Stable isotopes in helophytes reflect anthropogenic nitrogen pollution in entry streams at the Doñana World Heritage Site

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Abstract

Nitrogen (N) loading from anthropogenic activities is contributing to the eutrophication and degradation of wetlands worldwide. Doñana (southwestern Spain), includes a dynamic marshland protected as a UNESCO World Heritage Site, which has a catchment area exposed to increasing N inputs from intensive agriculture and poorly treated urban wastewaters. Identifying the sources of N entering this iconic wetland complex is vital for its conservation. To this end, we combined multiyear (2014-2016), spatially-explicit data on N concentration in water samples with measurements on the relative abundance of N stable isotopes (δ^{15}N) in *Bolboschoenus maritimus* and *Typha domingensis,* two dominant helophytes (i.e. emergent macrophytes) in the Doñana marsh and entry streams. Overall, plant tissues from entry streams showed higher δ^{15}N values than those from the marsh, particularly in those streams most affected by urban wastewaters. Isotopic values did not differ between plant species. Water samples affected by isotopically-enriched urban wastewaters and other diffuse organic N inputs (e.g. livestock farming) had relatively high Dissolved Inorganic Nitrogen (DIN) concentrations. In contrast, in streams mainly affected by diffuse N pollution from greenhouse crops, high DIN values were related to isotopically-depleted N sources (e.g., inorganic fertilizers). Thus, helophytes, in combination with other parameters such as N concentration in water or land cover, can be valuable indicators of anthropogenic pressures in Mediterranean wetlands. Helophytes have widespread distributions, and can be readily sampled even when water is no longer present. However, identification of specific N sources through helophyte δ^{15}N values is limited when key potential N sources are isotopically undistinguishable (e.g. fertilizers vs. atmospheric sources).

Key words

Nitrogen pollution, stable isotopes, helophytes, eutrophication, anthropogenic impact, wetland conservation

Abbreviations

DIN: Dissolved Inorganic Nitrogen

TN: Total Nitrogen

WHS: World Heritage Site

WWTP: Waste Water Treatment Plant

1. Introduction

Biogeochemical cycles have been severely altered worldwide by the over-enrichment of aquatic systems with nutrients, especially nitrogen (N). Human pressures, such as increasing use of chemical fertilizers in agriculture or land urbanization, are major and increasing causes of these alterations (Galloway et al., 2008; Tilman et al., 2002; Vitousek et al., 1997).

Wetlands play a key role in regulating the N cycle through different processes such as N sequestration (e.g. biomass production or sediment burial) or N removal (e.g. as N_2 by denitrification) (Costanza and D'Arge, 1997; Jordan et al., 2011; Kingsford et al., 2016). These processes represent a valuable ecosystem service both for society and wetlands, reducing the impact of excessive N inputs which otherwise would cause eutrophication, with adverse effects including cyanobacterial blooms, hypoxia, expansion of floating plants and, ultimately, loss of biodiversity (Compton et al., 2011; Green et al., 2017; Jenny et al., 2016; O'Neil et al., 2012). However, loss and degradation of natural wetlands is ongoing (Davidson, 2014), with major consequences for N regulation and other ecosystem services (Millenium Ecosystem Assessment, 2005).

N excess can originate from a variety of anthropogenic and natural processes. Point sources of excessive N loadings (e.g. chicken farms or wastewater treatment plants (WWTP)) are relatively easy to identify and manage (e.g. Carey and Migliaccio 2009). In contrast, diffuse N-sources (e.g. arable agriculture, atmospheric deposition) are more difficult to identify and control due to their uneven and widespread distribution within watersheds (Carpenter et al., 1998). Knowledge on the origin and spatial distribution of different N-sources is vital for effective management of N surplus in aquatic ecosystems.

Ratios of stable N isotopes ($^{15}N/^{14}N$, commonly expressed as $\delta^{15}N$ in ‰) vary among different N sources, providing a useful tool to identify the origin of N in aquatic systems (Heaton, 1986; Michener and Lajtha, 2007). For example, human wastewaters and animal waste N are typically enriched in $\delta^{15}N$ (10-20‰), while synthetic inorganic fertilizers have lower $\delta^{15}N$ values (-3 to 3‰) because they are derived from atmospheric nitrogen fixation ($\delta^{15}N$ -values close to zero).

Besides the specific N isotopic composition of different sources, common biogeochemical processes in aquatic systems (e.g. nitrification, denitrification, assimilation, fixation and mineralization) may also influence the δ^{15}N values of N compounds. For example, nitrate removal by denitrification results in isotopic enrichment of the heavier isotope (^{15}N) due to isotopic fractionation, thus increasing δ^{15}N values of residual nitrate (Mariotti et al., 1981; Minet et al., 2017). Therefore, the relative abundance of N isotopes (δ^{15}N) in N compounds is the result of mixed N sources and fractionation processes.

Numerous studies have monitored anthropogenic N loading from watersheds into coastal or inland waters by measuring δ^{15}N values in different biotic (e.g. plants, animal tissues) and abiotic (e.g. inorganic N, water) indicators (Cole et al., 2004; Karube et al., 2010; Kaushal et al., 2011; Vander Zanden et al., 2005). Aquatic plants are attractive indicators for tracing N inputs as they assimilate and/or fix N from the surrounding environment, integrating isotopic variability both spatially and temporally and thus reducing noise (Bannon and Roman, 2008; Cole et al., 2004; Kohzu et al., 2008; McIver et al., 2015; Wang et al., 2015). This may be particularly useful in Mediterranean wetlands, which are subject to high temporal variability in flooding patterns (Green et al. 2017), and subsequently in the sources and concentrations of N at a given moment of time. For instance, heavy rainfall events typically cause pulses of nutrients and organic matter in streams from catchment runoff (Bernal et al., 2013), or storm-water overflows from urban areas (Masi et al., 2017).

Aquatic plants can show a wide range of δ^{15}N values (15 to +20‰) depending on the available N sources, environmental conditions and physiological features (Kendall et al., 2008). For example, δ^{15}N values in submerged plants are useful indicators of wastewater inputs in temperate estuaries (Cole et al., 2004; McClelland et al., 1997; Savage and Elmgren, 2004).

Doñana, in south western Spain, is one of the most important wetland complexes in Europe and in the Mediterranean region, and is partly protected as a UNESCO World Heritage Site (WHS)(Green et al., 2018). However, these wetlands are under threat due to local human pressures and regional climate perturbations that act together, compromising water quantity and quality (Green et al., 2017). Impacts mainly originate outside the boundaries of the WHS, where economic development has been particularly intense in recent decades (Green et al., 2016; Serrano et al., 2006). Despite their importance, there is a lack of basic knowledge on the sources and levels of nutrient inputs entering the Doñana marshes (Espinar et al., 2015).

The goal of this study was to explore the variability of δ^{15}N values measured in helophytes (emergent aquatic plants) and N concentrations in surface waters to identify the major land-derived N sources and spatial distribution of N loading in the Doñana wetland complex. We compared δ^{15}N values measured in the two helophyte species (*B. maritimus* and *T. domingensis*) and N concentrations in entry streams and in the WHS marsh. We did this during two hydroperiods with contrasting precipitation patterns. We assessed whether the isotopic variation in plants and the N concentration in surface waters were higher in streams, owing to a higher impact of anthropogenic activities in the watersheds. We expected these parameters to be lower in the protected marsh due to the greater distance from intensive anthropogenic activities in the watersheds, and the strong N mitigation capacity of helophytes and microbial processes in the marsh (e.g. denitrification) (Hinshaw et al., 2017; Tortosa et al., 2011).

We also considered whether the stream in the watershed with the highest level of agricultural activity and urbanization ("El Partido") had higher δ^{15}N values and DIN concentrations. We expected this owing to the influence of urban wastewaters, and also due to the highly degraded state of the riparian vegetation, which is likely to reduce the N buffering capacity of the stream in response to diffuse N inputs from agricultural and livestock farming practices (Borja et al., 2009; Pinay et al., 2018).

2. Material and methods

2.1. Study area

Doñana is located in the estuary of the Guadalquivir River on the Atlantic coast in Southwestern Spain (37°0'N 6°37'W) (Fig.1) and is of international importance for biodiversity conservation (Green et al. 2017, 2018). The natural Doñana marshes are situated in a seasonal brackish floodplain (360 km^2) within a National Park declared in 1969 and later designated as a Biosphere Reserve, Ramsar Site, Special Protection Area for birds and WHS (Green et al. 2016). We studied the marsh system and entry streams ("La Rocina" and "El Partido", see Fig. 1) draining an area under different anthropogenic pressures such as agriculture, livestock, and urban wastewaters (WWF, 2017). The climate is subhumid Mediterranean with an Atlantic influence. Mean annual temperature is 17°C and the mean annual precipitation is 550 mm, ranging between years from 170 mm (2004-2005) to 1000 mm (1995-1996). Flooding dynamics in the marshes are highly dependent on seasonal and interannual variation in precipitation, mainly concentrated between October and April, with a dry season from May to September when the marshes dry out completely (Díaz-Delgado et al., 2016). However, data on annual water inputs are limited. Direct precipitation and entry streams are the main water sources in the marsh, which were estimated to contribute 70-190 hm^3/year and 20-140 hm^3/year, respectively (Castroviejo, 1993). Apart from direct precipitation, "La Rocina" and "El Partido" stream basins receive water inputs from the aquifer where mean discharges were estimated at 34 and 11 hm^3/year respectively, although flows have since been reduced due to groundwater extraction (Guardiola-Albert and Jackson, 2011; Manzano et al., 2005). Therefore, due to seasonal precipitation and groundwater abstraction, both streams are intermittent throughout the year.

More than half of "La Rocina" catchment area (400 km^2) is protected within the Doñana Natural Space (DNS). However, the northern area is used for intensive fruit culture (strawberry, blueberry, raspberry and blackberry) irrigated with groundwater, and fertilizer inputs have increased nitrate concentrations in "La Rocina" in recent decades (Tortosa et al., 2011). "El Partido" is a 39 km-long torrential stream which enters the protected area (DNS) 6 km before discharging into the Doñana marsh. "El Partido" catchment (308 km^2) has been subjected to channelization, deforestation, agricultural intensification and an increasing human population since the 1950s. This stream receives nutrient inputs from three different WWTP effluents (Fig.1) and also from agricultural and livestock farming runoff, especially during intense precipitation events when the stream flow may increase more than fifty-fold in a few hours (García-Novo et al., 2007; Mintegui Aguirre et al., 2011). Livestock farming pressure is notably higher in the “El Partido” than “La Rocina” watershed (see supplementary material). Moreover, it is likely that untreated waste from agricultural workers enters both catchments. Other water courses entering in the north-east of the marsh were not studied in detail, although we sampled water and helophytes close to the entry points.

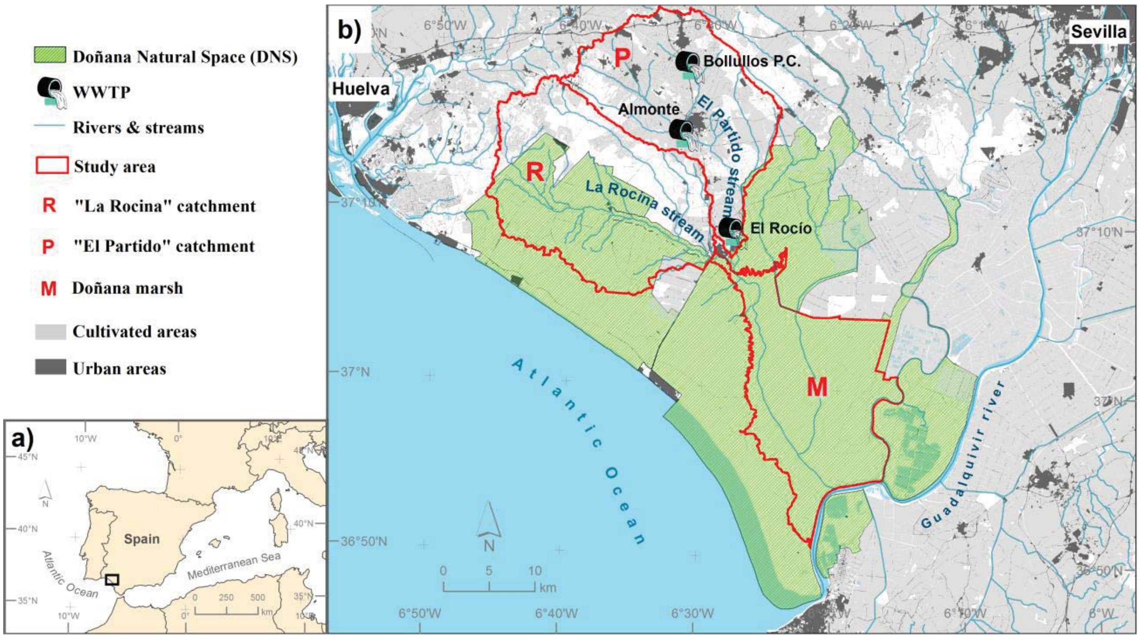


Figure 1. Location of (a) the Doñana wetlands in western Europe and (b) the limits of the Doñana Natural Space (DNS), the marsh (M) and the two catchment areas for the streams included in this study (“La Rocina” (R) and “El Partido” (P)). Two major anthropogenic pressures in this area are represented in the map (agriculture as `cultivated area´ and urban pollution as `WWTP´ (Waste Water Treatment Plants). Boundaries of “La Rocina” and “El Partido” catchments were delineated using a five metres digital terrain model (MDT05-PNOA) through digital aerial photogrammetry and automatic stereoscopic correlation by the Spanish

National Geographic Institute (http://pnoa.ign.es/). Marsh boundaries were delinated using Landsat time series inundation masks and photo interpretation. This work was carried out by the Remote Sensing Lab (LAST) at Doñana Biological Station (EBD-CSIC, Seville).

2.2. Field sampling

Our study period included the 2015-2016 hydrological years (where 2015 spans from September 2014 to August 2015, and 2016 from September 2015 to August 2016). We collected plant samples for δ^{15}N analysis from two abundant helophyte species: (1) *Bolboschoenus maritimus* (alkali bulrush) which is found across the intermittent, shallow marsh system (Espinar and Serrano, 2009; Lumbierres et al., 2017) and (2) *Typha domingensis* (southern cattail), mainly found along entry streams. We collected samples during the beginning of their growing season (April-May) when plants are actively uptaking inorganic N for biomass production, so δ^{15}N values measured in these new leaves can act as integrators of the surrounding environmental N isotopic signature (Dawson et al., 2002; Robinson, 2001). *B. maritimus* was sampled in 2015 and 2016 in the marsh and streams. *T. domingensis* was only sampled in 2016, to increase coverage of points in streams such as upstream/downstream of WWTPs. At each sampling site, we collected one to four replicates of green leaves. In the marsh, we collected leaves from *B.maritimus* plants which were separated by ≥10 m to avoid pseudoreplication due to sampling the same individual. In the streams, the area covered by *T. domingensis* and *B.maritimus* at each sampling site was comparatively smaller, so we collected samples separated by a shorter distance. To measure ambient N concentrations, we collected one surface-water sample per site on a monthly basis during the sampling period (Dec. 2014 – May 2015; Oct.2015- June 2016). However, the location and timing of water sampling varied between months and years, owing to changes in the spatial distribution of water. In 2016 water was scarce or absent in some areas due to low levels of precipitation. One of the advantages of using helophytes as indicators was that they could still be sampled under these conditions. As a result, there were some differences in the sets of sampling sites for helophytes and for water.

Furthermore, given the importance of the impact of the extensive strawberry culture and associated fertilizers, for reference we also collected a *T. domingensis* sample from a small catchment to the west which is entirely cultivated from strawberries (entry stream to Laguna Primera de Palos at 37º 10' 23.25'' N, 6º 53' 13.52'' O).

2.3. Laboratory analyses

2.3.1. Nitrogen stable isotope analyses

We combined the replicates of leaves collected at each sampling site into one composite sample. Leaves were cut into smaller pieces, dried at 60°C to constant weight, and then ball-milled to a fine powder in a mixer mill (Rerscht MM400, Germany). We weighed subsamples of powdered material (1.8 mg plants) and placed them in tin capsules for $\delta^{15}N$ determination at the Laboratory of Stable Isotopes of the EBD-CSIC (www.ebd.csic.es/lie/index.html). Samples were combusted at 1020ºC using a continuous flow isotope-ratio mass spectrometry system (Thermo Electron) by means of a Flash HT Plus elemental analyser interfaced with a Delta V Advantage mass spectrometer. Stable isotope ratios were expressed in the standard δ-notation (‰) relative to atmospheric N_2 ($\delta^{15}N$). Based on laboratory standards, the measurement error was ± 0.2‰. Standards used were IAEA-600 (Caffeine), LIE-P-22 (Casein, internal standard), LIE-BB (whale baleen, internal standard) and LIE-PA (razorbill feathers, internal standard). These laboratory standards were previously calibrated with international standards supplied by the International Atomic Energy Agency (IAEA, Vienna).

2.3.2. Dissolved and total nitrogen analyses

To measure dissolved N, we first filtered the samples in the laboratory on each sampling day through FILTER-LAB MFV5047 glass-fiber filters (0.45µm pore size) using a low-pressure vacuum pump. We then stored all samples (plants and water) in the freezer (-20°C) until analysis. To measure the concentration of three dissolved inorganic nitrogen (DIN) species, nitrate (NO_3^-), nitrite (NO_2^-) and ammonium (NH_4^+), we used standard colorimetric methods

(ISO 13395:1996 for nitrate and nitrite; ISO 11732:2005 for ammonium) on a multi-channel SEAL Analytical AA3 AutoAnalyzer (Norderstedt, Germany) at the Laboratory of Aquatic Ecology of EBD-CSIC (Seville, Spain). We analyzed total nitrogen (TN) by digestion with potassium (Nydahl, 1978). TN is the sum of the organic N, N-NO_3^-, N-$NO_2^-$and N-NH_4^+ in the water sample. Limit of detection for the analytical methods are: 0.004 μmol/L for N-NO_3^- and N-NO_2^-, 0.04 μmol/L for N-NH_4^+ and 40 μg/L for TN.

2.4. Statistical analysis

We performed a three-factor ANOVA to analyze the effects of habitat ((1) "La Rocina" stream, (2) "El Partido" stream and (3) the marsh), year (2015 or 2016) and plant species (*B. maritimus* and *T. domingensis*) on helophyte isotopic signatures (δ^{15}N). We checked normality of the dependent variable (δ^{15}N) and homoscedasticity of the model by the inspection of normal plots (Q-Q plots) and "Residuals vs Fitted" plots, respectively. We applied Tukey's post-hoc tests to identify significant differences between habitats. We also compared the coefficient of variation (CV) of δ^{15}N between these three habitats.

To analyze the effects of habitat on N concentrations in water, we first applied log or squared root transformation to improve normality. Because some parameters retained a highly skewed distribution after transformations, and we could not remove heteroscedasticity, we tested the differences in N concentrations between habitats within a given year using non-parametric tests (Kruskal-Wallis and post-hoc Wilcoxon tests).

We also used linear regression models to test the relationship between helophyte δ^{15}N values and the water N concentrations from the same sampling sites. These water samples were collected at specific periods which were representative of the usual flooding regime in the marsh, which normally starts at the beginning of the rainy period (Oct.-Dec.), reaching the maximum flooding extent during the winter (Feb.-Mar.) and decreasing during the spring (Apr.-

Jun.) until it completely dries up in summer (July-Aug.) (Díaz-Delgado et al., 2016). Accordingly, $\delta^{15}N$ values in 2015 were related to average water N concentration values from samples collected in December, February and May, and $\delta^{15}N$ values in 2016 with average N concentration values from December and April. We used equal numbers of sites for linear regression analyses in both years (n=15), but in 2015 the majority of them were located within the marsh, whereas in 2016 most of the selected sites were located within the entry streams. This was largely because in many sites at which we sampled plants in 2016, surface water was not available because of changes in the spatial distribution of water between years (Fig.2). We also included a categorical variable to control for helophyte species (*B. maritimus* and *T. domingensis*) in the models. We performed all the statistical analyses using R software (v 3.3.2). We used Sigmaplot (v 12) to make graphs.

2.5. Geospatial interpolation of N concentrations in the marsh

We used the set of data points collected in the marsh (i.e. water N concentrations) to assign values to the rest of unmeasured locations within the marsh boundaries by applying the Inverse Distance Weighting (IDW) method (Johnston et al. 2001, Kumar et al. 2007). We calculated each unknown point with a weighted average of the nine nearest values among the known points. As a result, we obtained different colored maps representing the DIN and TN concentrations using a three colour scale, with red tones representing the highest values and blue tones the lowest ones. We used ArcMap (10.2.1) to make the maps and geospatial interpolations.

2.6. Marsh flooding masks

We used different flooding masks to monitor the inundation of the Doñana marsh (Fig.2). These flooding masks were generated by the LAST-EBD using mid-infrared band 5 (1.55-1.75μm, TM and ETM+) and band 4 (0.8-1.1 µm, MSS) to produce final inundation masks based on 30 x 30 m pixels from Landsat images (see details in Bustamante et al. 2009; Díaz-Delgado et al. 2016).

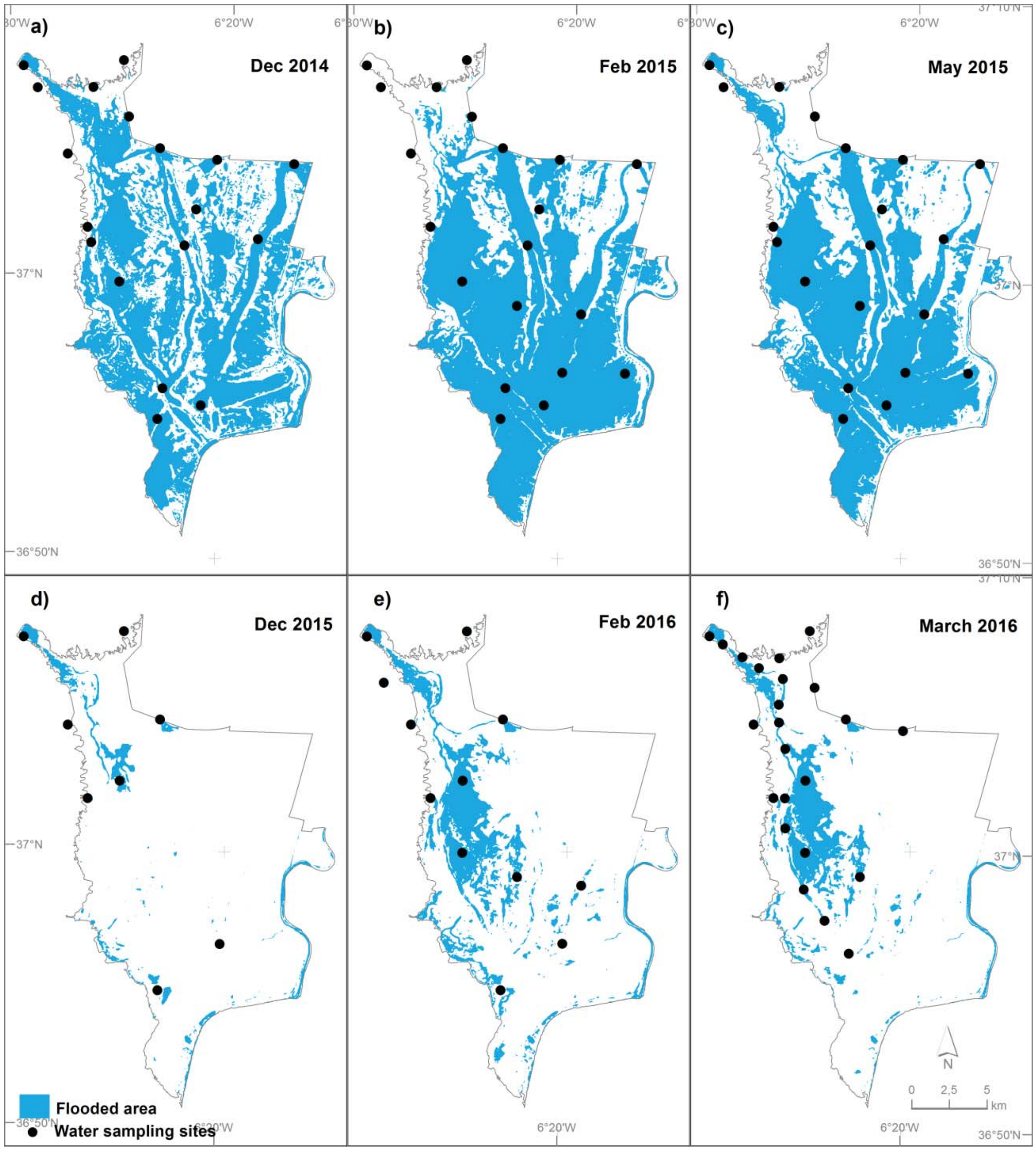


Figure 2. Extent of the flooded area in the Doñana marsh in two hydrological years (2015 and 2016). Inundation masks are based on Landsat 7 (ETM sensor) images acquired on 10 December 2014 (a), 19 May 2015 (c) and Landsat 8 (OLI) images from 20 February 2015 (b), 5 December 2015 (d), 23 February 2016 (e), 10 March 2016 (f). Dots represent sampling points

where we collected water samples. Dots in (f) show points where water was sampled in May 2016. We represent the flooded area in March instead of May 2016 because the former image is more similar to the flooding extent during our sampling in early May. After we completed water sampling, several days of intense precipitation reflooded the Doñana marsh, and the first satellite image in May was taken on the 21st after this major flooding event (no images were available in April).

3. Results

3.1. Spatial variation in plant $\delta^{15}N$ values

In a three-factor ANOVA, we analyzed simultaneously the effects of habitat ("La Rocina", "El Partido", marsh), year (2015 or 2016) and plant species on the isotopic signatures ($\delta^{15}N$) (Fig. 3, 4), and found that only habitat had a statistically significant effect ($F_{2, 74} = 18.79$, $P < 0.001$). Tukey tests revealed significantly higher $\delta^{15}N$ levels at "El Partido" stream than in the marsh ($P < 0.001$) and "La Rocina" ($P=0.005$). However, "La Rocina" stream and the marsh did not show a significant difference ($P = 0.217$). Neither plant species ($F_{1, 74}=1.143$, $P= 0.288$) nor year ($F_{1, 74}=0.017$, $P= 0.897$) had significant effects on $\delta^{15}N$. There was also a difference in the coefficient of variation (CV) between habitats, with higher values in streams (2015: $CV_{Partido}=33.22\%$, $CV_{Rocina}=47.35\%$, $CV_{marsh}=31.39\%$; 2016: $CV_{Partido}=40.40\%$, $CV_{Rocina}=35.28\%$, $CV_{marsh}=32.47\%$). Our additional sample of *T. domingensis* from the nearby Laguna Primera de Palos catchment dedicated entirely to strawberry culture had a relatively low $\delta^{15}N$ value of +6.03‰.

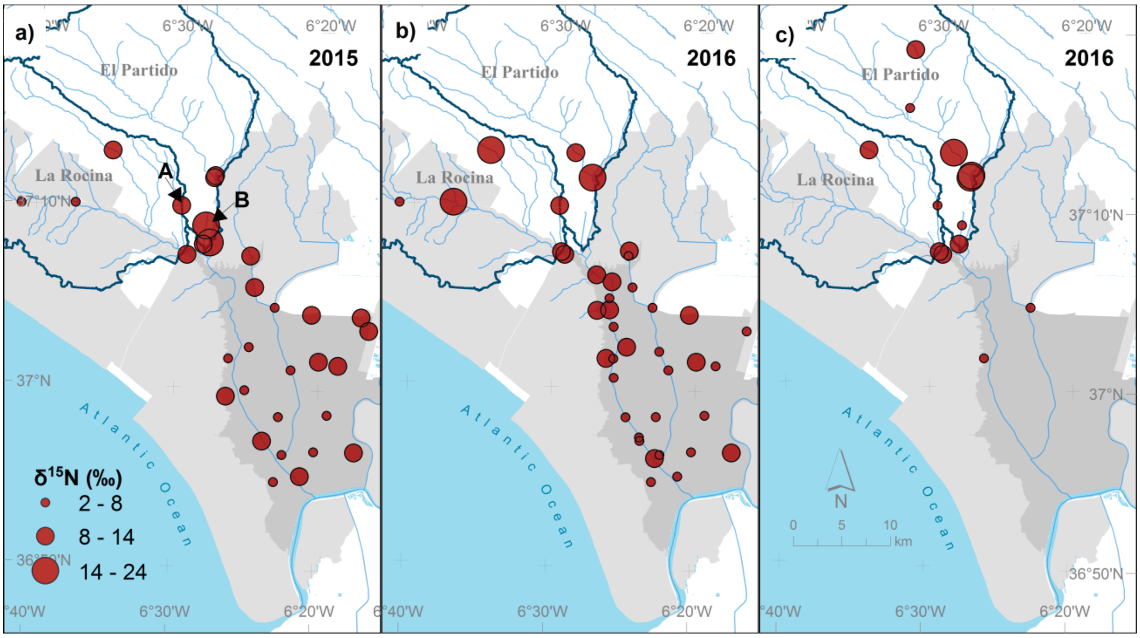


Figure 3. Variability of nitrogen stable isotopes obtained from helophytes. (a) *B. maritimus* collected in April-May 2015. (b) *B. maritimus* and (c) *T. dominguensis* collected in April-May 2016. Dot size represents the isotopic values at each site (δ^{15}N (‰)). In map (a) A and B indicate the points with the highest N concentration in relation to the measured δ^{15}N values, as shown in Figure 5. A corresponds to a water leak from a broken pipe transporting groundwater for human consumption. B is the outflow of El Rocío WWTP.

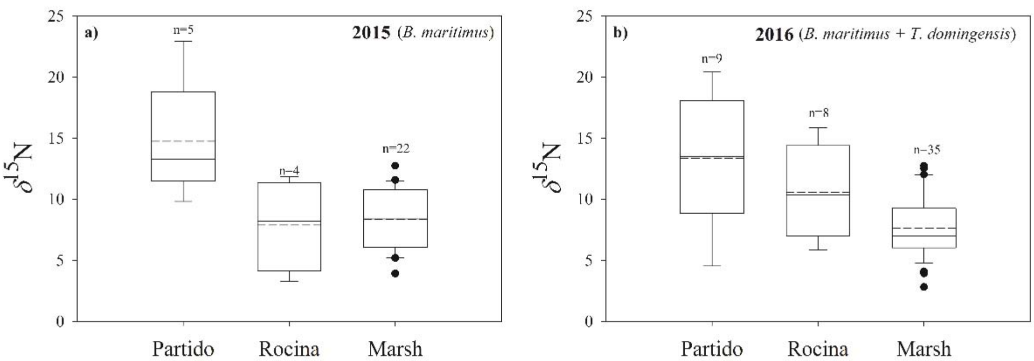


Figure 4. Box plots of (a) isotopic signatures (δ^{15}N, (‰)) of *B. maritimus* in 2015 and (b) *B. maritimus* + *T. domingensis* in 2016 in each habitat ("El Partido" stream, "La Rocina" stream and the marsh). The solid horizontal line shows the median of δ^{15}N values. The dashed

horizontal line shows the mean of $\delta^{15}N$ values ($Partido_{2015}$= 14.75±2.19 (s.e.); $Rocina_{2015}$= 7.87±1.86; $Marsh_{2015}$=8.35±0.55; $Partido_{2016}$= 13.35±1.79; $Rocina_{2016}$= 10.54±1.31; $Marsh_{2016}$= 7.67±0.41). The bottom and top of the box show the 25th and 75th percentiles, respectively. The whiskers are drawn out to the 10th and 90th percentiles (Cleveland method). Extreme values outside these percentiles are marked as outliers.

3.2. Spatial variation in N concentrations in surface waters

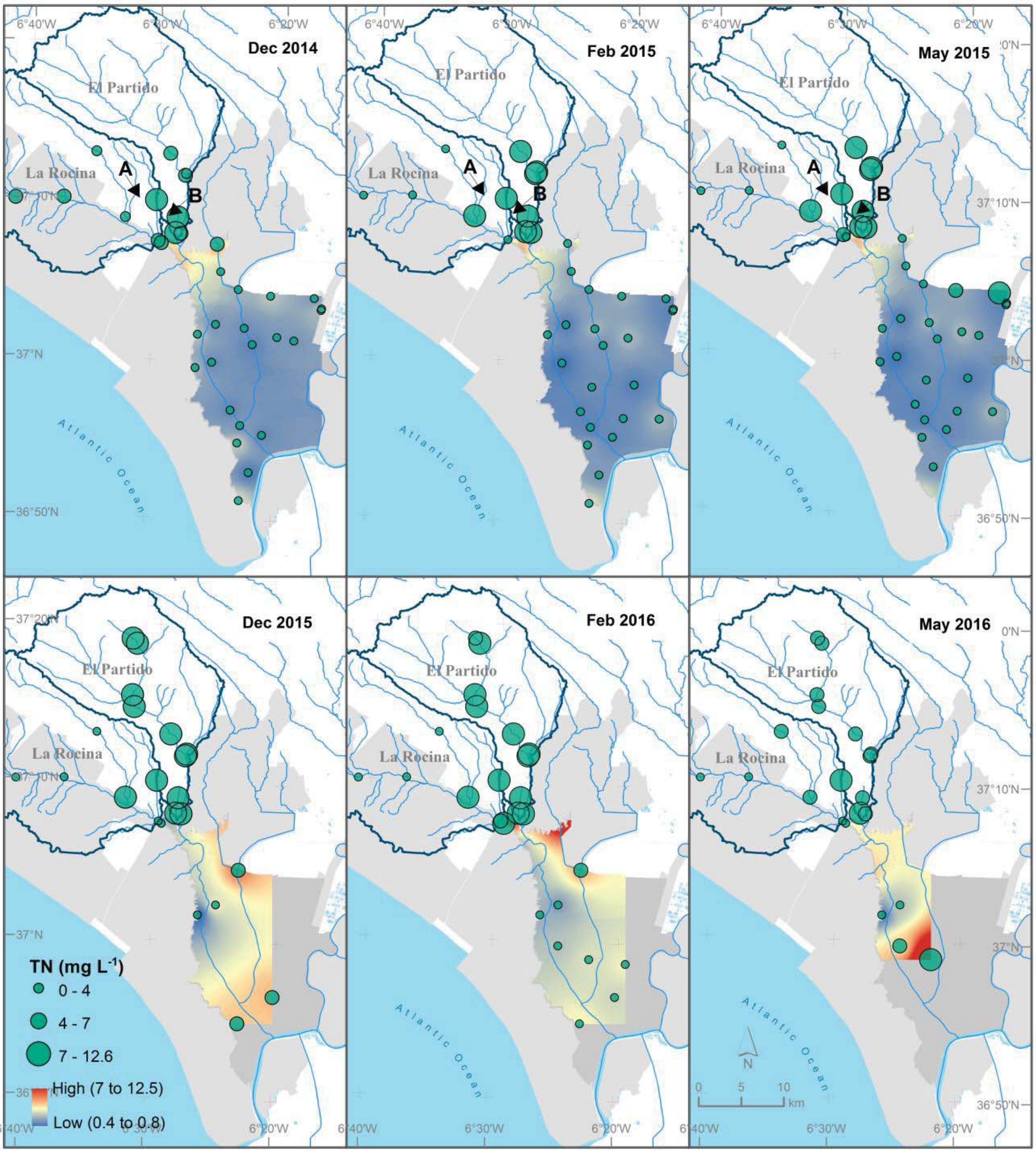
Water N concentrations were much higher in entry streams than in the marsh, the difference often being several orders of magnitude in the case of NO_3^-, NO_2^- and NH_4^+concentrations (Table 1, Fig. 5). Differences between the two streams and the marsh were statistically significant, except for the differences regarding NO_2^- and NH_4^+concentrations in 2016 between “La Rocina” and the marsh (Table 1). Although NO_3^-, NO_2^- and NH_4^+ levels were also considerably higher at “El Partido” compared to “La Rocina” in both years, these differences were not statistically significant (Table 1), although sample sizes were small.

Table 1. Comparison of the concentration of different dissolved inorganic N (DIN) species plus Total N (TN) among two streams (“La Rocina” and “El Partido”) and the Doñana marsh in 2015 and 2016 using a Kruskal-Wallis test. Cells with different letters (“a” and “b”) indicate significant differences (α=0.05) among medians within each N variable group. Median values were calculated for each sampling point using N concentration data excluding summer months (June, July and August). The `Sample size´ column refers to the number of sampling points. Each year we collected a different number of samples (one to twelve) per sampling point, because some points dried out faster, or were less accessible, than others (Fig. 2).

Year	Variable	Median (mg N L^{-1}) [25% -75% percentile]			Sample size (n)			df	χ^2	P
		El Partido stream	La Rocina stream	Marsh	El Partido stream	La Rocina stream	Marsh			
2015	$N\text{-}NO_3^-$	2.528 ^{b} [2.319 – 2.864]	0.746 ^{b} [0.408 – 2.227]	0.008 ^{a} [0.002 - 0.021]	6	6	28	2	19.204	<0.001
	$N\text{-}NO_2^-$	0.268 ^{b} [0.250 – 0.288]	0.013 ^{b} [0.008 – 0.026]	0.005 ^{a} [0.003 - 0.008]	6	6	28	2	19.571	<0.001
	$N\text{-}NH_4^+$	1.619 ^{b} [0.390 – 2.257]	0.051 ^{b} [0.023 – 0.175]	0.018 ^{a} [0.015 - 0.027]	6	6	28	2	13.104	0.001
	TN	8.070 ^{b} [7.774 – 9.078]	3.445 ^{b} [2.875 – 5.664]	1.944^{a} [1.729 - 3.074]	6	6	28	2	16.928	<0.001
2016	$N\text{-}NO_3^-$	3.186 ^{b} [3.014– 3.226]	0.418 ^{b} [0.169 – 2.663]	0.001 ^{a} [0.001 - 0.008]	10	6	11	2	16.542	<0.001
	$N\text{-}NO_2^-$	0.257 ^{b} [0.191 – 0.340]	0.027 ^{ab} [0.008 – 0.069]	0.005 ^{a} [0.002 - 0.008]	10	6	11	2	18.920	<0.001
	$N\text{-}NH_4^+$	0.637 ^{b} [0.574 – 0.889]	0.099 ^{ab} [0.071 – 0.182]	0.037 ^{a} [0.028 - 0.060]	10	6	11	2	17.805	<0.001
	TN	8.773 ^{b} [7.988 – 9.284]	3.476 ^{a} [2.379 – 7.229]	3.982 ^{a} [3.416 - 4.423]	10	6	11	2	10.751	0.005

Geospatial interpolation suggests that, within the marsh, the N concentrations are highest in areas close to the mouth of entry streams (Fig.5). This is more obvious for DIN than for Total N, and is especially obvious in December 2014 when we found particularly high DIN concentrations in both the north-west and north-east areas of the marsh (Fig. 5b).

a)



Dec 2014
Feb 2015
May 2015
Dec 2015
Feb 2016
May 2016
El Partido
La Rocina
A
B
Atlantic Ocean
TN (mg L⁻¹)
0 - 4
4 - 7
7 - 12.6
High (7 to 12.5)
Low (0.4 to 0.8)
N
0 5 10 km
6°40'W
6°30'W
6°20'W
37°10'N
37°N
36°50'N
37°20'N

356

b)

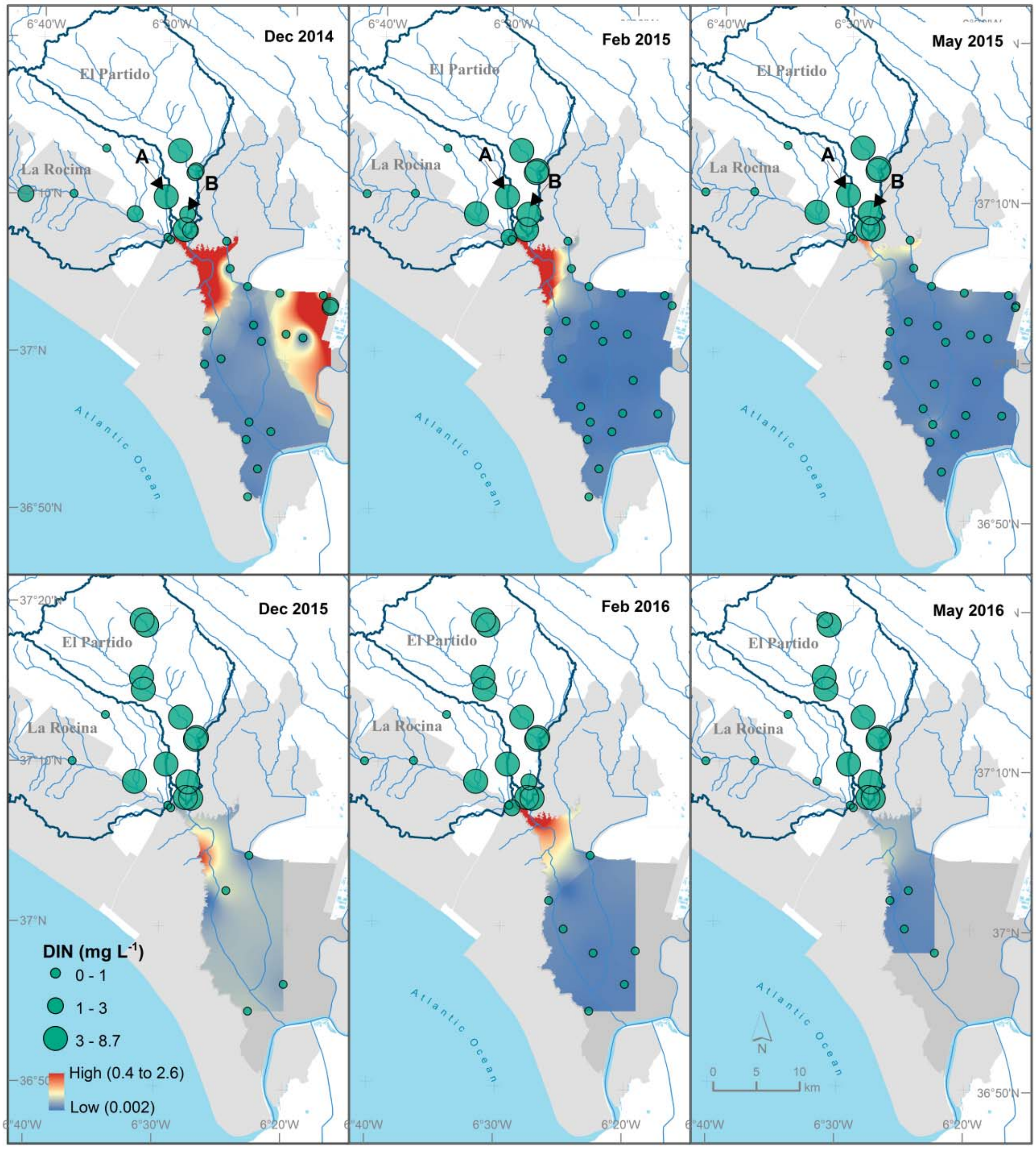


Figure 5. (a) Total N (TN) and (b) dissolved inorganic nitrogen (DIN) (mg N L^{-1}) variability in two hydrological years (2015 and 2016) in the Doñana marsh and entry streams from the "La Rocina" and "El Partido" catchments. Dot size represents the average values of all samples collected in December, February and May each year. The colour gradient shows the result of TN and DIN data interpolation in the marsh. Points A and B are explained in Figure 3. DIN is the sum of NO_3^-, NO_2^- and NH_4^+ concentrations.

3.3. Relationship between $\delta^{15}N$ and N concentrations

Linear regression models revealed that isotopic values in plants were related to N concentration in surface waters. In 2015, we found a significant positive relationship between $\delta^{15}N$ values of *B. maritimus* and the concentration of three of the four measured N species (NO_2^-, NO_3^-, TN) (Table 2, Fig. 6). In 2016, all the relationships were again positive, but we only found a significant relationship between the helophyte $\delta^{15}N$ values (*B.maritimus* + *T. domingensis*) and the NO_2^- in a model corrected for the partial effect of plant species (Table 2, Fig. 6).

Table 2. Results of linear regression models with the isotopic signatures ($\delta^{15}N$ (‰)) as the dependent variable and the mean N concentrations of different N species in water samples (NO_3^-, NO_2^-, NH_4^+, TN) as predictor variables. In 2016 \`plant species´ was also included as a fixed factor. Concentrations were log transformed. In 2015, log transformations did not entirely eliminate heterocedasticity (Fig. 6) so p-values (P) should be treated with some caution.

Year	Response variable	Adj.R^2	Explanatory variables	df	Estimate + SE	F	P
2015	$\delta^{15}N$ (*B.maritimus*)	0.401	Log N-NO_2^-	12	1.749± 0.561	9.709	0.008
		0.311	Log N-NO_3^-	12	0.750±0.223	6.870	0.022
		0.011	LogN-NH_4^+	12	1.039±0.970	1.148	0.305
		0.324	Log NT	12	2.632±0.977	7.258	0.019
2016	$\delta^{15}N$ (*B.maritimus* + *T. domingensis*)	0.140	Log N-NO_2^- (species)†	18	1.201 ±0.531	2.633	0.036
		0.081	Log N-NO_3^- (species)†	18	0.730 ±0.383	1.883	0.180
		-0.02	Log N-NH_4^+ (species)†	18	0.800 ±0.661	0.795	0.241
		0.077	Log NT (species)†	18	0.084±0.076	1.837	0.187

† These models were corrected for the partial effect of plant species (*B.maritimus* and *T. domingensis*), but the species effect was not statistically significant.

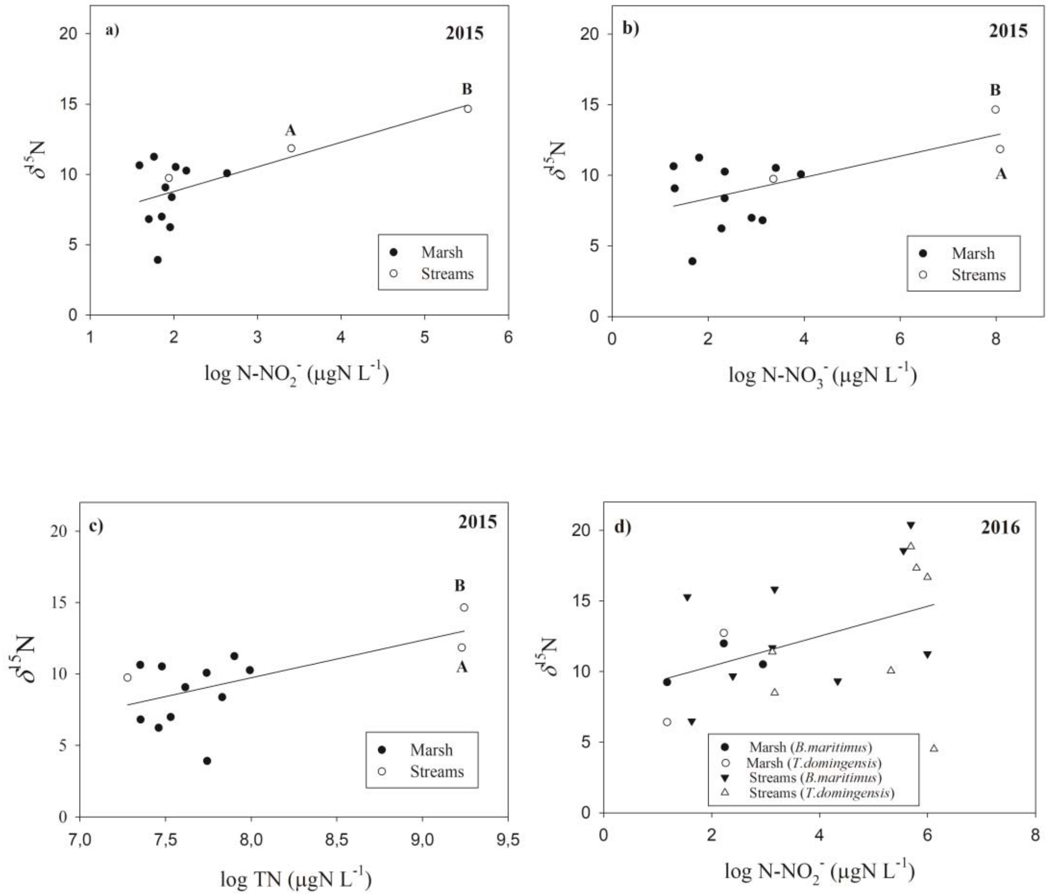


Figure 6. Relationship between $\delta^{15}N$ (‰) of helophytes (*B. maritimus* and *T.domingensis*) and mean of surface water N concentrations (NO_2^-, NO_3^- and TN in μg N L^{-1}) collected in 2015 (a,b,c) and 2016 (d) in the Doñana marsh and entry streams. In 2015 we only measured $\delta^{15}N$ in *B.maritimus* and sampled water three times per point. In 2016 we measured $\delta^{15}N$ in *B.maritimus* and *T.domingensis* and sampled water two times per point. See Fig. 3 for location of A and B sampling points.

4. Discussion

We provide the first study of N isotopic values ($\delta^{15}N$) in aquatic plants in combination with N concentrations in surface waters of the Doñana wetland complex, and show that land-use changes from natural habitats to urban use and intensive agriculture over recent decades have led to N pollution in entry streams, as well as surface waters of the Doñana marsh within the WHS.

The high spatio-temporal variability observed in N concentrations and δ^{15}N values in our study are indicative of different N sources (anthropogenic and natural) typically occurring in mixed agricultural and urban landscapes (Carpenter et al., 1998). Anthropogenic N inputs are reflected by the generally high water N concentrations and mean helophyte δ^{15}N values in the streams compared to the marsh, especially within "El Partido" watershed which is most likely affected by isotopically-enriched N sources due to strong human pressures such as urban wastewaters, animal farming and of crops with manure fertilization (Heaton 1986; Mayer et al. 2002; Wigand et al. 2007; Inglett and Reddy 2006). In contrast, we found relatively low δ^{15}N values in helophytes of "La Rocina" watershed, pointing to the dominance of isotopically-depleted inorganic N sources, most likely fertilizers used for irrigated agriculture (Bol et al., 2005; Vitòria et al., 2004).

4.1. Excessive N loading in Doñana entry streams

In the last decades, several studies have reported anthropogenic N pollution in surface and groundwater of the Doñana region, particularly regarding contamination by DIN related to the intensive use of fertilizers and the discharge of urban wastewaters into the stream (Arambarri et al., 1996; Olias et al., 2008; Serrano et al., 2006; Tortosa et al., 2011). In this study we found that, regardless of the sampling period, N concentrations in the streams were much higher compared to the marsh (Fig. 5). Particularly, we found the highest N concentrations in "El Partido" stream, which are likely related to the discharge of continuous effluents from three WWTPs (Fig.1) and their frequent noncompliance with the EU Wastewater Treatment Directive (91/271/EEC). Indeed, we recorded higher N concentrations downstream of WWTP entry points than upstream. The influence of human waste-waters in the north-west area of the Doñana marsh is also confirmed by the presence of high genetic diversity of *E. coli* virulence genes

(Cabal et al., 2017). Wastewater inputs have been directly linked with botulism outbreaks that cause waterbird mortalities in Spanish wetlands (Anza et al., 2014), and such mortalities have often been reported from Doñana.

Moreover, we generally observed higher DIN concentrations in both streams during low flow and high temperature conditions. Under such conditions, many aquatic plants and invertebrates may be highly sensitive to DIN concentrations (Corriveau et al., 2010). We recorded NH_4^+ concentrations in "El Partido" that often exceeded guidelines for good ecological status (>1 mg NH_4^+/L) based on reference values established under the Water Framework Directive (WFD) for some Spanish rivers (Real Decreto 817/2015). We also detected high NO_2^- concentrations in streams (1 - 2.4 mg L^{1-}), likely to have toxic or even lethal effects in aquatic organisms (Kocour Kroupova et al., 2016). Although not included in our study, the Guadiamar river (flowing into the north-east area of the Doñana marsh) is also affected by N pollution from anthropogenic activities in the Guadiamar watershed (Alonso et al., 2004).

We recorded much lower N concentrations in the marsh than in the streams, especially away from the north-west and north-east areas close to the mouths of the streams, suggesting that the marsh is providing an ecosystem service of water purification, by reducing the N content in the water from polluted streams. This was predictable given the abundance of helophytes such as *B. maritimus* (Gottschall et al., 2007; Jan Vymazal, 2013; Lumbierres et al., 2017). Nevertheless, this is likely to come at a cost of reduced biodiversity and limited resilience of the marsh to further eutrophication. For example, N surplus increases the probability of harmful cyanobacteria blooms in the marsh, causing strong negative impacts such as mass mortalities of fish and waterfowl (Lopez-Rodas et al., 2008). The reductions in water inputs and increases in temperature associated with climate change make such events more likely, emphasizing the need to take action to reduce anthropogenic nutrient inputs (Green et al. 2017). The areas of the marsh with the highest DIN concentrations (Fig. 5) also have the highest chlorophyll-*a* concentrations, confirming eutrophication effects (authors unpublished data). Moreover, water inputs into the marsh are driven by monthly and interannual rainfall variations which strongly affect N concentrations in the surface water. During prolonged dry periods, entry streams are practically the only water input into the marsh. There was less precipitation, and consequently a smaller flooded area, in 2016 than in 2015 (Fig.2). This probably explains the observed higher TN values in the marsh in 2016 (Fig.5), as a result of decreasing dilution capacity of the water column. Moreover, during dry years vegetation growth is limited in the marsh, which leads to reduced N removal capacity by denitrification in the sediment (Hinshaw et al., 2017).

In a future scenario of decreasing precipitation and increasing temperatures combined with land-use intensification, we can expect a loss in the capacity of the vegetation and microbial communities in the streams and marsh to remove N from surface waters, and an increase in harmful effects of eutrophication in the Doñana system. Thus, local measures to control N and P pollution such as reduced fertilizer leaching, green filters or tertiary wastewater treatments are necessary to improve the conservation status in Doñana and increase ecosystem resilience. Reduced groundwater extraction for agriculture would also help to maintain groundwater discharge into streams and hence dilute nutrient concentrations (Green et al. 2017).

4.2. Helophyte δ^{15}N as an indicator of anthropogenic pressures

As with submerged aquatic plants, high δ^{15}N values in helophytes are likely to indicate dominant organic sources of N from wastewaters, manure or bird guano, because of their high N isotopic signatures (Diebel and Vander Zanden, 2012; González-Bergonzoni et al., 2017). Between the three studied areas (“La Rocina”, “El Partido” and the marsh) we found higher δ^{15}N values in helophytes collected in “El Partido” (Fig.3, 4). We suggest this is strongly linked to a higher agricultural, urban and livestock farming pressure in this watershed in comparison to “La Rocina”, or indeed the marsh. This is despite the high density of ungulates (domestic and wild) and of colonial waterbirds (Gortázar et al., 2008; Ramo et al., 2013) in the marsh, whose excreta also have high signatures (Bedard-Haughn et al., 2003). We would expect high δ^{15}N in locations in the marsh with bird colonies, although we did not sample these areas so as to avoid disturbance.

The presence of WWTPs in "El Partido" watershed is likely to be the most influential N source explaining the high δ^{15}N values in helophytes, as WWTPs effluents are continuously discharging isotopically enriched N compounds into the stream. Diffuse N inputs such as agricultural land runoff mostly depend on the precipitation patterns, which in the Doñana region are highly variable (Díaz-Delgado et al., 2016).

"La Rocina" stream does not receive urban wastewaters, and other human pressures such as livestock farming, urban and industrial activities are limited (supplementary material). However, there is strong agricultural pressure in the watershed because it drains a large berry culture area, causing NO_3^- contamination of surface and ground water (Olias et al., 2008; Tortosa et al., 2011). The low δ^{15}N values we recorded in "La Rocina" watershed are in line with those recorded by Tortosa et *al.* (2011) in dissolved NO_3^- ($\delta^{15}N\text{-}NO_3^-$), suggesting that helophytes are indicating a surplus of isotopically-depleted inorganic N fertilizers, normally ranging from -4 to +6‰ (Vitòria et al., 2004). The likely influence of inorganic fertilizers in our samples is supported by the low δ^{15}N value recorded in our reference stream whose catchment is 100% strawberry fields.

However, δ^{15}N values recorded in helophytes do not provide a complete means to distinguish the anthropogenic or natural origin of N. On the one hand, when only measuring δ^{15}N values, N sources are undistinguishable when they show similar values (e.g. inorganic fertilizers vs. atmospheric N precipitation). On the other hand, plants are not conservative tracers of N due to N fractionation occurring during N assimilation, which together with other biological, chemical and physical N cycling processes in the system results in ^{15}N enrichment of the original N source. Therefore, δ^{15}N values in helophytes do not reflect only the N sources but also the N fractionation processes (Robinson, 2001). In our study, we did not quantify the contribution of N fractionation processes, but we would expect them to have an influence, and to vary according to sampling locations and time. A previous study carried out in "La Rocina" stream found that potential denitrification (i.e. ^{15}N enrichment) increased at those locations with higher organic matter content in the sediments (Tortosa et al., 2011). Furthermore, the presence of vegetation in wetlands can increase denitrification rates (Hinshaw et al., 2017; Valiela and Cole, 2002).

4.3. Relationship between water N concentrations and plant $\delta^{15}N$ values

The relationship we found between water N concentrations and helophyte $\delta^{15}N$ values (Fig.6) was weaker than those recorded in submerged estuarine plants (McClelland et al., 1997; Savage and Elmgren, 2004). Indeed, variation in water N concentration explained relatively little of the variation of $\delta^{15}N$ values. At some points, we found high N concentrations and high $\delta^{15}N$ values coincided, e.g. downstream from WWTPs. On the other hand, there were other points with high water N concentrations but low $\delta^{15}N$ values in "La Rocina" stream where high N concentrations are largely due to inorganic fertilizers.

4.4. Conclusions

Long-term conservation programs for a complex wetland system such as Doñana necessarily require a combination of different monitoring tools to better understand the impacts of different human pressures and climate change, such as N pollution (Green et al. 2017). Stable isotope tracers such as $\delta^{15}N$ in biota can be a useful indicator of anthropogenic nitrogen in monitoring programs. Helophytes are of particular interest in shallow Mediterranean wetlands, because they are widespread and abundant plant species that can integrate information when there is much temporal variability in precipitation, water levels and N sources, and because they can be sampled even when surface water is not available during dry periods. Strong spatio-temporal variability in standing water is typical of aquatic systems with a Mediterranean climate (Cook et al., 2016; Gasith and Resh, 1999), which also typically receive anthropogenic N inputs from fertilizers (organic and inorganic) and WWTP effluents in the watershed.

Our results suggest that high δ^{15}N values recorded in helophytes along "El Partido" stream are linked to WWTP effluent discharge, together with seasonal runoff of organic N from agricultural areas. Thus, if wastewater treatment is improved in the Doñana catchment, we would expect the δ^{15}N values in stream helophytes to be reduced in the future, as observed in estuarine macrophytes when treatment of urban waters was enhanced (McClelland et al. 1997).

However, helophytes are not completely effective at distinguishing between N sources with either low δ^{15}N values (such as inorganic fertilizers or atmospheric N deposition) or high δ^{15}N values (such as manure or wastewaters) especially in highly mixed and anthropized landscapes such as the Doñana watershed. Furthermore, biogeochemical processes such as denitrification or ammonia volatilization may influence δ^{15}N values in helophytes due to ^{15}N enrichment of residual inorganic N in the sediment. Thus, further information or methodologies are desirable to detect the N origin within a wetland system more precisely. Future studies on N pollution in Mediterranean wetlands should include additional indicators that allow improved discrimination between N sources with similar δ^{15}N values. For example, multiple isotopic approach (Meghdadi and Javar, 2018), together with information on atmospheric N deposition or microbial activity rates, may improve determination of anthropogenic contamination in surface and ground waters.

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Figure

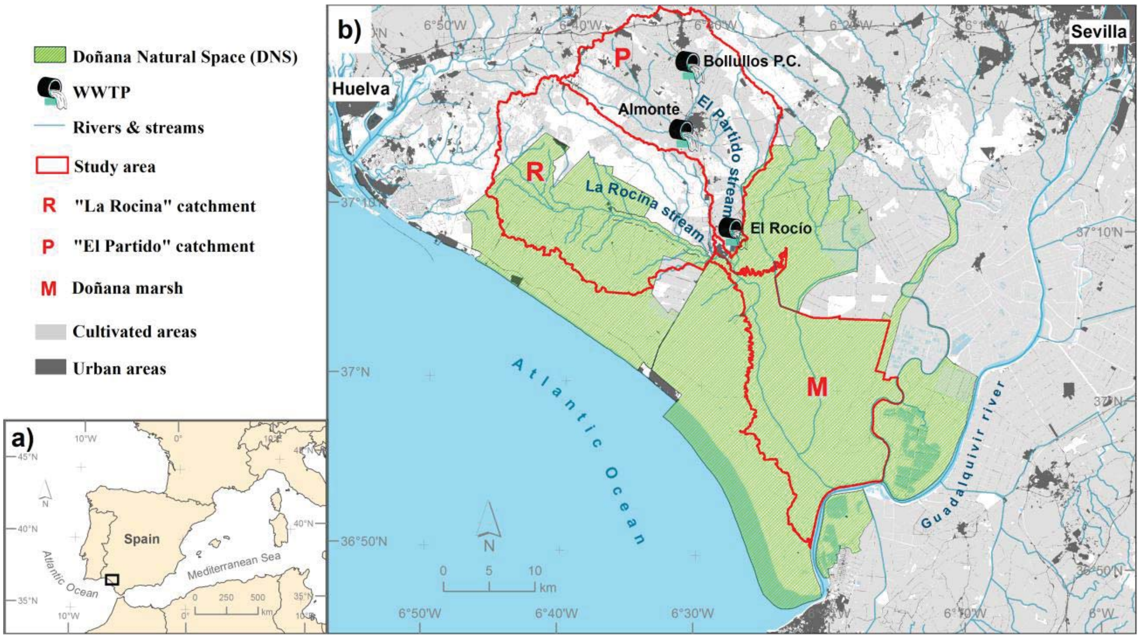


Figure 1. Location of (a) the Doñana wetlands in western Europe and (b) the limits of the Doñana Natural Space (DNS), the marsh (M) and the two catchment areas for the streams included in this study ("La Rocina" (R) and "El Partido" (P)). Two major anthropogenic pressures in this area are represented in the map (agriculture as `cultivated area´ and urban pollution as `WWTP´ (Waste Water Treatment Plants). Boundaries of "La Rocina" and "El Partido" catchments were delineated using a five metres digital terrain model (MDT05-PNOA) through digital aerial photogrammetry and automatic stereoscopic correlation by the Spanish National Geographic Institute (http://pnoa.ign.es/). Marsh boundaries were delinated using Landsat time series inundation masks and photo interpretation. This work was carried out by the Remote Sensing Lab (LAST) at Doñana Biological Station (EBD-CSIC, Seville).

Figure

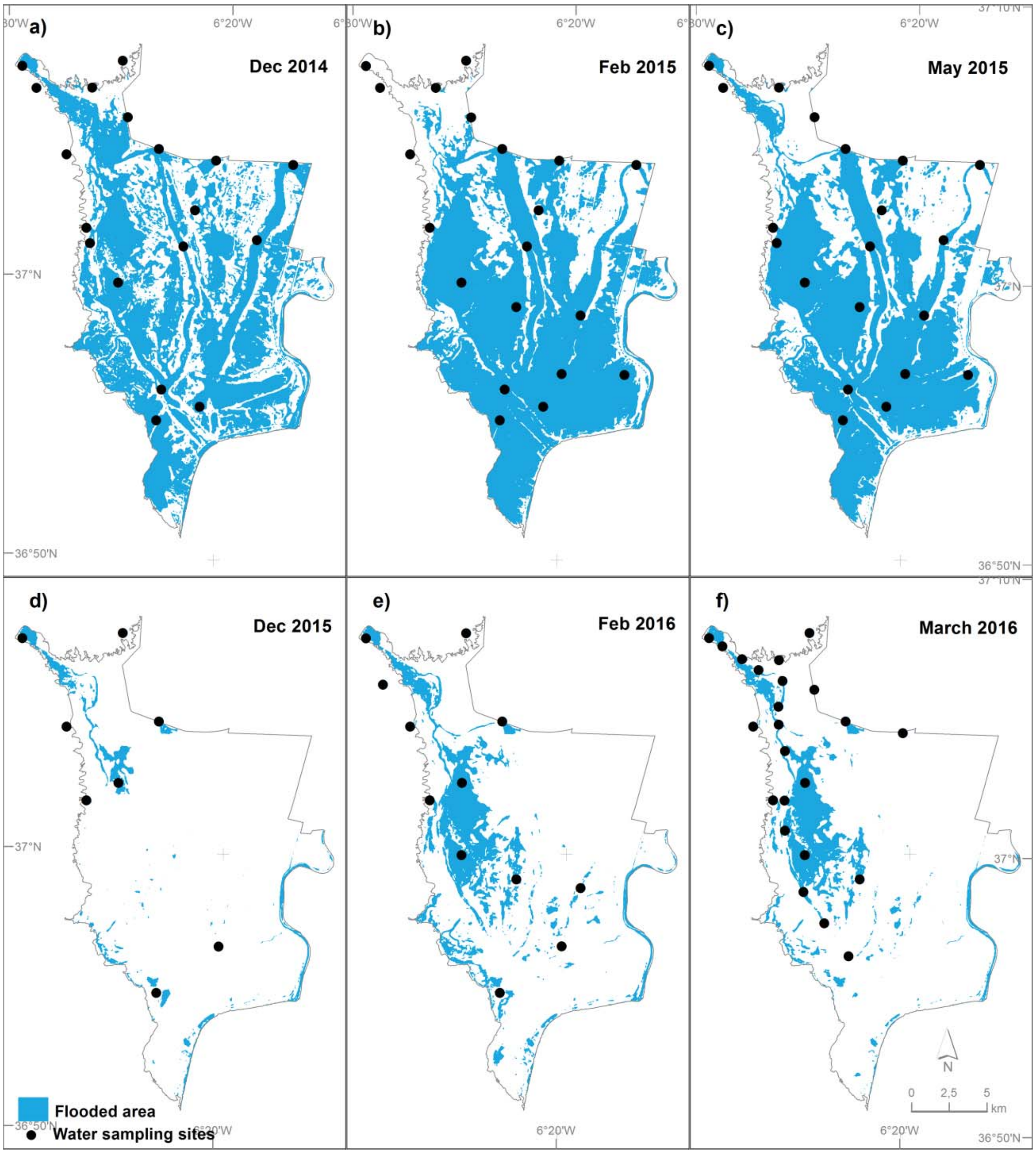


Figure 2. Extent of the flooded area in the Doñana marsh in two hydrological years (2015 and 2016). Inundation masks are based on Landsat 7 (ETM sensor) images acquired on 10 December 2014 (a), 19 May 2015 (c) and Landsat 8 (OLI) images from 20 February 2015 (b), 5 December 2015 (d), 23 February 2016 (e), 10 March 2016 (f). Dots represent sampling points where we collected water samples. Dots in (f) show points where water was sampled in May 2016. We represent the flooded area in March instead of May 2016 because the former image is more similar to the flooding extent during our sampling in early May. After we completed water

sampling, several days of intense precipitation reflooded the Doñana marsh, and the first satellite image in May was taken on the 21^{st} after this major flooding event (no images were available in April).

Figure

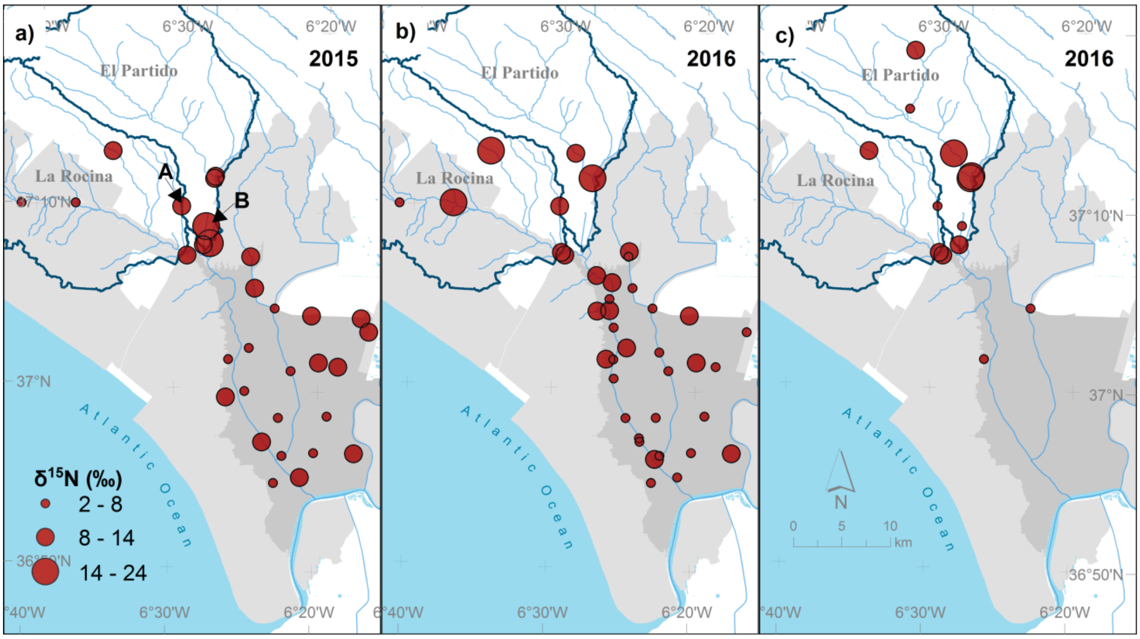


Figure 3. Variability of nitrogen stable isotopes obtained from helophytes. (a) *B. maritimus* collected in April-May 2015. (b) *B. maritimus* and (c) *T. dominguensis* collected in April-May 2016. Dot size represents the isotopic values at each site (δ^{15}N (‰)). In map (a) A and B indicate the points with the highest N concentration in relation to the measured δ^{15}N values, as shown in Figure 5. A corresponds to a water leak from a broken pipe transporting groundwater for human consumption. B is the outflow of El Rocío WWTP.

Figure

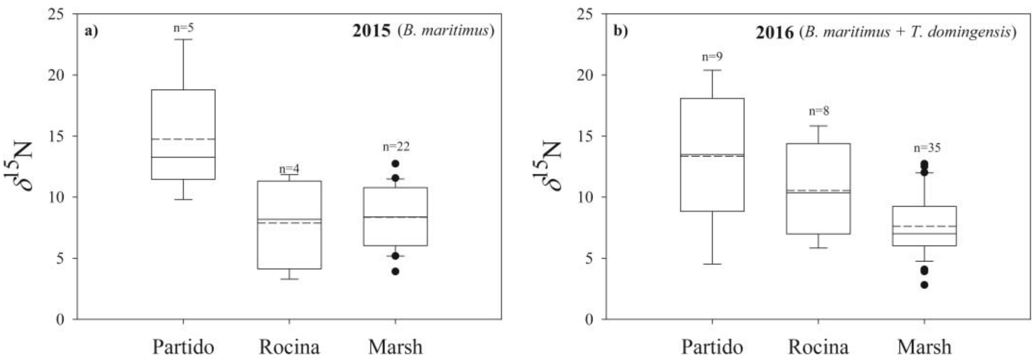
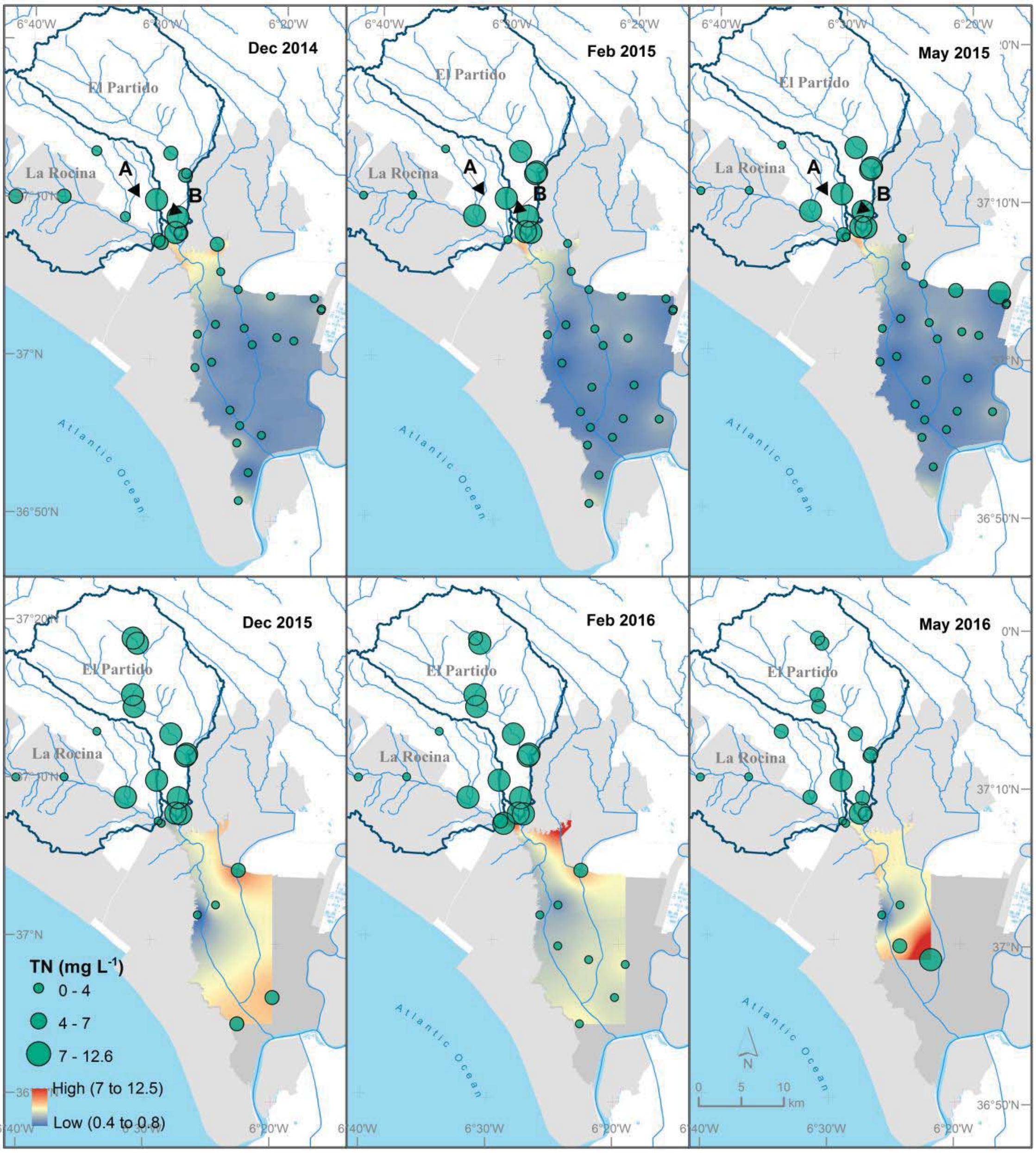


Figure 4. Box plots of (a) isotopic signatures (δ^{15}N, (‰)) of *B. maritimus* in 2015 and (b) *B. maritimus* + *T. domingensis* in 2016 in each habitat ("El Partido" stream, "La Rocina" stream and the marsh). The solid horizontal line shows the median of δ^{15}N values. The dashed horizontal line shows the mean of δ^{15}N values ($Partido_{2015}$= 14.75±2.19 (s.e.); $Rocina_{2015}$= 7.87±1.86; $Marsh_{2015}$=8.35±0.55; $Partido_{2016}$= 13.35±1.79; $Rocina_{2016}$= 10.54±1.31; $Marsh_{2016}$= 7.67±0.41). The bottom and top of the box show the 25th and 75th percentiles, respectively. The whiskers are drawn out to the 10th and 90th percentiles (Cleveland method). Extreme values outside these percentiles are marked as outliers.

Figure

a)


Dec 2014
Feb 2015
May 2015
Dec 2015
Feb 2016
May 2016
El Partido
La Rocina
A
B
Atlantic Ocean
6°40'W
6°30'W
6°20'W
37°10'N
37°N
36°50'N
37°20'N
TN (mg L^{-1})
0 - 4
4 - 7
7 - 12.6
High (7 to 12.5)
Low (0.4 to 0.8)
N
0 5 10 km

b)

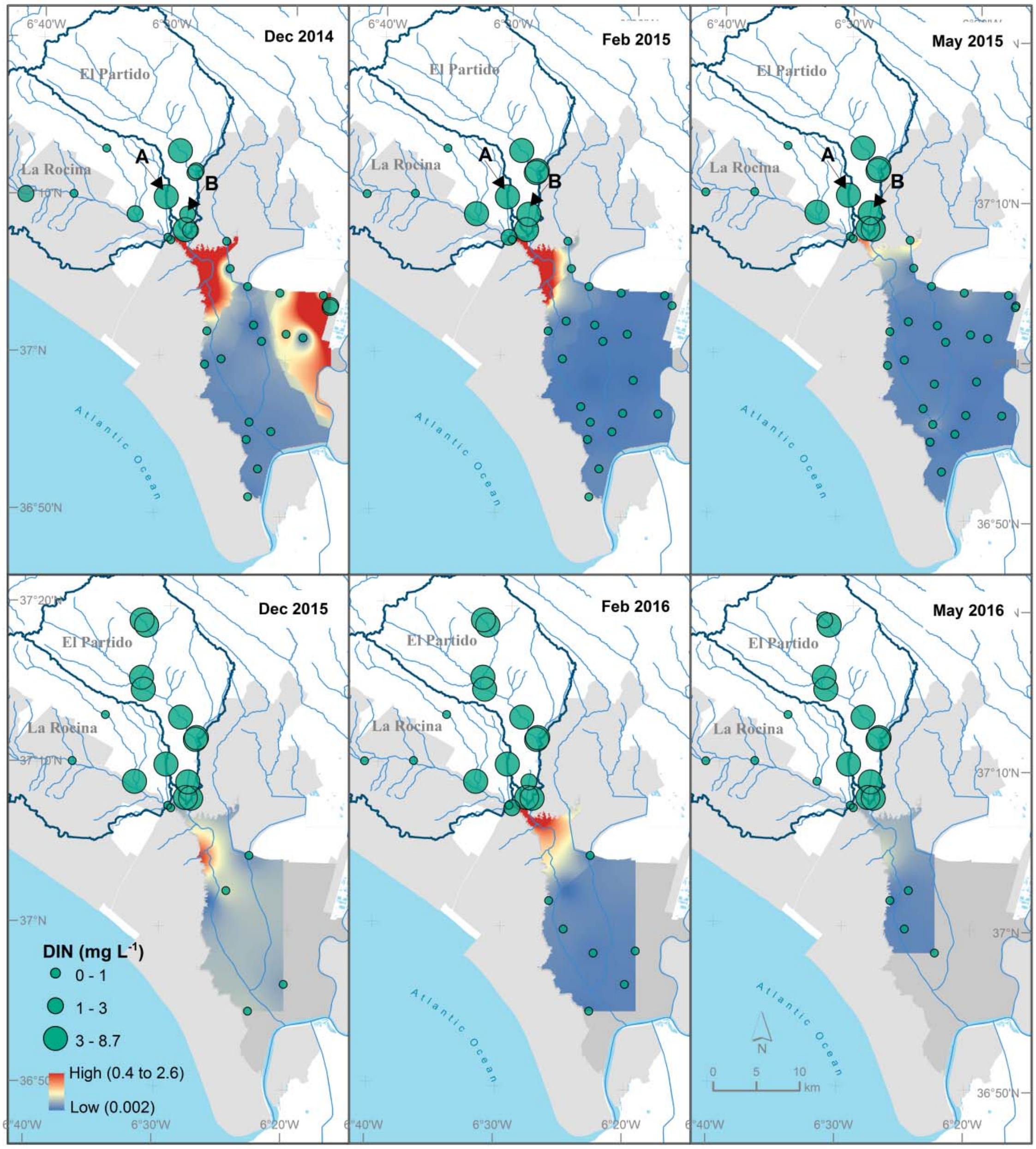


Figure 5. (a) Total N (TN) and (b) dissolved inorganic nitrogen (DIN) (mg N L^{-1}) variability in two hydrological years (2015 and 2016) in the Doñana marsh and entry streams from the "La Rocina" and "El Partido" catchments. Dot size represents the average values of all samples collected in December, February and May each year. The colour gradient shows the result of TN and DIN data interpolation in the marsh. Points A and B are explained in Figure 3. DIN is the sum of NO_3^-, NO_2^- and NH_4^+ concentrations.

Figure

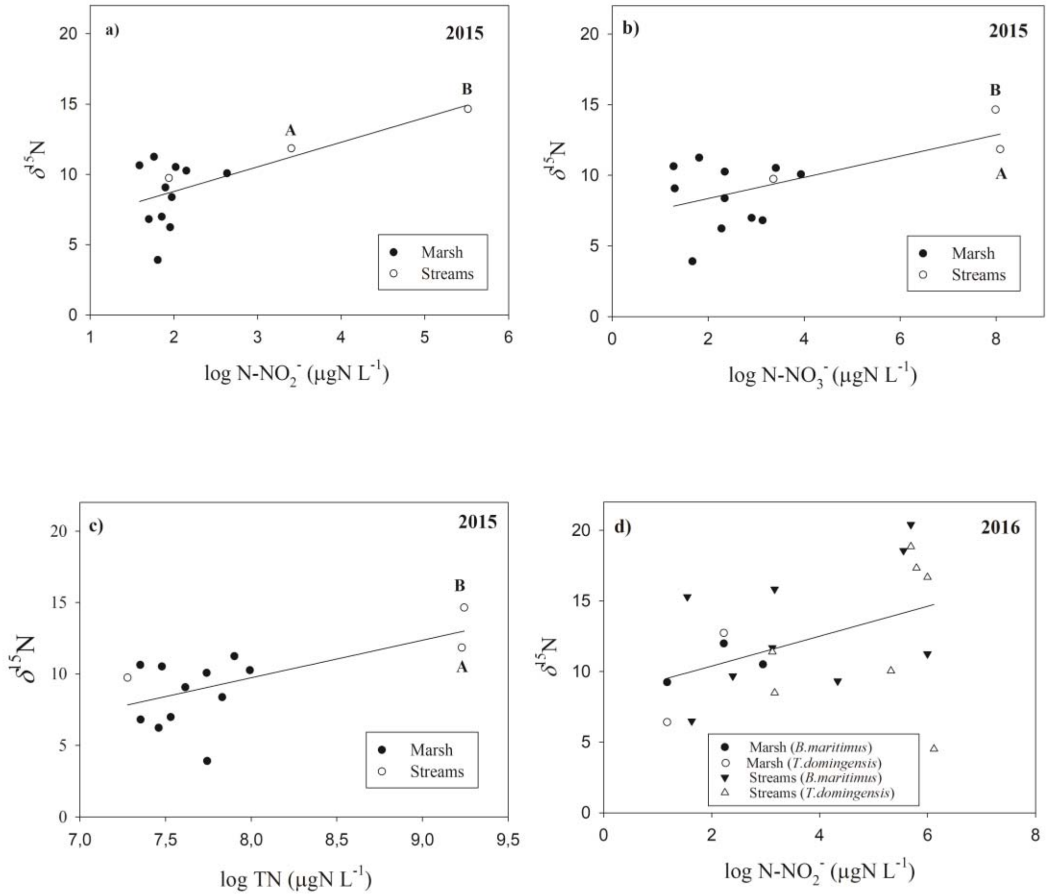


Figure 6. Relationship between δ^{15}N (‰) of helophytes (*B. maritimus* and *T.domingensis*) and mean of surface water N concentrations (NO_2^-, NO_3^- and TN in μg N L^{-1}) collected in 2015 (a,b,c) and 2016 (d) in the Doñana marsh and entry streams. In 2015 we only measured δ^{15}N in *B.maritimus* and sampled water three times per point. In 2016 we measured δ^{15}N in *B.maritimus* and *T.domingensis* and sampled water two times per point. See Fig. 3 for location of A and B sampling points.

Table

Table 1. Comparison of the concentration of different dissolved inorganic N (DIN) species plus Total N (TN) among two streams ("La Rocina" and "El Partido") and the Doñana marsh in 2015 and 2016 using a Kruskal-Wallis test. Cells with different letters ("a" and "b") indicate significant differences (α=0.05) among medians within each N variable group. Median values were calculated for each sampling point using N concentration data excluding summer months (June, July and August). The `Sample size´ column refers to the number of sampling points. Each year we collected a different number of samples (one to twelve) per sampling point, because some points dried out faster, or were less accessible, than others (Fig. 2).

Year	Variable	Median (mg N L^{-1}) [25% -75% percentile]			Sample size (n)			df	χ^2	P
		El Partido stream	La Rocina stream	Marsh	El Partido stream	La Rocina stream	Marsh			
2015	N-NO_3^-	2.528^{b} [2.319 – 2.864]	0.746^{b} [0.408 – 2.227]	0.008^{a} [0.002 - 0.021]	6	6	28	2	19.204	<0.001
	N-NO_2^-	0.268^{b} [0.250 – 0.288]	0.013^{b} [0.008 – 0.026]	0.005^{a} [0.003 - 0.008]	6	6	28	2	19.571	<0.001
	N-NH_4^+	1.619^{b} [0.390 – 2.257]	0.051^{b} [0.023 – 0.175]	0.018^{a} [0.015 - 0.027]	6	6	28	2	13.104	0.001
	TN	8.070^{b} [7.774 – 9.078]	3.445^{b} [2.875 – 5.664]	1.944^{a} [1.729 - 3.074]	6	6	28	2	16.928	<0.001
2016	N-NO_3^-	3.186^{b} [3.014– 3.226]	0.418^{b} [0.169 – 2.663]	0.001^{a} [0.001 - 0.008]	10	6	11	2	16.542	<0.001
	N-NO_2^-	0.257^{b} [0.191 – 0.340]	0.027^{ab} [0.008 – 0.069]	0.005^{a} [0.002 - 0.008]	10	6	11	2	18.920	<0.001
	N-NH_4^+	0.637^{b} [0.574 – 0.889]	0.099^{ab} [0.071 – 0.182]	0.037^{a} [0.028 - 0.060]	10	6	11	2	17.805	<0.001
	TN	8.773^{b} [7.988 – 9.284]	3.476^{a} [2.379 – 7.229]	3.982^{a} [3.416 - 4.423]	10	6	11	2	10.751	0.005

Table 2. Results of linear regression models with the isotopic signatures ($\delta^{15}N$ (‰)) as the dependent variable and the mean N concentrations of different N species in water samples (NO_3^-, NO_2^-, NH_4^+, TN) as predictor variables. In 2016 `plant species´ was also included as a fixed factor. Concentrations were log transformed. In 2015, log transformations did not entirely eliminate heterocedasticity (Fig. 6) so p-values (P) should be treated with some caution.

Year	**Response variable**	**Adj.R^2**	**Explanatory variables**	**df**	**Estimate + SE**	**F**	**P**
2015	$\delta^{15}N$ (*B.maritimus*)	0.401	Log N-NO_2^-	12	1.749± 0.561	9.709	0.008
		0.311	Log N-NO_3^-	12	0.750±0.223	6.870	0.022
		0.011	LogN-NH_4^+	12	1.039±0.970	1.148	0.305
		0.324	Log NT	12	2.632±0.977	7.258	0.019
2016	$\delta^{15}N$ (*B.maritimus* + *T. domingensis*)	0.140	Log N-NO_2^- (species)†	18	1.201 ±0.531	2.633	0.036
		0.081	Log N-NO_3^- (species)†	18	0.730 ±0.383	1.883	0.180
		-0.02	Log N-NH_4^+ (species)†	18	0.800 ±0.661	0.795	0.241
		0.077	Log NT (species)†	18	0.084±0.076	1.837	0.187

† These models were corrected for the partial effect of plant species (*B.maritimus* and *T. domingensis*), but the species effect was not statistically significant.

Supplementary Material

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