trace nitrate pollution sources and nitrogen cycling in  
87 aquatic ecosystems (Mayer et al. 2002; Nestler et al. 2011; Kaushal et al., 2011; Soto et al. 2019).  
88 Ratios of stable N isotopes ( $^{15}\text{N}/^{14}\text{N}$ , expressed as  $\delta^{15}\text{N}$  in ‰) vary among different nitrate  
89 sources. Nitrate derived from human wastewaters or manure are usually more enriched in  $\delta^{15}\text{N}_{\text{NO}_3}$   
90 (+10 to +20 ‰) than nitrate from most synthetic fertilizers (-3 to +3 ‰), atmospheric deposition  
91 (-15 to +7 ‰) or natural soils (-6 to +9 ‰) (Kendall 1998; Bateman and Kelly, 2007). However,  
92 distinguishing between nitrate sources with wide and overlapping  $\delta^{15}\text{N}_{\text{NO}_3}$  ranges (e.g. synthetic  
93 fertilizers vs. atmospheric deposition), or identifying the influence of different transformation  
94 processes is not always possible if only  $\delta^{15}\text{N}_{\text{NO}_3}$  is used.

95 A simultaneous dual nitrate isotope approach ( $\delta^{15}\text{N}_{\text{NO}_3}$  and  $\delta^{18}\text{O}_{\text{NO}_3}$ ) offers the advantage of a  
96 more precise distinction between sources and processes, since  $\delta^{18}\text{O}_{\text{NO}_3}$  (i.e. the ratio  $^{18}\text{O}/^{16}\text{O}$ )  
97 shows a greater resolution for the origin of certain sources that overlap for  $\delta^{15}\text{N}_{\text{NO}_3}$  (Craine et al.,  
98 2015). For example, while synthetic fertilizers and nitrate atmospheric deposition show  
99 overlapping  $\delta^{15}\text{N}_{\text{NO}_3}$ , the  $\delta^{18}\text{O}_{\text{NO}_3}$  values of synthetic fertilizers (around +23 ‰) (Michalski et al.,  
100 2015) are considerably higher than those of atmospheric deposition (ranging from +60 ‰ to +98  
101 ‰) (Kendall et al., 2008). Furthermore, overlapping of  $\delta^{15}\text{N}_{\text{NO}_3}$  values may also occur when there  
102 are changes in  $\delta^{15}\text{N}_{\text{NO}_3}$  for one of the sources due to transformation processes (e.g. nitrification,  
103 denitrification, mineralization, ammonia volatilization or assimilation) (Kendall et al., 2008).  
104 Hence, nitrate removal by denitrification or assimilation may produce  $^{15}\text{N}_{\text{NO}_3}$  enrichment in the  
105 residual nitrate of an originally  $^{15}\text{N}_{\text{NO}_3}$ -depleted source (e.g. synthetic fertilizers), which can make  
106 it undistinguishable from another  $^{15}\text{N}_{\text{NO}_3}$  enriched, untransformed source (e.g. human  
107 wastewaters) (Kendall, 1998). Such fractionating processes also produce  $^{18}\text{O}_{\text{NO}_3}$  enrichment,  
108 resulting in comparatively higher  $\delta^{18}\text{O}_{\text{NO}_3}$  values in the residual nitrate than in the  $^{15}\text{N}_{\text{NO}_3}$   
109 enriched, untransformed sources (Mariotti et al., 1988; Granger et al., 2004; Søvik and Mørkved,  
110 2008). Thus, nitrate isotopic composition in most aquatic systems is the result of simultaneous  
111 transformations and nitrate source mixing which are often undistinguishable from each other  
112 without the application of multi-isotopic approaches such as the dual nitrate isotope approach  
113 (Kendall et al., 2008, Otero et al., 2009, Yue et al., 2017). The latter has been used to study nitrate  
114 transport and transformations in numerous watersheds worldwide. However, there is a lack of  
115 isotope studies in arid and semiarid areas subjected to warm temperatures, strong rainfall variation  
116 and water scarcity, such as the Doñana wetland (Custodio et al., 2009; Tortosa et al., 2011, Wong  
117 et al., 2018). Given climate change and the ongoing nutrient inputs from anthropogenic activities,  
118 vulnerability to eutrophication is expected to continue increasing in Mediterranean wetlands  
119 (Green et al. 2017). In this context, it is critical to improve our understanding of natural nitrate  
120 removal processes (e.g. denitrification) which can reduce eutrophication.

121 In this study we aimed to identify (and quantify) the main anthropogenic nitrate sources and  
122 specific transformation processes in the Doñana watershed using the dual nitrate stable isotope  
123 approach ( $\delta^{15}\text{N}_{\text{NO}_3}$  and  $\delta^{18}\text{O}_{\text{NO}_3}$ ). In combination with nitrate concentrations in surface waters and  
124 land use data, we expect to gain information on the relationships between agricultural practices  
125 and the nitrate input into these Mediterranean streams. Given results for  $\delta^{15}\text{N}$  in emergent aquatic  
126 vegetation in our study area (Paredes et al. 2019), we hypothesized that sampling sites affected  
127 by upstream wastewater treatment plant discharges would show higher inputs from urban sources  
128 than sites mainly affected by agricultural practices. Moreover, we hypothesized that  
129 biogeochemical processes occurring either in the water column, sediments, riparian zone or  
130 groundwater would partly explain nitrate isotopic variability transport and transformation in our  
131 study area. Finally, mixing of surface waters with different nitrate isotopic compositions may  
132 explain the remaining variability of the nitrate isotopic values.

133

## 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 (0.45 $\mu$ m pore size) using a low-pressure vacuum pump. We stored all filtered samples in the freezer (-20°C) prior to isotopic analyses ( $\delta^{15}\text{N}_{\text{NO}_3}$  and  $\delta^{18}\text{O}_{\text{NO}_3}$ ) and  $\text{NO}_3^-$  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 inhabitants (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

187 western area of the Doñana watershed (Fig. 1). Nitrate sources are uncertain at these sites since  
188 different anthropogenic point and diffuse nitrate inputs are contributing simultaneously to their  
189 surface waters. All sites are influenced by both surface and groundwaters since they are located  
190 on a sandy permeable terrain connected to the underlying unconfined aquifer, except for “El  
191 Rocío” marsh (MRSro) which is located over silty-clay impermeable deposits where the aquifer  
192 is confined below (Serrano et al. 2006).

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

197

### 198 **2.3. Stable isotope and nitrate concentration analyses**

199 We measured the nitrate concentration ( $\text{NO}_3^-$ ) using standard colorimetric methods (ISO  
200 13395:1996). We also measured  $\text{NO}_2^-$  (ISO 13395:1996) and  $\text{NH}_4^+$  (ISO 11732:2005)  
201 concentrations. All dissolved inorganic N measurements were carried out on a multi-channel  
202 SEAL Analytical AA3 AutoAnalyzer (Norderstedt, Germany), at the Laboratory of Aquatic  
203 Ecology of EBD-CSIC (Seville, Spain). Limits of detection for the analytical methods were 0.004  
204  $\mu\text{mol L}^{-1}$  for N- $\text{NO}_3^-$  and N- $\text{NO}_2^-$  and 0.040  $\mu\text{mol L}^{-1}$  for N- $\text{NH}_4^+$ .

205 We measured  $\delta^{15}\text{N}$  and  $\delta^{18}\text{O}$  values of dissolved nitrate using the Cd reduction method proposed  
206 by McIlvin et al. (2005). This method (ISO 9001: 2008 certification) is based on the reduction of  
207 nitrate to  $\text{N}_2\text{O}$  (g) by Cd and its subsequent pre-concentration by means of a gas purification  
208 system connected to an IRMS, to perform, once concentrated, the measurement of the isotopic  
209 ratio of the  $\delta^{18}\text{O}_{\text{N}_2\text{O}}$  and  $\delta^{15}\text{N}_{\text{N}_2\text{O}}$ . The  $\text{N}_2\text{O}$  was analyzed using a Pre-Con coupled to a Finnigan  
210 MAT 253 Isotope Ratio Mass Spectrometer (IRMS) (Thermo Scientific). In the case of the  
211 presence of nitrite, sulfamic acid was added to the water samples to remove  $\text{NO}_2^-$ , in order to  
212 avoid any interference in the measurement of the nitrogen and oxygen isotopic composition of

213 dissolved nitrate (Granger and Sigman, 2008). Following Coplen (2011), several international  
214 and laboratory (CCiT) standards were interspersed among samples to normalize the results. For  
215 the  $\delta^{15}\text{N}_{\text{NO}_3}$  and  $\delta^{18}\text{O}_{\text{NO}_3}$  analysis the employed standards were USGS-32, USGS-34, USGS-35  
216 and results were referenced to the international scale (AIR for  $\delta^{15}\text{N}$  and V-SMOW for  $\delta^{18}\text{O}$ ). The  
217 reproducibility ( $1\sigma$ ) of the samples, calculated from the standards systematically interspersed in  
218 the analytical batches, was  $\pm 1.0\text{‰}$  for  $\delta^{15}\text{N}_{\text{NO}_3}$  and  $\pm 1.5\text{‰}$  for  $\delta^{18}\text{O}_{\text{NO}_3}$ . Samples for the isotopic  
219 analyses were processed at the “MAIMA” Research group laboratory and analyzed at the “Centres  
220 Científics i Tecnològics” of the “Universitat de Barcelona” (UB).

221

222

## 223 **2.4. Identification of nitrate sources**

224 To identify nitrate sources we plotted all the measured  $\delta^{15}\text{N}_{\text{NO}_3}$  vs.  $\delta^{18}\text{O}_{\text{NO}_3}$  values together with  
225 reference isotope values from major potential watershed sources: chemical fertilizers (Vitòria et  
226 al., 2004), soil nitrate from nitrification and wastewaters and/or organic fertilizers from Widory  
227 et al. (2004) (Table SP1, Sup. Mat.). According to Kendall et al. (2008) during nitrification there  
228 is a large fractionation in the  $^{15}\text{N}$  during the transformation of  $\text{NH}_4^+$  to  $\text{NO}_2^-$ , ( $\epsilon_{\text{NH}_4/\text{NO}_2} = -38$  to -  
229  $14\text{‰}$ ) and negligible  $^{15}\text{N}$  fractionation in the transformation of  $\text{NO}_2^-$  to  $\text{NO}_3^-$ , but in N-limited  
230 systems, since the transformation is complete, the final  $\text{NO}_2^-$ , and therefore  $\text{NO}_3^-$ , will show a  
231 small  $^{15}\text{N}$  isotopic effect. We have assumed a complete nitrification, and therefore the average  
232  $\delta^{15}\text{N}$  considered for ammonium derived fertilizers ranges between  $-5$  and  $+5\text{‰}$ . Regarding  
233 oxygen, nitrification can incorporate two atoms of O from water and one atom of O from  $\text{O}_2$  in  
234 some cases, and all oxygen atoms from water in others (Snider et al 2010, Venkiteswaran et al.,  
235 2019). Additionally, the  $\delta^{18}\text{O}_{\text{O}_2}$  values vary depending on the productivity of the system  
236 (Wassenaar et al 2010, Venkiteswaran et al 2015), and its analysis (see Wassenaar and Koehler  
237 1999 for details) may be of great relevance for a better interpretation of nitrate isotope results. In  
238 our case, for simplicity, the expected  $\delta^{18}\text{O}_{\text{NO}_3}$  derived from nitrification of  $\text{NH}_4^+$  (either from soil,  
239 manure or fertilizer) was calculated following *Eq. 1* (Mayer et al. 2001), and using the range of

240  $\delta^{18}\text{O}_{\text{H}_2\text{O}}$  values of the studied samples (Table SP2, Sup. Mat.) and a  $\delta^{18}\text{O}_{\text{O}_2}$  of +23.5‰ (Kroopnick  
241 and Craig, 1972).

242 
$$\delta^{18}\text{O}_{\text{NO}_3} = \frac{1}{3} \cdot \delta^{18}\text{O}_{\text{O}_2} + \frac{2}{3} \delta^{18}\text{O}_{\text{H}_2\text{O}} \quad \text{Eq. 1}$$

243

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

249 We estimated proportional contributions of these nitrate sources from the watershed into the  
250 dissolved riverine nitrate of each sampling location by using dual isotope values introduced into  
251 Bayesian isotope mixing model approach (MixSIAR; Stock and Semmens 2016, Moore and  
252 Semmens 2008). Potential sources and their expected isotope values are described in Table SP1.  
253 We combined the isotopic composition of primary sources of soil and fertilizer  $\text{NH}_4^+$  due to their  
254 overlap in isotope values and their subsequent lack of source discrimination. The variable ‘site’  
255 was included as fixed effect into the models of three chains of 100,000 iterations, a burn-in of  
256 50,000 and a thinning of 50. Using this modelling approach, three scenarios were evaluated  
257 because denitrification processes seem to be a main driver of isotopic variation in these temporary  
258 systems (see Results and Discussion). For this reason, fractionation factors (and SD) associated  
259 to denitrification processes at a level of 25%, 50% and 75% ( $\pm 10\%$ ) of the fraction denitrified  
260 according to the model below (see Section 2.5.) were used in each scenario.

261

## 262 **2.5. Denitrification processes**

263 Denitrification typically produces a coupled increase in  $\delta^{15}\text{N}_{\text{NO}_3}$  and  $\delta^{18}\text{O}_{\text{NO}_3}$ , with a slope ranging  
264 from 0.5 to 1 (Böttcher et al., 1990; Wunderlich et al., 2013). Since the initial isotopic composition  
265 can be different depending on nitrate origin, we roughly estimated how this process could shift

266 the isotopic values of the main potential sources in our system by representing two shaded areas  
267 in the  $\delta^{15}\text{N}_{\text{NO}_3}$  vs.  $\delta^{18}\text{O}_{\text{NO}_3}$  graph (Fig.2). Each shaded area corresponded to the theoretical values  
268 of samples that have undergone denitrification with (1) inorganic fertilizer/soil nitrate origin  
269 (green) and (2) sewage/manure origin (light purple). Since denitrification slopes may differ, these  
270 two theoretical areas partially overlap.

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

$$273 \quad \varepsilon_{P/S} = \ln \frac{\delta_s - \delta_{s,0}}{\ln f} \quad \text{Eq. 2,}$$

274 where  $\varepsilon_{P/S}$  is the isotopic fractionation,  $\delta_s$  and  $\delta_{s,0}$  are the isotopic composition of the residual (s)  
275 and initial (s,0) nitrate, and  $f$  is the remaining nitrate fraction. Both  $\varepsilon^{15}\text{N}_{\text{NO}_3}$  and  $\varepsilon^{18}\text{O}_{\text{NO}_3}$  can be  
276 modeled.

277 Denitrification percentages were estimated using an average isotopic fractionation of  $\varepsilon^{15}\text{N}_{\text{NO}_3/\text{N}_2}$   
278 = -15‰ (Böttcher et al., 1990) and a  $\varepsilon^{18}\text{O}_{\text{NO}_3/\text{N}_2} / \varepsilon^{15}\text{N}_{\text{NO}_3/\text{N}_2}$  ratio of 0.7. Different denitrification  
279 models were calculated based on the initial isotope values ( $\delta^{15}\text{N}_{\text{NO}_3}$  and  $\delta^{18}\text{O}_{\text{NO}_3}$ ) of the original  
280 nitrate source:

281 (1) For water samples affected by nitrate inputs from synthetic fertilizers: since the original  
282  $\text{NO}_3^-$  could be a mixing of sources such as nitrified  $\text{NH}_4^+$  fertilizers and  $\text{NO}_3^-$  fertilizers, we  
283 performed two different models using the same initial  $\delta^{15}\text{N}_{\text{NO}_3}$  value (+4‰) but two different  
284  $\delta^{18}\text{O}_{\text{NO}_3}$  values (+6‰ and +11‰, respectively). The latter initial values ( $\delta^{15}\text{N}_{\text{NO}_3}$  and  $\delta^{18}\text{O}_{\text{NO}_3}$ )  
285 represented different proportions of  $\text{NO}_3^-$  fertilizers vs. nitrified  $\text{NH}_4^+$  fertilizers (Fig. 4) based  
286 on values reported in an area with greenhouse cultivation, a predominant use of synthetic  
287 fertilizers and no denitrification, and that had an average  $\delta^{15}\text{N}_{\text{NO}_3}$  of +4‰ and  $\delta^{18}\text{O}_{\text{NO}_3}$  values up  
288 to +11‰ (Vitòria et al., 2004).

289 (2) For water samples affected by nitrate inputs derived from wastewaters: since some of  
290 these samples also followed a denitrification trend (WWTP\_alm, WWTP\_roc), two  
291 denitrification models were calculated using different initial  $\delta^{15}\text{N}_{\text{NO}_3}$  (+7‰ and +16‰,  
292 representing the bibliographic range for wastewater) and a  $\delta^{18}\text{O}_{\text{NO}_3}$  of +6‰ (representing the

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

295

## 296 **2.6. Effects of seasonal changes in $\delta^{15}\text{N}_{\text{NO}_3}$ and $\delta^{18}\text{O}_{\text{NO}_3}$**

297 To observe whether there was any temporal trend in the nitrate isotopic composition and  
298 concentrations over our study period (February to June), we pooled the data for all sites and both  
299 years (2015 and 2016) by month (Fig. 3). Additionally, we plotted the isotopic values ( $\delta^{15}\text{N}_{\text{NO}_3}$ ,  
300  $\delta^{18}\text{O}_{\text{NO}_3}$ ) of each site collected in 2016 together with the precipitation and temperature data. We  
301 only represented the isotopic data of those sampling sites with two or more samples collected  
302 during 2016 (Table 2). Meteorological data was collected from the Almonte Meteorological  
303 Station (37° 08' 53" N, 06° 28' 35" W, near El Rocío town).

### 3. Results and discussion

#### 3.1. Isotopic values and nitrate concentrations

Isotopic values ( $\delta^{15}\text{N}_{\text{NO}_3}$ ,  $\delta^{18}\text{O}_{\text{NO}_3}$ ) 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  $\text{NO}_3$  L<sup>-1</sup> with a median of 14.4 mg  $\text{NO}_3$  L<sup>-1</sup>, being generally higher in winter than in spring-summer. Isotopic values for  $\delta^{15}\text{N}_{\text{NO}_3}$  were higher during spring, ranging between +11.3 ‰ and +27.9 ‰ with a median of +19.1 ‰. Isotopic values for  $\delta^{18}\text{O}_{\text{NO}_3}$  ranged between +1.9 ‰ and +29.3 ‰ with a median of +11.4 ‰. However, no clear temporal trend was observed for  $\delta^{18}\text{O}_{\text{NO}_3}$  during the study period (Fig. 3).

Nitrite concentrations varied between 0.1 and 3.4 mg  $\text{NO}_2$  L<sup>-1</sup> with a median of 0.8 mg  $\text{NO}_2$  L<sup>-1</sup>. Ammonium concentrations varied between 0.001 and 8.6 mg  $\text{NH}_4$  L<sup>-1</sup> with a median of 2.2 mg  $\text{NH}_4$  L<sup>-1</sup> (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,  $\delta^{15}\text{N}_{\text{NO}_3}$  and  $\delta^{18}\text{O}_{\text{NO}_3}$  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

330 the two sites along the Marín stream: WWTP\_roc and STmar) and in MRSro to show nitrate  
331 isotopic values similar to the reference values for urban wastewaters (Widory et al., 2004).  
332 However, most of these samples (except for WWTP\_alm) also showed higher  $\delta^{15}\text{N}_{\text{NO}_3}$  and  
333  $\delta^{18}\text{O}_{\text{NO}_3}$  values than expected (Fig. 2). Thus, these results suggest that one or more fractionating  
334 processes, in addition to mixing, may have shifted isotopic data to higher values (Lamb et al.,  
335 2012; Viana and Bode, 2013).

336

### 337 3.2.2. Coupled $^{15}\text{N}_{\text{NO}_3}$ and $^{18}\text{O}_{\text{NO}_3}$ enrichment: denitrification vs. assimilation

338 We suggest that the coupled increase of  $\delta^{15}\text{N}_{\text{NO}_3}$  and  $\delta^{18}\text{O}_{\text{NO}_3}$  values observed in the two ponds  
339 (PDpal, PDmim), La Cañada stream (STca) and some samples influenced by the Almonte WWTP  
340 (WWTP\_alm, STpar1, STpar2) and the El Rocío WWTP (WWTP\_roc and STmar) may be  
341 strongly linked to common biological processes, such as denitrification and/or assimilation, which  
342 produce coupled increase in the isotopic values of the original nitrate source due to discrimination  
343 of heavier isotopes ( $^{15}\text{N}$ ,  $^{18}\text{O}$ ) over lighter ones ( $^{14}\text{N}$ ,  $^{16}\text{O}$ ) (Granger et al., 2004, 2008). Moreover,  
344 the correlation slopes in our data (slope<sub>PDpal, PDmim, STca, STmar</sub> = 0.7 and slope<sub>STpar2</sub> = 0.73; Fig. 2)  
345 matched the enrichment slopes reported during both these processes, ranging between 0.5 and 1  
346 for denitrification (Böttcher et al., 1990; Wunderlich et al., 2013) and closer to 1 for assimilation  
347 (Granger et al., 2004). However, we suggest that denitrification has a stronger effect on nitrate  
348 isotopic fractionation than assimilation in some of these sites since large increases of  $\delta^{15}\text{N}_{\text{NO}_3}$  and  
349  $\delta^{18}\text{O}_{\text{NO}_3}$  values are most likely the result of high isotopic fractionation ( $\epsilon$ ), typically occurring  
350 during denitrification ( $\epsilon$  = -5 to -40‰) but not during assimilation ( $\epsilon$  = -4 to -10‰) (Kendall and  
351 Aravena, 2000; Nikolenko et al., 2018). In the particular case of the two ponds (PDpal, PDmim),  
352 a combination of both (denitrification and assimilation) can explain their high coupled isotope  
353 enrichments, but the levels of algal production ( $\text{chl}_{\text{aPDpal}} = 22.7 \pm 11.4 \mu\text{g L}^{-1}$ ;  $\text{chl}_{\text{aPDmim}} = 3.4 \pm 2$   
354  $\mu\text{g L}^{-1}$ ) and the presence of a oxygenated water column ( $\text{DO}_{\text{PDpal}} = 9.7 \pm 1.2 \text{ mg L}^{-1}$ ;  $\text{DO}_{\text{PDmim}} =$   
355  $11.8 \text{ mg L}^{-1}$ ) indicated that any denitrification process probably mainly occurred before entering



356 the pond. The high connection between the aquifer and surface waters can result in the discharge  
357 of groundwater into these ponds (with potential high levels of denitrified nitrate pool – see below)  
358 and the assimilation of remaining nitrate in their standing waters.

### 359 3.2.3. Denitrification

360 Denitrification was likely to cause nitrate isotopic increase in the study sites, so we estimated  
361 percentages of denitrification using an average isotopic fractionation value  $\epsilon^{15}\text{N}_{\text{NO}_3/\text{N}_2} = -15\text{‰}$   
362 (Böttcher et al., 1990). Although this should be considered only as a rough estimate, both ponds  
363 (PDpal and PDmim) showed the highest estimates of denitrification percentages among all sites  
364 (40% to 80%), especially in samples collected during spring/summer (Fig. 4). Estimated  
365 denitrification percentages for the rest of the sampling sites (STca, WWTP\_alm, STpar1, STpar2,  
366 WWTP\_roc and STmar) showed generally lower values than for the ponds (10-60%). Although  
367 our results showed that all nitrate concentrations kept below the maximum recommended for  
368 surface waters ( $50 \text{ mg L}^{-1} \text{ NO}_3^-$ ), we could expect that real nitrate inputs may be considerably  
369 higher since a large proportion is removed by denitrification, according to the estimated  
370 denitrification percentages. Thus, water quality monitoring programs measuring only nitrate  
371 concentrations in surface water are most likely underestimating the real amount of nitrate exported  
372 from the watershed into the streams and ponds. Although we did not study where the nitrate  
373 reduction processes take place within our study sites, we assume that it simultaneously occurs:  
374 (1) in the water column and sediments of streams and ponds (Tortosa et al. 2011), (2) in the  
375 riparian groundwater zone prior to reaching the surface waters (Sebilo et al., 2003; Griffiths et  
376 al., 2016), and/or (3) in the deeper groundwater system affected by nitrate leaching from the  
377 intensive use of fertilizers (Kim et al., 2015; Otero et al., 2009). In addition, denitrification may  
378 often occur within WWTPs as observed in the sample collected at WWTP\_roc (directly from the  
379 effluent pipe) where both  $\delta^{15}\text{N}_{\text{NO}_3}$  and  $\delta^{18}\text{O}_{\text{NO}_3}$  values were increased compared to the reference  
380 values for WWTP nitrate sources (Fig. 2). Overall, the degree to which denitrification may take  
381 part in each compartment would depend on the particular site characteristics and the  
382 environmental conditions. Thus, we further evaluate the relative contribution of each source into

383 the surface waters by including different scenarios of denitrification in these Mediterranean  
384 wetlands and streams. This information may be particularly relevant for management of surface  
385 waters with similar characteristics to the study area, since denitrification constitutes a key process  
386 in the attenuation of nitrate.

#### 387 3.2.4. *Source mixture tracking and quantification*

388 Mixing of multiple nitrate sources (e.g. fertilizers, wastewaters, precipitation), is a common  
389 process in watersheds and has a direct effect on nitrate isotopic composition (Kendall et al. 2008).  
390 Mixing can also occur between transformed and untransformed nitrate from either the same  
391 source or different sources. Although denitrification seems to be the most important process  
392 explaining nitrate isotopic variation within our sites, we cannot rule out the possibility that mixing  
393 also played a key role in the observed nitrate isotopic values. For example, isotopic values of  
394 samples collected in STmar fell completely within the denitrification overlapping area (Fig. 2),  
395 which could suggest that the nitrate source could be either organic or inorganic, or a mix of the  
396 two. Indeed, this site not only receives water from El Rocío WWTP effluent but also from the  
397 upstream drainage area where a high percentage of land is dedicated to intensive greenhouse crops  
398 (Fig. SP1, Sup. Mat.). Moreover, areas affected by one main nitrate source, such as synthetic  
399 fertilizers from agricultural practices in La Rocina, Los Sotos and Laguna de Palos watersheds  
400 (without any WWTP influence), could exhibit mixing of waters of the same nitrate source but  
401 with different level of isotopic fractionation (e.g. non-denitrified surface waters mixing with  
402 denitrified groundwaters). Therefore, we followed a Bayesian approach in order to quantify the  
403 relative contribution of the nitrate sources (i.e. soil and fertilizer  $\text{NH}_4^+$ , nitrate-based fertilizers,  
404 organic fertilizer and wastewaters) at a given site under different denitrification scenarios. Some  
405 of the samples fell outside the mixing polygon formed by the potential nitrate sources in the first  
406 scenario that considers a 25% level of denitrification (Figure SP3), which indicates that the other  
407 two scenarios at 50% and 75% level are more likely.

408 Agricultural practices are important sources for nitrate contamination when considering scenarios  
409 of 50 to 75% level of denitrification (Table 3). The nitrate derived from soil and  $\text{NH}_4^+$  fertilizers  
410 contributed from 37 to 89% at the level of 75% denitrified nitrate, from 4 to 33 % at the level of  
411 50%, and from 0 to 3% at the level of 25%. In addition, nitrate based fertilizers contributed to the  
412 mixture around 3-50%, 1-6% and ~1%, respectively, when we consider there is no extensive  
413 recycling by bacteria in the soil. Under certain conditions of high microbial activity and sufficient  
414 residence time in the unsaturated area, these nitrate-based fertilizers can also be recycled in the  
415 soil in a process abbreviated as MIT (Mineralization - Immobilization – Turnover; Mengis et al.,  
416 2001). During this process, the isotopic composition of the N is approximately constant, but the  
417  $\delta^{18}\text{O}_{\text{NO}_3^-}$  loses its characteristic isotopic signal of + 23 ‰ and will have the same  $\delta^{18}\text{O}_{\text{NO}_3^-}$  that  
418 nitrified ammonium-based fertilizers. Unfortunately, we do not have direct measurements of  
419 microbial activity from soils in the sampled region, and we kept both type of synthetic fertilizers  
420 separately in the model. Our estimations of source partitioning are based on nitrate-based  
421 fertilizers not recycled in the soil from now on in the text. In contrast, the wastewater sources took  
422 a predominant role under the scenarios of lower level of denitrification processes, except for  
423 STpar1 that kept its importance in all cases. As expected, the contribution of the nitrate from  
424 atmospheric deposition was relatively low.

425

426 Furthermore, direct relationships between the proportional contributions of each sources and land  
427 use cover were evaluated (Fig.5). The contribution of soil and fertilizer ammonium positively  
428 correlated with the percent cover of agricultural crops (without including greenhouses) at a 75%  
429 level of denitrification ( $R^2=0.37$ ), but not significantly ( $p>0.05$ ). Greenhouse cover percentage  
430 was also correlated with nitrate-based fertilizer contributions in all scenarios ( $R^2=0.46-0.53$ ,  
431  $p<0.05$ ). There is clearly a direct link between the agricultural practices in the watershed and their  
432 contamination inputs to the riverine nitrate. For the other sources, we also found a relationship  
433 with land uses or with the drainage area for all scenarios (all cases were significant,  $p<0.05$ ).  
434 There was a negative correlation between the drainage area and the contribution of atmospheric

435 deposition, which indicates a potential dilution effect with groundwater water sources in the  
436 watershed. At last, but not least, a positive relation of wastewater sources and urban cover  
437 suggested a direct link with the WWTPs from the area. Said that, these connections with land use  
438 practices were evaluated at the same level of denitrification, which is likely not the case.

439 Overall, our results indicate that at least between 50-75% of the nitrate that inputs this aquatic  
440 ecosystem is denitrified before entering the surface waters, and that there is a direct link between  
441 nitrate sources and watershed land uses. Thus, we suggest that future research in Mediterranean  
442 temporary streams needs to consider the confounding effect of both denitrification and mixing for  
443 source tracking and quantification across time and space, so as to facilitate more effective nitrate  
444 pollution management of these surface waters. Future studies of nitrate pollution in Mediterranean  
445 systems should implement tools such as Bayesian stable isotope mixing models (e.g. Soto et al.,  
446 2019, Yi et al., 2017), but while considering important processes such as denitrification to  
447 estimate the relative contribution of the main nitrate sources.

#### 448 *3.2.5. Other processes affecting nitrate isotopic composition*

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

451

452 *(1) Ammonia volatilization* commonly results from application of urea and manure during  
453 agricultural practices within the watershed (Bouwman et al., 2002), causing strong enrichment of  
454  $^{15}\text{N}$  in the residual  $\text{NH}_4^+$  ( $\epsilon = -25\text{‰}$ ) which oxidizes to  $^{15}\text{N}$ -enriched nitrate without causing  
455 variation in the  $\delta^{18}\text{O}$  values (Nikolenko et al., 2018). Several samples from WWTP\_alm and  
456 STpar1 showed variability in the  $\delta^{15}\text{N}_{\text{NO}_3}$  that was not coupled with shifts in the  $\delta^{18}\text{O}_{\text{NO}_3}$ ,  
457 suggesting that these samples were influenced by ammonia volatilization processes prior to  
458 nitrification.

459

460

461 (2) *Seasonal variations* in climatic conditions (temperature and precipitation) and anthropogenic  
462 activities (e.g. agricultural cycles) can strongly influence nitrate inputs into watersheds. Although  
463 our sampling was not systematic, we assume that seasonality is an important factor affecting  
464 nitrate transformation and mixing processes in our study area. Our results revealed that most sites  
465 showed increasing coupled  $\delta^{15}\text{N}_{\text{NO}_3}$  and  $\delta^{18}\text{O}_{\text{NO}_3}$  values from winter to spring (Fig. SP2 Sup.  
466 Mat.). Similarly, when combining all sites from the watershed,  $\delta^{15}\text{N}_{\text{NO}_3}$  isotopic values increased  
467 from winter to late spring (February to June) whereas nitrate concentrations decreased (Fig. 3).  
468 These results could be partly related to higher denitrifying microbial activity due to gradual  
469 temperature increase (Chen et al. 2009). Furthermore, in the Mediterranean area precipitations  
470 are often concentrated within a short period of time. Intense precipitations over a short period can  
471 cause considerable watershed runoff and rapid transport of nitrate from the agricultural areas, or  
472 inputs of atmospherically-derived nitrate, into the receiving streams or ponds, which may result  
473 in abrupt changes of  $\delta^{15}\text{N}_{\text{NO}_3}$  and  $\delta^{18}\text{O}_{\text{NO}_3}$  values (Divers et al., 2014; Soto et al., 2019). In the  
474 Doñana watershed the amount of nitrate loading into the stream and the predominant nitrate  
475 sources after a particular heavy rain event would be linked to the ongoing specific agricultural  
476 activities at that moment, for example, fertigation of berries from December until June or  
477 application of manure for agricultural land conditioning during summer months. In contrast,  
478 during prolonged periods of scarce precipitations (late spring, summer and early fall) base-flow  
479 in streams remains low, or even ceases completely in some cases, whereas WWTP effluents are  
480 continuous throughout the year, thus we expect that nitrate isotopic composition downstream of  
481 WWTPs would mainly reflect urban wastewaters (Lin et al. 2019). During dry periods the relative  
482 contribution of groundwater to the streams may also be important (Custodio et al., 2009).  
483 Denitrification may occur in groundwaters due to the infiltration of nitrates from the agricultural  
484 practices (Rodríguez and Stefano, 2012), hence stream water would probably reflect a high  
485 proportion of  $^{15}\text{N}_{\text{NO}_3}$  and  $^{18}\text{O}_{\text{NO}_3}$  enriched groundwater. Dry periods are predicted to increase in  
486 the Mediterranean region during coming decades lowering the water table (Guardiola et al. 2011;  
487 Cramer et al., 2018), and nitrate removing processes such as denitrification could be negatively  
488 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 ( $\delta^{15}\text{N}_{\text{NO}_3}$  and  $\delta^{18}\text{O}_{\text{NO}_3}$ ), 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.  $^{15}\text{N}_{\text{NO}_3}$ ,  $^{18}\text{O}_{\text{NO}_3}$  and  $^{11}\text{B}$ , biological indicators) are recommended to aid development of management and conservation strategies against anthropogenic nitrogen pollution and eutrophication.

## 514 **Acknowledgements**

515 We thank Miguel Ángel Bravo and Sarai López for their invaluable field assistance and the staff  
516 of the Stable Isotope Lab (LIE-EBD), Remote Sensing Lab (LAST-EBD), and Aquatic Ecology  
517 Lab (LEA-EBD). We also thank the logistic and technical support for fieldwork provided by  
518 Doñana ICTS-RBD. We would also like to thank the CCiT of the Universitat the Barcelona for  
519 the isotopic analyses support. Thanks to Marta Peña for her support and advices with the artwork.

520

## 521 **Funding**

522

523 The project leading to these results has received funding from “la Caixa” Foundation (ID  
524 100010434) under agreement LCF/BQ/SO14/52250006, the Severo Ochoa Program for Centers  
525 of Excellence in R+D+I [SEV-2012-0262], the PACE-ISOTEC (CGL2017-87216-C4-1-R)  
526 project financed by the Ministerio de Ciencia, Innovación y Universidades Español and by the  
527 AEI/FEDER of the European Union, and partially by the MAG (2017 SGR 1733) project financed  
528 by the Generalitat de Catalunya. Irene Paredes was supported by a PhD contract from  
529 International Scholarship Program “La Caixa-Severo Ochoa 2014”.

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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 <sup>2</sup> )	Agricultural (%)		Forested (%)	Grassland (%)	Urban <sup>†</sup> (%)	Water (%)
			Greenhouses	Other crops				
Primera de Palos	PDpal*	2.8	85.20	0.20	0.44	0.22	3.39	0.0
	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
El Partido	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 2.**  $\delta^{15}\text{N}_{\text{NO}_3}$  and  $\delta^{18}\text{O}_{\text{NO}_3}$  values and  $\text{NO}_3^-$  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	$\delta^{15}\text{N}_{\text{NO}_3}$ (‰)	$\delta^{18}\text{O}_{\text{NO}_3}$ (‰)	$\text{NO}_3^-$ ( $\text{mg}_{\text{NO}_3} \text{L}^{-1}$ )
WWTP_roc†	1	Urban wastewaters	06/05/2015	+26.7	+16.5	12.1
			18/02/2016	+17.5	+5.9	18.3
WWTP_alm†	4	Urban wastewaters*	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
			15/03/2016	+11.3	+17.6	16.9
PDpal†	4	Chemical fertilizers	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
			11/05/2015	+27.9	+28.0	9.0
PDmim	3	Uncertain	18/02/2016	+17.5	+20.5	14.8
			26/04/2016	+25.2	+24.0	9.0
			06/05/2015	+13.4	+15.0	13.3
STca	2	Uncertain	25/02/2016	+13.7	+18.1	19.2
			06/05/2015	+20.5	+17.5	13.2
STmar	4	Uncertain	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
			06/05/2015	+16.1	+9.6	10.2
STpar2	5	Uncertain	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
			06/05/2015	+20.5	+1.9	10.3
STpar1	5	Uncertain	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
			06/05/2015	+20.5	+1.9	10.3
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  $\text{NH}_4^+$ , fertilizer  $\text{NO}_3^-$ , 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.

<b><u>25% denitrified</u></b>									
Site	n	Soil and fert $\text{NH}_4^+$	SD	$\text{NO}_3$ 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

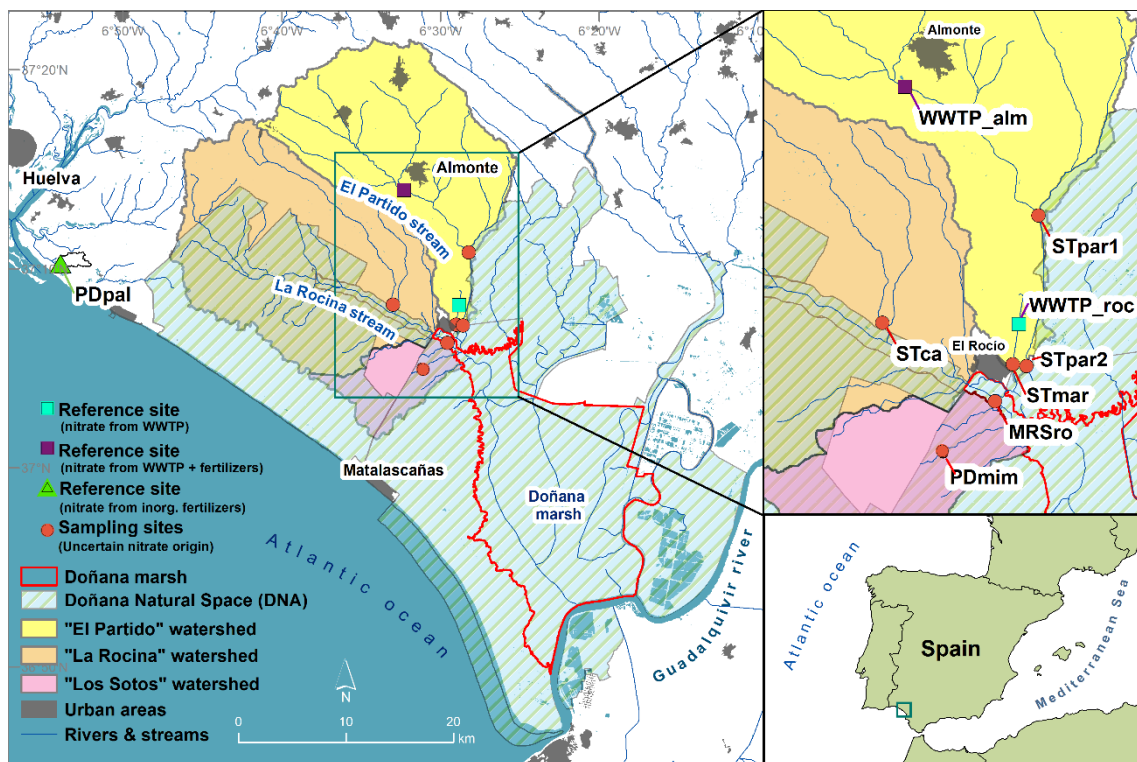
<b><u>50% denitrified</u></b>									
Site	n	Soil and fert $\text{NH}_4^+$	SD	$\text{NO}_3$ fert	SD	Wastewater	SD	Atm Dep	SD
WWTP_roc†	1	0.15	0.11	0.04	0.05	0.7	0.104	0.08	0.04
WWTP_alm†	4	0.09	0.13	0.01	0.03	0.86	0.131	0.02	0.02
PDpal†	4	0.11	0.11	0.04	0.15	0.44	0.105	0.35	0.1
PDmim	3	0.09	0.11	0.04	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

<b><u>75% denitrified</u></b>									
Site	n	Soil and fert $\text{NH}_4^+$	SD	$\text{NO}_3$ 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

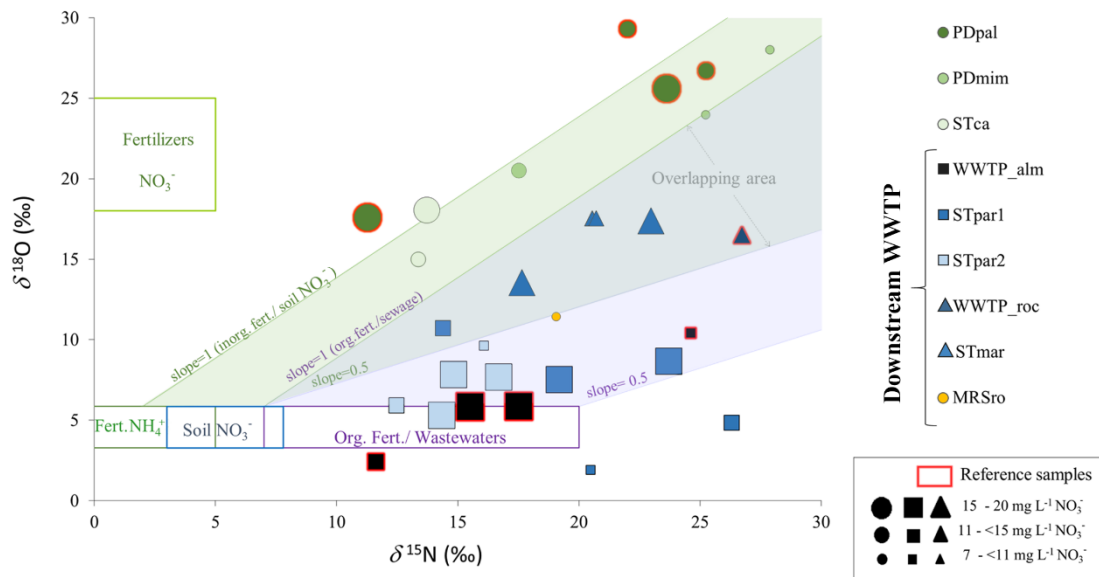
### Caption Figure 1: Study area

**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.



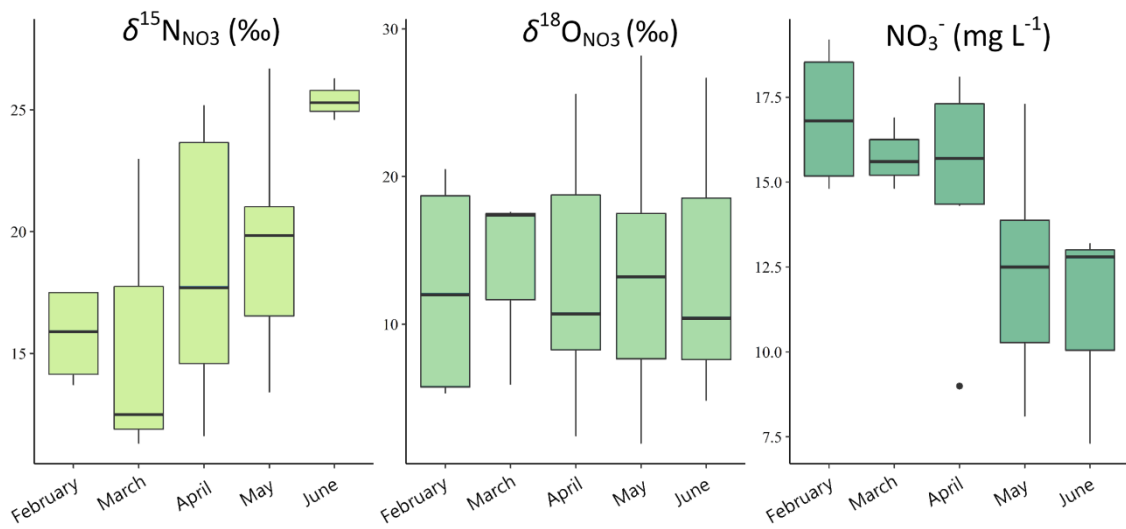
**Caption Figure 2: Isotopic data**

**Figure 2.**  $\delta^{15}\text{N}$  vs.  $\delta^{18}\text{O}$  values of the samples together with published reference data (in boxes, Table SP3, Sup. Mat.) from chemical fertilizers (Vitória 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  $\text{NO}_3^-$  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.



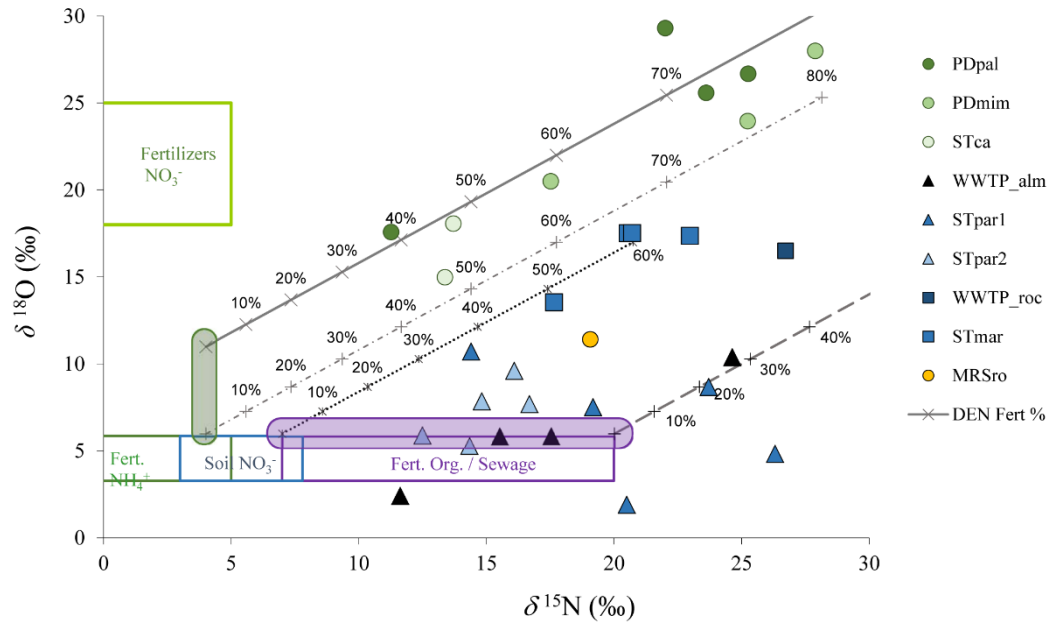
**Caption Figure 3: Temporal variation**

**Figure 3.** Temporal variation of isotopic values ( $\delta^{15}\text{N}$  and  $\delta^{18}\text{O}$ ) and  $\text{NO}_3^-$  concentrations. Each boxplot contains pooled data for all sites and both years (2015 and 2016) by month (February to June).



**Figure 4.**  $\delta^{15}\text{N}$  vs.  $\delta^{18}\text{O}$  values of the samples together with the modelled denitrification trends.

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



**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).

