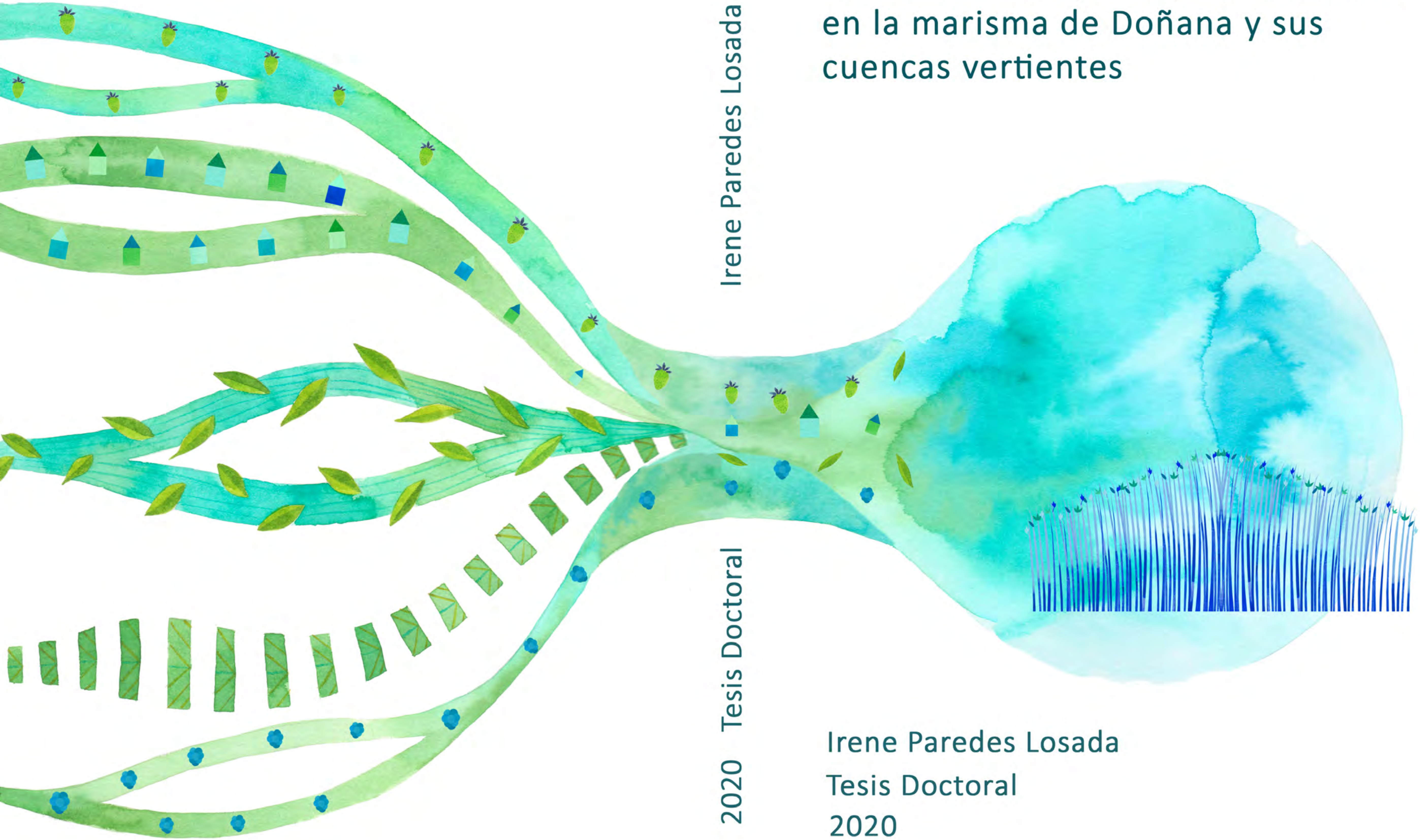
# Presiones antrópicas y eutrofización en la marisma de Doñana y sus cuencas vertientes



Irene Paredes Losada

2020 Tesis Doctoral

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2020

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# Presiones antrópicas y eutrofización en la marisma de Doñana y sus cuencas vertientes

Memoria presentada por la Licenciada en Ciencias Ambientales Irene Paredes Losada para optar al título de Doctora por la Universidad de Sevilla

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"la Caixa"

GOBIERNO DE ESPAÑA

MINISTERIO DE ECONOMÍA Y COMPETITIVIDAD

EXCELENCIA SEVERO OCHOA

*A todas aquellas personas que respetan*
*y luchan por la conservación de los*
*ecosistemas acuáticos,*

*A mi familia,*

# Índice

# Resumen

## Presiones antrópicas y eutrofización en la marisma de Doñana y sus cuencas vertientes

Los humedales figuran entre los ecosistemas más complejos y productivos del mundo, son hábitats cruciales para la biodiversidad y proporcionan una amplia variedad de servicios ecosistémicos fundamentales para el bienestar humano. Sin embargo, desde mediados del siglo XX, la intensificación de la agricultura y el aumento de las aguas residuales han provocado que muchos humedales estén severamente afectados por un aumento de la contaminación y pérdida de la calidad de las aguas. La eutrofización, definida como el enriquecimiento excesivo de nutrientes en los sistemas acuáticos, es una de las causas principales de la contaminación del agua a nivel mundial y que provoca efectos negativos como la anoxia, mortalidad de peces y malos olores. En la región mediterránea, donde la combinación de las presiones humanas y el cambio climático está provocando una reducción alarmante en la disponibilidad y calidad del agua dulce, los humedales son especialmente vulnerables a los procesos de eutrofización.

Doñana, situada al suroeste de España, es un conjunto icónico de humedales temporales reconocido como una de las áreas con mayor biodiversidad en Europa y la región del Mediterráneo. Sin embargo, tanto el área central protegida como Parque Nacional, Patrimonio de la Humanidad y Reserva de la Biosfera por la UNESCO, como los arroyos de las cuencas vertientes, también situados dentro de los límites de la Reserva de la Biosfera, están altamente amenazados por presiones humanas sobre la cantidad y calidad del agua. El fuerte desarrollo de la agricultura y las aguas residuales municipales pobremente tratadas son las principales causas de la pérdida de cantidad y calidad de las aguas, y del consecuente incremento progresivo de la eutrofización en este humedal a lo largo de las últimas décadas. Sin embargo, la escasa disponibilidad de datos históricos sobre la calidad de las aguas superficiales y la baja resolución espacio-temporal

de los mismos dificultan la posibilidad de realizar un análisis detallado del proceso de eutrofización. Además, hasta la fecha se dispone de muy poca información sobre el origen específico de los nutrientes. En este contexto, la presente Tesis contribuye a aumentar el conocimiento sobre los patrones espacio-temporales en las concentraciones de nutrientes y a identificar el posible origen de los nutrientes para abordar las causas de la eutrofización, tanto en la marisma del Parque Nacional como en los arroyos de las cuencas vertientes, mediante el análisis de concentraciones de nutrientes (nitrógeno y fósforo), clorofila-*a*, composiciones isotópicas del nitrógeno, oxígeno e hidrógeno en plantas acuáticas emergentes (castañuela -*Bolboschoenus maritimus* y espadaña -*Typha domingensis*) ($\delta^{15}N$), nitratos disueltos ($\delta^{15}N$, $\delta^{18}O$) y agua ($\delta^{2}H$), y otros parámetros físico-químicos e hidrológicos del agua (ej. conductividad, profundidad de la columna de agua). Para ello, se usaron datos recogidos durante múltiples muestreos de campo entre 2013 y 2016, principalmente durante el periodo húmedo (octubre-junio) cuando la disponibilidad de agua fue mayor.

Los resultados de esta Tesis muestran que, a pesar de las múltiples figuras de protección que se han ido implementado en Doñana con el objetivo de garantizar la protección de este sistema único, las actividades humanas en las cuencas vertientes se han intensificado a lo largo de las últimas décadas incrementando la presión antrópica sobre los humedales. Como ejemplo, nuestros resultados sobre la cuantificación de la evolución de cultivos bajo plástico demuestra que el área cubierta por invernaderos en las cuencas de la Rocina, los Sotos y el Partido aumentó un 487% desde 1995 hasta 2016. Esto explicaría que las concentraciones de nutrientes observadas en los arroyos sean consistentemente superiores que en la marisma. Esto no indica que en la marisma no exista un problema de eutrofización, sino que su intensidad es menor aunque no menos preocupante. El arroyo del Partido es con diferencia el que mostró los valores más altos de concentraciones de nutrientes, donde un 63% de las todas las muestras (n=91) se clasificaron dentro de las peores clases de calidad del agua según las referencias usadas en la presente Tesis. Concretamente, el

93% de las concentraciones de fosfatos ($PO_4$) y fósforo total (TP), el 71% del nitrógeno total (TN) y el 69% de nitritos ($NO_2$) superaron los niveles umbrales a partir de los cuales las condiciones no son aptas para la vida piscícola. Los arroyos en las cuencas de la Rocina y los Sotos presentaron valores más bajos que en el Partido, aun así, alrededor de un tercio de las muestras recogidas (n= 78) tampoco permitirían la vida piscícola. Las muestras recogidas en la cuenca del Guadiamar indicaron mejorar calidad del agua que los demás arroyos. Los resultados isotópicos confirmaron además que el nitrógeno en los arroyos y las entradas a la marisma tiene un origen principalmente antrópico (fertilizantes y aguas residuales urbanas), mientras que en el interior la marisma parece tener un origen más natural, proveniente del propio ecosistema, aunque sería necesario un estudio más detallado para confirmarlo. Además, los resultados isotópicos de N y O sugieren que la desnitrificación parece ser un proceso predominante en los puntos de arroyos y lagunas muestreados. Esto indicaría por un lado, que existe un mecanismo importante de atenuación natural de la eutrofización en el sistema, y por otro lado, que la cantidad de nitratos derivados de la contaminación difusa y puntual en la cuenca podría estar subestimada.

**Desde un punto de vista de la gestión, la presente Tesis arroja luz al problema de la eutrofización en Doñana desde una perspectiva amplia, integrando en un mismo estudio información espacio-temporal de la marisma del Parque Nacional y los arroyos vertientes, y abordando por primera vez la caracterización de las distintas fuentes de contaminación por nutrientes mediante las aproximaciones isotópicas.** Esto último es esencial a la hora de determinar las actuaciones prioritarias que ayuden a reducir el exceso de nutrientes que reciben los ecosistemas acuáticos. Las actuaciones más relevantes podrían variar en función de si se determina que las fuentes de nutrientes principales provienen, por ejemplo, de la agricultura o las aguas residuales. Es necesario que trabajos científicos como esta Tesis sean tenidos en cuenta a la hora de elaborar medidas adecuadas para frenar el problema de la eutrofización, y de la calidad de las aguas en general, en Doñana y su entorno.

# Abstract


## Human pressures and eutrophication in the Doñana marsh and its watershed

Wetlands are among the world's most complex and productive ecosystems, are crucial habitats for biodiversity, and provide a wide range of ecosystem services essential to human well-being. However, since the middle of the 20th century, intensification of agriculture and increased urban wastewater inputs have severely impacted many wetlands by increased pollution and loss of water quality. Eutrophication, defined as the excessive enrichment of nutrients in aquatic systems, is one of the major causes of water pollution globally, resulting in negative effects such as anoxia, fish mortality and bad odours. In the Mediterranean region, where the combination of human pressures and climate change is causing an alarming reduction in the availability and quality of freshwaters, wetlands are particularly vulnerable to eutrophication processes.

Doñana, located in southwest Spain, is an iconic temporary wetland complex recognized as one of the most biodiverse areas in Europe and the Mediterranean region. However, water quantity and quality in both the central area (protected as a National Park, and UNESCO World Heritage Site) and the watershed streams (located within the boundaries of the UNESCO Biosphere Reserve), are highly threatened by human pressures. The intense development of agriculture and poorly treated municipal wastewater are among the main causes behind the increase of eutrophication in this wetland over the last few decades. However, the limited availability of historical data on surface water quality and the low spatial-temporal resolution of these data make it difficult to carry out a detailed analysis of the eutrophication process. In addition, very little information is available until now on the specific origin of nutrients. The present Thesis advances our knowledge about the spatial-temporal patterns in nutrient concentrations and the possible origin of the nutrients in order to address the causes of eutrophication,

both in the National Park marshes and in the watershed streams. This work is based on analysing concentrations of nutrients (nitrogen and phosphorus), chlorophyll-*a*, isotopic compositions of nitrogen, oxygen and hydrogen in emerging aquatic plants (*Bolboschoenus maritimus* and *Typha domingensis*) ($\delta^{15}N$), dissolved nitrates ($\delta^{15}N$, $\delta^{18}O$) and water ($\delta^{2}H$), and other physico-chemical and hydrological parameters of water (e.g. conductivity, water column depth). For this purpose, we collected field samples between 2013 and 2016, mainly during the wet period (October-June) when water availability was higher.

The results of this Thesis show that, despite the multiple protective status assigned to Doñana with the aim of guaranteeing the protection of this unique system, human activities in the river basins have intensified over the last few decades, with a negative impact on water quality in the wetlands. For example, we find that the area covered by greenhouses in the Rocina, Sotos and Partido basins increased by 487% from 1995 to 2016. This helps to explain why nutrient concentrations we observed in the streams were consistently higher than in the marshes. The Partido stream has by far the highest values of nutrient concentrations, where 63% of all samples (n=91) were classified within the worst water quality classes. In particular, 93% of phosphate ($PO_4$) and total phosphorus (TP) concentrations, 71% of total nitrogen (TN) and 69% of nitrites ($NO_2$) exceeded the threshold levels above which conditions are unsuitable for fish life. The streams in the Rocina and Sotos basins showed lower concentrations than in the Partido, yet about one third of the samples collected (n= 78) were not compatible with fish life either. Samples collected in the Guadiamar basin indicated better water quality than the other streams. Eutrophication is also occurring in the marsh but remains less intense.

The isotope results also confirmed that the nitrogen in the streams and their entrances to the marshes is mainly of anthropic origin (fertilizers and urban waste water), while in the interior the marsh seems to have a more natural origin, coming from the ecosystem itself, although a more detailed study would be needed to confirm this. Furthermore, the isotopic results of N and O suggest that denitrification is a predominant process in the streams and lagoons sampled. This would indicate, on the one hand, that there is an important natural attenuation mechanism of eutrophication in the system, and on the other hand, that the amount of nitrates entering from diffuse and point source pollution in the basin may be underestimated by our nitrate data.

**From a management point of view, the present Thesis sheds light on the problem of eutrophication in Doñana from a broad perspective, integrating in the same study spatio-temporal information of the National Park marsh and the entry streams, and addressing for the first time the characterization of the different sources of nutrient pollution through stable isotope approaches.** The latter is essential when determining the priority actions that will help to reduce the excess of nutrients that the aquatic ecosystems receive. The most urgent actions depending on whether the main sources of nutrients are identified as coming from e.g. agriculture or wastewater. Scientific research such as this Thesis needs to continue to help the development of appropriate measures to curb the problem of eutrophication in the iconic Doñana wetland complex.

# Introducción

## La situación alarmante de los humedales en el mundo

### *Definición de humedal*

Definir de forma breve y concisa qué es un humedal resulta complicado dada la gran variedad de humedales que existen en el mundo y el enorme dinamismo espacio-temporal que dificulta el establecimiento de sus límites. Actualmente, la definición más conocida a nivel mundial es la establecida en los Artículos 1 y 2.1 del Convenio de Ramsar sobre los Humedales (1971) (Caja 1). Sin embargo, esta definición no se basa en un concepto ecológico de humedal sino en una unidad paisajística (ej. marisma, turbera) que dependiendo del país o región puede dar lugar a diferentes significados. Además, no incluye las áreas saturadas no encharcadas (criptohumedales) y las encharcadas de forma intermitente. Desde un punto de vista más ecológico, el National Research Council (NRC, 1995) estableció una definición de carácter más científico donde, a nivel institucional, el humedal se reconocía por primera vez como un ecosistema y suprimía el concepto erróneo de que los humedales son exclusivamente zonas de transición, ecotonos o fronteras entre ecosistemas acuáticos y terrestres, ya que también existen humedales rodeados únicamente por zonas más secas (Mushet et al., 2015) (Caja 2).

***Caja 1. Definición de humedal según el Convenio de Ramsar sobre los Humedales (1971)***

Se considerarán humedales las extensiones de marismas, pantanos, turberas o superficies cubiertas de agua sean estas de régimen natural o artificial, permanentes o temporales estancadas o corrientes, dulces, salobres o saladas incluidas las extensiones de agua marina cuya profundidad en marea baja no exceda de los seis metros. Además podrán comprender zonas de bordes fluviales o de costas adyacentes al humedal, así como las islas o extensiones de agua marina de una profundidad superior a los seis metros en marea baja, cuando se encuentra dentro del humedal.

***Caja 2. Definición de humedal según en National Research Council (1995)***

Un humedal es un ecosistema que depende de una constante o recurrente inundación con aguas poco profundas o en saturación en o cerca de la superficie del sustrato. Las características esenciales mínimas de un humedal son la inundación o saturación recurrente en o cerca de la superficie del terreno y la presencia de características físicas, químicas y biológicas reflejo de la inundación o saturación recurrente. Las características comunes del diagnóstico son suelos hídricos y vegetación higrófila. Estas características estarán presentes excepto donde factores fisicoquímicos, bióticos o antrópicos específicos los han eliminado o impedido su desarrollo.

## *Distribución y servicios ecosistémicos*

Los humedales se encuentran distribuidos por todo el mundo y los estudios más recientes estiman que actualmente ocupan un 8% de la superficie terrestre aproximadamente, aunque se cree que la superficie real es aún mayor (Davidson et al., 2018; Fig. 1). Estos ecosistemas figuran entre los más complejos y productivos del mundo, y suponen un hábitat fundamental para una inmensa diversidad de especies de microorganismos, plantas, insectos, reptiles, anfibios, aves, peces y mamíferos. Un buen ejemplo son los humedales continentales, que a pesar del bajo porcentaje de la superficie terrestre que ocupan, albergan más de un 40% de la biodiversidad mundial (Mitra et al., 2003). Además, los humedales desempeñan un papel clave en los procesos hidrológicos a nivel de cuenca, como por ejemplo la recarga de los acuíferos (Kadykalo and Findlay, 2016). Los humedales también tienen un enorme valor social y económico ya que proporcionan una amplia variedad de servicios ecosistémicos fundamentales para el bienestar humano, como por ejemplo el abastecimiento de materias primas y alimentos, la regulación hídrica, control de la erosión o el desarrollo de la cultura local y la educación ambiental (Mitsch et al., 2015).

### *Amenazas*

Los humedales se encuentran entre los ecosistemas más amenazados en el mundo. La extensión global de los humedales se estima que ha disminuido entre un 33% (Hu et al., 2017) y un 71% (Davidson, 2014) desde principios del siglo XX, y que actualmente su pérdida y degradación continúan aumentando de forma alarmante a nivel mundial (Gardner et al. 2015). Desde finales de los años 70 se calcula la tasa de desaparición de humedales naturales ha sido tres veces superior a la de los bosques durante ese periodo (Ramsar Convention on Wetlands., 2018). Esto es un reflejo de la gran presión a la que están sometidos estos ecosistemas, principalmente debido al enorme conflicto de intereses relacionados con el desarrollo agrícola, urbano y turístico que afectan negativamente a su conservación. En la actualidad, aproximadamente el 40% de la superficie de los continentes está ocupada por cultivos o pastos (Foley et al. 2005). Una de las principales consecuencias de la expansión e intensificación de las actividades humanas a nivel mundial sobre los humedales es la pérdida de la calidad del agua debido al aumento de contaminantes en el agua (ej. pesticidas, farmacéuticos, metales pesados, microplásticos o nutrientes).

## La eutrofización: una amenaza global para los humedales

Desde hace décadas, la eutrofización representa una de las principales causas de degradación de la calidad del agua en los humedales a nivel mundial. La eutrofización es un proceso natural que se define como el enriquecimiento excesivo de nutrientes en los sistemas acuáticos, y cuya terminología proviene de la palabra griega "*εὐτροφία*" (eutrophia, i.e. "*buen estado de nutrición*"). También se usa en la literatura científica el término inglés "*cultural eutrophication*", que atribuye el aumento global de este proceso a la actividad humana (Schindler et al. 2016). Según Steffen et


**Figura 1.** Distribución de humedales en el mundo según las estimaciones de “ocurrencia de agua” entre 1984 y 2018 calculadas a partir de imágenes Landsat (imagen adaptada de Global Surface Water Explorer, 2019).

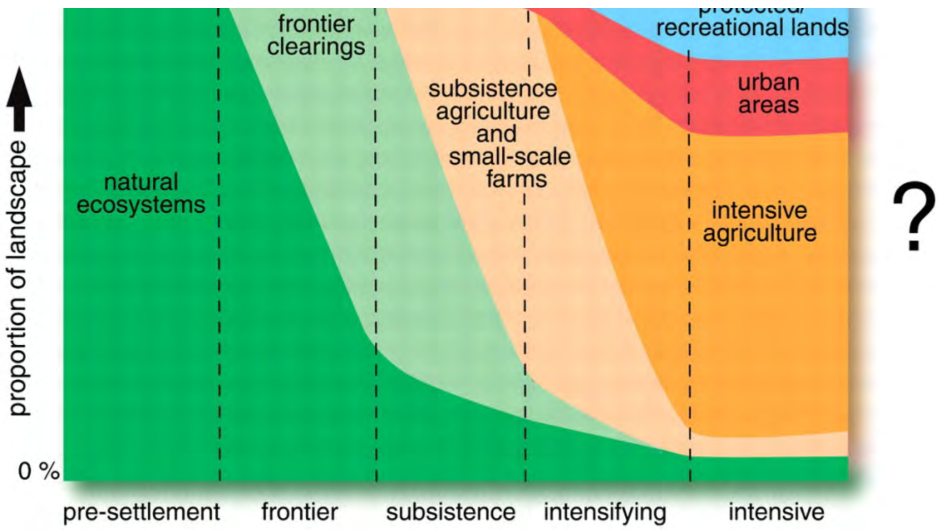


**Figura 2.** Evolución de la superficie terrestre ocupada por distintos usos desde el Paleolítico hasta la actualidad (Foley et al., 2005).

al. (2015) las perturbaciones humanas han alterado completamente los ciclos biogeoquímicos del nitrógeno y fósforo, sobrepasándose los límites planetarios hasta el punto de que el riesgo de que los daños sobre el Sistema Tierra sean irreversibles es muy alto (Fig. 3). Concretamente, el fuerte desarrollo de la industria, la agricultura y las áreas urbanas a partir de la segunda mitad del siglo XX propició un aumento desmesurado del aporte de nutrientes (principalmente N y P) a los sistemas acuáticos, y desde entonces es uno de los mayores problemas de calidad de las aguas continentales y costeras a nivel mundial, cuyos efectos se ven además acentuados por el cambio climático (Moss et al. 2011, Downing 2014; Fig. 4). En los últimos 50 años, el uso de fertilizantes a nivel mundial se estima que aumentó aproximadamente un 700% mientras que la agricultura de regadío un 70% (Foley et al. 2005). Según Lassaletta et al. (2014), más de la mitad del nitrógeno que se usa actualmente en la fertilización de los cultivos a nivel mundial acaba incorporándose a los ecosistemas naturales. La consecuencia más directa de la eutrofización es el incremento de la tasa de producción primaria, a veces alcanzando densidades extremadamente altas. En particular, el crecimiento masivo de ciertas especies de cianobacterias supone un riesgo importante para la salud humana y la vida silvestre ya que pueden producir toxinas, algunas de ellas incluso con efectos letales. El crecimiento excesivo de productores primarios también afecta indirectamente a las condiciones físico-químicas de los ecosistemas acuáticos: fuertes fluctuaciones de oxígeno entre el día y la noche, disminución de la penetración de la luz en la columna de agua por aumento de la turbidez o condiciones de hipoxia debido a procesos aerobios de descomposición de la materia orgánica. Una medida indispensable para el control de la eutrofización es la reducción de la entrada externa de nutrientes (principalmente N y P) desde las fuentes contaminantes a los sistemas acuáticos (Martin, 2013, Hamilton et al. 2016). En algunos sistemas también se han aplicado con éxito métodos que reducen la concentración de nutrientes directamente en la columna de agua (Lürling & Oosterhout, 2013; Schindler et al. 2016; Magalhães et al. 2017.

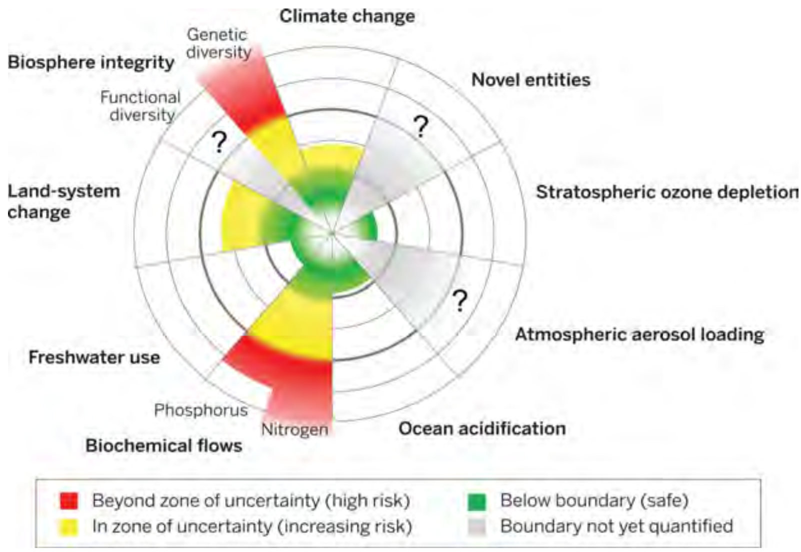


**Figura 3.** Variables control que definen los Límites Planetarios (*planetary boundaries*) según Steffen et al. (2015). Los Límites Planetarios son niveles establecidos para medir la perturbación humana sobre la Tierra, más allá de los cuales su funcionamiento puede verse profundamente alterado. Las zonas en verde indican un nivel seguro, en amarillo se representa una zona de incertidumbre (riesgo creciente) y en rojo es la zona de alto riesgo. Los ciclos de nitrógeno y fósforo ya han sobrepasado el límite planetario (círculo de la línea más gruesa).

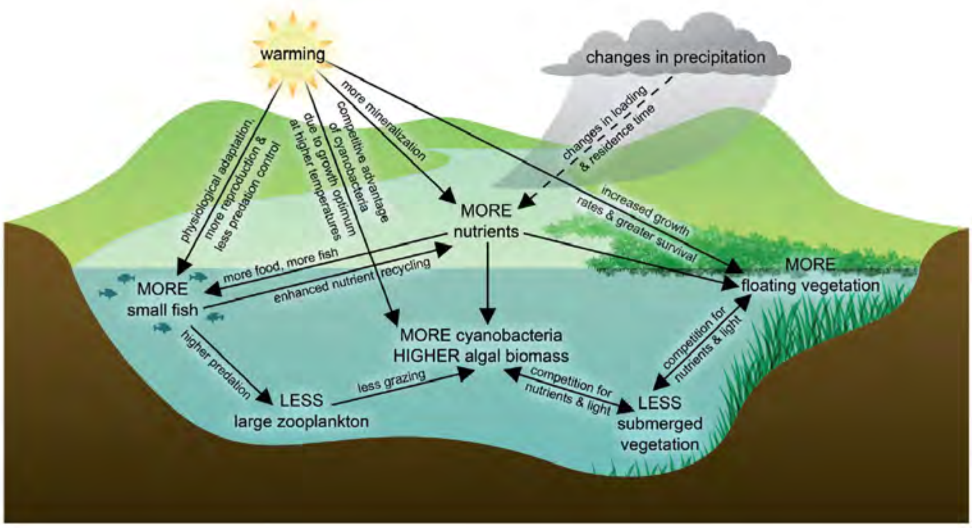


**Figura 4.** Esquema ilustrando el proceso de eutrofización y los efectos del cambio climático (Moss et al. 2011).

## El caso particular de los humedales mediterráneos

Los humedales mediterráneos representan el conjunto de ecosistemas con mayor biodiversidad de la cuenca mediterránea y proporcionan servicios ecosistémicos de vital importancia para la región. Ocupan entre un 2-3 % del total del área de los 27 países incluidos dentro de la Cuenca del Mediterráneo (Geijzendorffer et al., 2019). Sin embargo, a pesar de la existencia de medidas nacionales e internacionales para la conservación de los humedales (ej. Convención de Ramsar), la situación en la cuenca mediterránea es particularmente alarmante. Los humedales mediterráneos se encuentran altamente amenazados debido a las continuas presiones socio-económicas, la inestabilidad política de muchos países y el rápido aumento de la población desde 1990 (41% en zonas costeras). Además, la combinación de las presiones humanas junto con el cambio climático en esta región (descenso de las precipitaciones y el aumento de las temperaturas) está provocando una reducción alarmante en la disponibilidad y calidad del agua dulce, tanto para el consumo humano como para el propio funcionamiento de los ecosistemas acuáticos (MedECC, 2019; Fig. 5). La tasa de desaparición de los humedales mediterráneos es superior respecto a la media mundial, especialmente en las zonas costeras donde las presiones humanas (ej. urbanización, turismo) y ambientales (ej. incremento del nivel del mar) provocan un mayor impacto. Según el último informe elaborado por el Observatorio de Humedales Mediterráneos (MWO2, 2018), se estima que desde principios del siglo XX se ha perdido alrededor del 50% de los humedales mediterráneos naturales, y los que quedan están en su gran mayoría degradados (ej. Doñana) o son artificiales (Mitraki et al. 2004; Fig. 6). Además, en la Cuenca del Mediterráneo existe una fuerte desigualdad en políticas de control de la calidad del agua entre los países de la Unión Europea (zona norte) y el resto de países mediterráneos (zona sur y este), ya que los primeros cuentan con un mayor soporte normativo de obligatorio cumplimiento (ej. Directiva Marco del Agua 2000/60/CE), mientras que los segundos apenas cuentan con normas consolidadas en materia de calidad del agua, ni con la obligatoriedad de cumplirlas (MWO2, 2018).

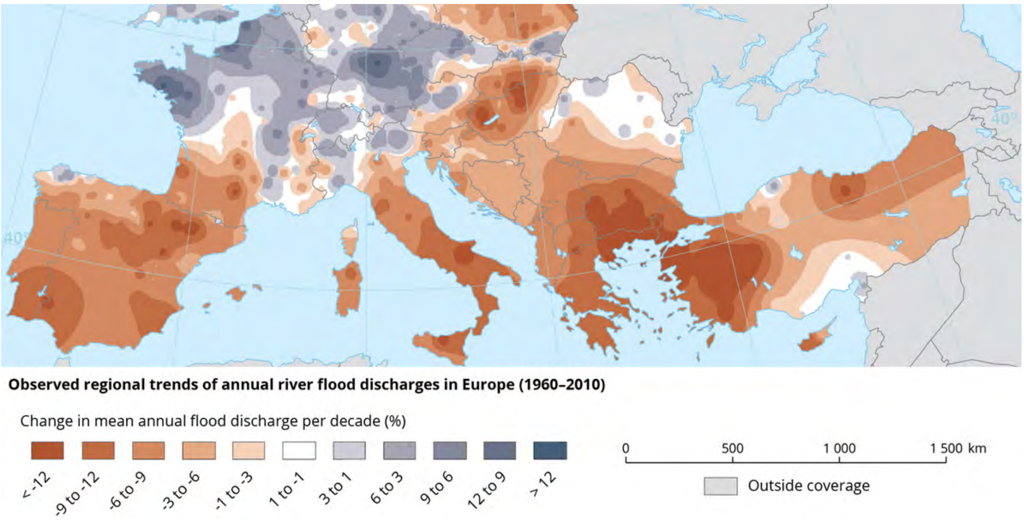


**Figura 5.** Tendencias regionales del caudal anual de los ríos en la zona mediterránea europea (1960-2010). El color azul indica incremento de caudal y el rojo descenso (se mide el porcentaje de cambio de la media anual de los caudales por década). Nota: en la zona mediterránea europea existe un claro descenso de los caudales de los ríos en las últimas décadas, lo que concuerda con las tendencias del cambio climático para esta región. Imagen adaptada de European Environmental Agency (EEA 2019a).


**Figura 6.** Lago Koronia (Grecia), en el pasado fue el cuarto más grande en superficie de agua en Grecia. En 1975 fue designado Humedal de Importancia Internacional Ramsar. Sin embargo, las crecientes presiones humanas (agricultura de regadío, ganadería, pesca, caza y aguas residuales no tratadas) pusieron en peligro la integridad del ecosistema. Como consecuencia la superficie de agua disminuyó de 47 km$^2$ en 1970 a 30 km$^2$ en 1995 y el lago pasó a ser hipereutrófico, con floraciones de cianobacterias tóxicas y muertes masivas de peces cada vez más frecuentes (Mitraki et al. 2004). Esta pérdida severa de cantidad (ilustrada en las imágenes satélite) y calidad del agua han provocado un impacto tan grande sobre el humedal que su recuperación, aun estableciendo medidas adecuadas, probablemente necesitará más tiempo de lo que tardó en degradarse. Las imágenes satélite pertenecen al 31 de diciembre en todos los casos. Fuente: Google Earth.

# La eutrofización en humedales mediterráneos españoles: panorama actual y perspectivas futuras

## *Panorama actual*

España es el tercer país del mundo con mayor número de Humedales de Importancia Internacional por el Convenio de Ramsar. Entre ellos se encuentran aquellos que albergan las mayores poblaciones de aves acuáticas en Europa (ej. Doñana), convirtiéndose en áreas de vital importancia para su supervivencia. La conservación de estos humedales también es fundamental para el mantenimiento de numerosas actividades socioeconómicas como la pesca, el cultivo de arroz, el turismo o el suministro de agua (SEO-Birdlife, 2018). Sin embargo, la biodiversidad en la mayoría de ellos se encuentra gravemente amenazado por múltiples presiones humanas desde hace décadas, de entre los que destacan importantes humedales como Doñana, la Albufera de Valencia, el Delta del Ebro o el Mar Menor (Vicente & Miracle, 1992; Sanchez-Ramos et al. 2016; Ibañez & Caiola 2018; Green et al. 2018; Fig. 7). Entre los problemas principales de estos humedales destaca la progresiva eutrofización que se viene registrando durante las últimas décadas, cuyas causas comunes son: la intensificación de la agricultura en las cuencas y el aumento de las aguas residuales urbanas, en muchos casos pobremente tratadas. El Mar Menor, una de las lagunas costeras más grandes e importantes del Mediterráneo y de Europa, es uno de los humedales más profundamente alterados por la eutrofización en España debido las grandes cantidades de nutrientes y materia orgánica derivados de la intensa actividad agraria y agropecuaria en la cuenca (García-Pintado et al. 2007; WWF-ANSE, 2018; Ruiz Fernández et al. 2019). En el caso del humedal de Doñana, hace 30 años que se encuentra en el Registro de Montreux, sin embargo, su situación desde entonces ha ido a peor. Los procesos de eutrofización afectan tanto a las áreas más protegidas (Parque Nacional) como a su entorno (cuencas vertientes, arroyos). Algunos de los efectos más destacados en Doñana por exceso de nutrientes en el sistema han sido la mortandad masiva de fauna por cianobacterias tóxicas ocurrida en 2001 y 2004 (López-Rodas et al. 2008) y la expansión del helecho invasor *Azolla filiculoides* en la marisma desde 2001 por aumento de los fosfatos en el agua (Espinar et al. 2015). Doñana también ha sufrido mortandades por el botulismo, favorecido por la entrada de aguas residuales (Anza et al. 2014).

Estos ejemplos son una indicación clara de que los planes de conservación de humedales y la gestión de los recursos hídricos en España no han conseguido llevar a cabo medidas exitosas para mejorar el estado de aquellos humedales más amenazados y degradados. Esta situación es incompatible con el cumplimiento de los objetivos de conservación requeridos por Ramsar y por las Directivas Europeas sobre calidad del agua y estado de los ecosistemas acuáticos (SEO-Birdlife, 2018). Sin embargo, esta situación podría revertirse, pues en muchas regiones europeas y a pesar de que los niveles siguen siendo altos, existe una reducción en la carga de nutrientes de las aguas superficiales como resultado de las medidas de gestión (EEA 2019b), que a su vez están sustentadas por un cada vez mayor conocimiento de los ecosistemas acuáticos y las sinergias con las presiones antrópicas y el cambio climático.

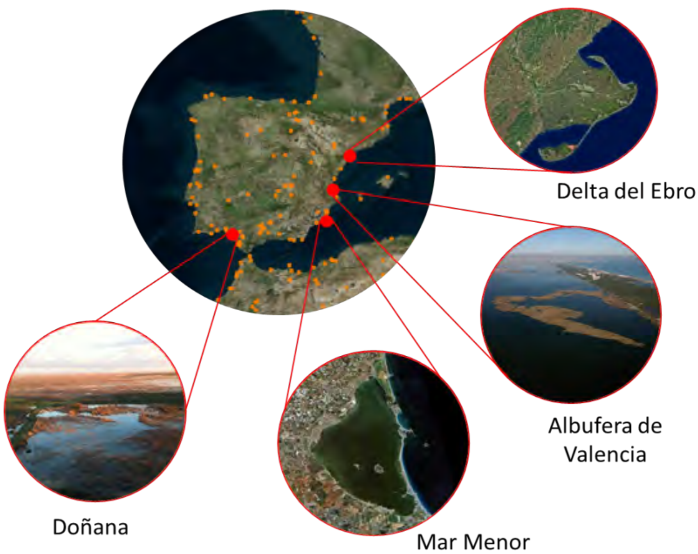


**Figura 7.** Ejemplo de cuatro Humedales de Importancia Internacional Ramsar más destacados en España (Doñana, Mar Menor, Albufera de Valencia y Delta del Ebro) que se encuentran sometidos a severas presiones humanas desde hace décadas, principalmente por el fuerte desarrollo de la agricultura intensiva y el crecimiento de la población en el entorno. La eutrofización es uno de los problemas más destacados a los que se enfrentan. Los puntos naranjas representan otros Humedales Ramsar.

# Área de estudio

La recolección de muestras y el trabajo de campo destinados a la realización de la presente Tesis Doctoral se llevó a cabo en: 1) la marisma del Parque Nacional de Doñana; 2) en los principales arroyos de las cuencas vertientes a la marisma (Rocina, Partido, Sotos y Guadiamar), situados fuera de los límites del Parque Nacional; 3) en la Laguna Primera de Palos, situada a 30 km al oeste del Parque Nacional (Fig. 8). Esta sección se divide en tres partes, la primera es una introducción general sobre Doñana y su área de influencia socioeconómica, la segunda parte es la descripción detallada de las áreas de estudio específicas donde se recogieron muestras para la presente Tesis doctoral, y la tercera parte es un resumen de la metodología general donde se describen los principales indicadores de calidad del agua usados en esta Tesis.

## Doñana y su área de influencia socioeconómica

Doñana es un conjunto de humedales emblemático y de mayor extensión en Europa. Se sitúa al suroeste de la Península Ibérica junto a la desembocadura del río Guadalquivir y forma parte de las provincias de Huelva, Sevilla y Cádiz. El clima de esta región es mediterráneo sub-húmedo, con otoños e inviernos lluviosos y no muy fríos, y veranos calurosos y secos. La precipitación media anual es 550 mm, con grandes variaciones interanuales entre 170 y 1000 mm, y la temperatura media anual de 17°C (Díaz-Delgado et al., 2016) .

El interés ecológico de Doñana reside en que reúne la mayor parte de los ecosistemas fluviales, forestales, litorales y marismeños propios de esta zona. Los hábitats principales que caracterizan a este humedal son la marisma, una llanura de inundación temporal formada por sustratos arcillosos, y el manto eólico, una zona de arenas que puede llegar a albergar más de 3000 lagunas temporales en años lluviosos (Díaz-Paniagua et al. 2010).

Doñana integra una biodiversidad única en el contexto europeo e internacional con más de 4000 especies de fauna y flora europea y africana, incluyendo tanto especies amenazadas a nivel mundial como especies endémicas de la península Ibérica. Destaca principalmente por ser un espacio de extraordinaria importancia para la cría, invernada y paso de miles de aves acuáticas y terrestres de toda Europa y África.

La conservación del patrimonio natural de Doñana comenzó en 1963 con la adquisición de cerca de 7,000 ha por parte del Fondo Mundial para la Conservación de la Naturaleza (WWF), que cedió al Consejo Superior de Investigaciones Científicas (CSIC), creándose en 1964 la Reserva Biológica de Doñana (RBD). Posteriormente, en 1969 se creó el Parque Nacional de Doñana (PND) que, tras varias ampliaciones, actualmente ocupa 54,252 ha. En 1989 la Junta de Andalucía creó la figura del Parque Natural de Doñana como zona de protección alrededor del Parque Nacional (68,236 ha) pero con un menor grado de protección ambiental. A partir de 2006, cuando la gestión del Parque Nacional fue transferida a la Comunidad Autónoma de Andalucía, ambos Parques, Nacional y Natural, se unificaron para formar el "Espacio Natural de Doñana" (END), un ente único de gestión para la conservación de este entorno natural, que actualmente ocupa alrededor de 122,000 ha.

Además, Doñana también sustenta designaciones de protección a nivel internacional, entre otras, la de Reserva de la Biosfera por la UNESCO (1980, límites ampliados en 2013), Zona Húmeda de Importancia Internacional según el Convenio Ramsar (1982), Zona de Especial Protección para las Aves de la Red Natural 2000 (ZEPA, 1987), Patrimonio de la Humanidad por la UNESCO (1994), Hábitats de Interés Comunitario (HIC, 1997), Lugar de Importancia Comunitaria (LIC, 2006) Zona de Especial Conservación (ZEC, 2012) y Lista Verde de Áreas Protegidas UICN (2015).

Doñana también ha sido tradicionalmente un lugar muy ligado al aprovechamiento de recursos a través de la pesca, la caza y la recolección de materias primas. En la actualidad el Plan Rector de Uso y Gestión considera como aprovechamientos tradicionales compatibles el carboneo, el coquineo, la apicultura, la recogida de piñas y la ganadería extensiva. En el Área de Influencia Socioeconómica (200.601,86 ha), formada por los términos municipales que aportan terreno al Parque Nacional (Almonte, Hinojos, Aznalcázar y La Puebla del Río; Tabla 1), la actividad económica principal corresponden al sector agrícola, seguido en menor medida del sector servicios, industria y construcción. Dentro del sector agrícola conviven tanto la agricultura tradicional (cereal, olivo y vid) como la agricultura moderna de regadío del fresón y otros frutos rojos, que representan actualmente unos de los principales cultivos en cuanto a superficie y economía de la zona, así como también frutales y arrozal.

**Tabla 1.** Población y superficie (ha) de los municipios incluidos en el Área de Influencia Socioeconómica del Parque Nacional de Doñana. Fuente: INE y Organismo Autónomo de Parques Nacionales.

| Municipio | Provincia | Población (2018) | Superficie(ha) (2015) | % del municipio dentro del Parque Nacional |
|---|---|---|---|---|
| Almonte | Huelva | 24.013 | 86.011,96 | 33,49 |
| Hinojos | Huelva | 3.909 | 32.033,71 | 30,52 |
| Aznalcázar | Sevilla | 4.495 | 45.042,04 | 32,33 |
| Puebla del Río | Sevilla | 11.879 | 37.514,15 | 0,21 |
| **Total** | | **44.296** | **200.601,86** | |

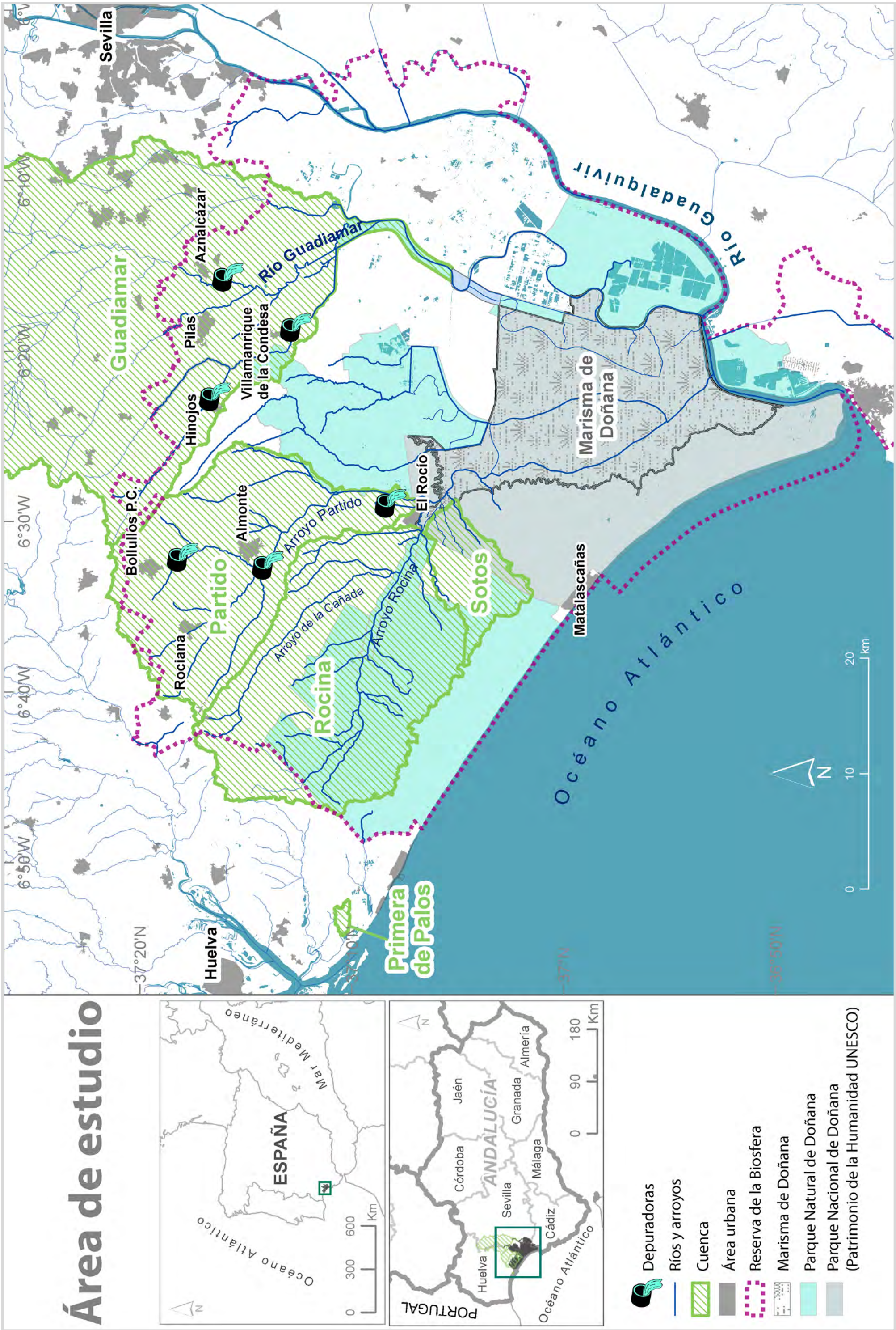


**Figura 8.** Área de estudio.

# Área de estudio

## *Marisma*

La marisma de Doñana, tal y como la conocemos hoy en día, representa solo el 30% (36.000 ha) de la marisma original que había principios de siglo XX, mientras que el otro 70% fue transformado en área de cultivo (Zorrilla-Miras et al. 2014). La marisma es un relieve prácticamente llano formado a partir de la deposición de sedimentos cuaternarios continentales derivados de la dinámica fluvio-mareal en el estuario del Guadalquivir (Machuca et al. 1992). El sustrato más superficial (5-15m) está formado principalmente por arcillas y algo de arenas que hacen de éste una capa impermeable, sin apenas conexión con las aguas subterráneas en esta zona (sistema acuífero número 27, subunidad Almonte-Marisma). Únicamente existe conexión a través de los llamados *ojos*, que son pequeñas y aisladas surgencias de agua subterránea situadas normalmente en los bordes de la marisma. La microtopografía de la marisma, formada por pequeñas elevaciones (*paciles*, *quebradas*, *vetas*, *levé*) y depresiones (*lucios*, *caños*, *hondones*), juega un papel hidrológico y ecológico muy relevante (Mintegui et al. 2004), propiciando la presencia de una gran diversidad de fauna y flora, algunas de ellas incluso amenazadas de extinción (ej. cerceta pardilla, avetoro, malvasía). De hecho, los principales grupos de vegetación que encontramos en la marisma se dividen según el nivel altimétrico: en la marisma baja predominan dos especies de vegetación helófita, la castañuela (*Bolboschoenus maritimus*) y el bayunco (*Schoenoplectus litoralis*); en la marisma alta nos encontramos principalmente con plantas carnosas como el almajo común (*Arthrocnemum macrostachyum*) y el almajo dulce (*Suaeda vera*) (Espinar et al. 2002).

La dinámica hidrológica de la marisma ha sido muy cambiante a lo largo de la historia, debido principalmente a actuaciones antrópicas. Actualmente el funcionamiento hidrológico de la marisma depende en mayor medida del régimen pluviométrico local y de las entradas de aguas superficiales por la zona norte, a través de los arroyos vertientes. De forma general, se distinguen cuatro fases hidrológicas principales: (1) fase de llenado en la que la marisma comienza a encharcarse durante las primeras lluvias del otoño-invierno; (2) fase de inundación en la que una gran extensión se convierte en un lago somero; (3) fase de vaciado donde el comienzo de la primavera provoca un aumento de los procesos de evaporación y menores precipitaciones que van reduciendo el área inundada; (4) fase seca en la que la marisma se seca completamente, convirtiéndose en un desierto de arcilla quebrada, normalmente entre julio y agosto. Sin embargo, esta dinámica puede variar enormemente entre años, dependiendo del momento en que ocurran las precipitaciones y la cantidad de las mismas.

En relación a las presiones humanas, la marisma siempre ha tenido una densidad poblacional baja, debido en gran medida al carácter "insalubre" que históricamente se asociaba a los humedales. Lo anterior favoreció al aislamiento que ha sido clave en la conservación de este territorio a lo largo de la historia. En la actualidad, las actividades humanas dentro de la marisma se limitan principalmente a la ganadería, la investigación científica y, en menor medida, al turismo de naturaleza.

### *Cuencas vertientes*

En la presente Tesis se recogieron datos de diferentes arroyos localizados dentro de las principales cuencas vertientes a la marisma de Doñana: Partido, Rocina, Sotos y Guadiamar (Fig. 8). Todas estas cuencas son a su vez parte de la cuenca del Guadalquivir. Respecto a la litología, estas cuencas se asientan principalmente sobre limos arenosos calcáreos, arenas, areniscas y gravas, todos ellos de origen sedimentario, finos y con poca cohesión (Sendra et al. 2002), lo que proporciona permeabilidad entre las aguas superficiales y subterráneas e incrementa la vulnerabilidad de las cuencas frente a procesos erosivos.

La **cuenca del Partido** cubre una extensión de unas 30.800 ha, tiene un desnivel absoluto de aproximadamente 137 m y su principal curso de agua es el arroyo del Partido. Este arroyo constituye uno de los principales aportes hídricos a la marisma de Doñana. Debido a la proximidad con el océano y la inexistencia de barreras que impidan la entrada de tormentas desde el Atlántico, el arroyo del Partido presenta un comportamiento torrencial que puede llegar a multiplicar de veinte hasta treinta veces la media del caudal estimado durante el periodo estival (5 $m^3 s^{-1}$). Estos eventos extremos van ligados a una fuerte erosión de los cauces, cuyas márgenes no ofrecen apenas protección debido al mal estado de conservación de la vegetación de ribera y de los cauces. Lo anterior se debe a un cúmulo de actuaciones humanas como los desmontes, las canalizaciones, las parcelaciones del cauce, el sobrepastoreo, el vertido de escombros en ciertos puntos y la ocupación intensiva que han contribuido al deterioro general de este sistema fluvial durante las últimas décadas (Borja et al. 2009). Una de las mayores consecuencias ha sido el incremento en el transporte de sólidos a la marisma, lo que ha formado un cono de deyección que en el futuro podría llegar a cerrar el paso de agua entre la marisma del Rocío y el Caño de la Madre. La eliminación de la vegetación de ribera también ha dado lugar a que especies exóticas invasoras como *Arundo donax* (cañaverales) colonicen buena parte de las orillas de los cauces (Borja et al. 2015).

En relación a los usos del suelo en la cuenca, desde el último cuarto del siglo XX se han producido importantes transformaciones como la sustitución progresiva de los cultivos tradicionales por otros más tecnificados e intensivos (cultivos de regadío bajo plástico), la extracción de áridos (canteras) o la sustitución de monte natural por plantaciones de eucaliptos. Además, desde la década de los noventa la implantación de cultivos bajo plásticos alrededor de los cauces ha contribuido a la impermeabilización de las laderas así como la utilización de productos químicos con efecto impermeabilizante en el cultivo de olivares y frutales, provocando como consecuencia la reducción de la infiltración del agua y el aumento de la escorrentía, lo que acelera aún más el proceso erosivo y la acumulación de sedimentos en el cauce (Borja et al. 2009).

La **cuenca de la Rocina** cubre una extensión de 40.000 ha, tiene un desnivel absoluto de aproximadamente 115 m y su principal curso de agua es el arroyo de la Rocina, que forma parte del Parque Nacional de Doñana bajo la figura de "Zona de Protección del Arroyo de la Rocina". El arroyo de La Rocina discurre en dirección este-sureste y desemboca en la marisma de Doñana a la altura del Rocío, constituyendo uno de sus principales aportes de agua. Su caudal, sobre todo en el tramo bajo, es de origen freático la mayor parte del año, mientras que la escorrentía por precipitaciones sólo es importante con lluvias intensas. Esto contribuye a prolongar el periodo de inundación de la marisma más allá de la finalización del periodo de lluvias (Custodio et al. 2009). Una de las características más remarcables de este arroyo es que presenta uno de los bosques autóctonos de ribera mejor conservados del entorno de Doñana. Este bosque de ribera proporciona importantes servicios ecosistémicos en la cuenca, como por ejemplo, reduciendo la erosión de las márgenes o proporcionando un hábitat de calidad a especies de fauna y flora amenazadas (ej. lince ibérico). Estos bosques se mantienen principalmente por la existencia de aguas freáticas y profundas del acuífero de Doñana. A excepción del arroyo de la Rocina, el resto de afluentes en la cuenca se encuentran en un estado de conservación peor, sobre todo los que se localizan en la zona de cabecera y la margen izquierda (ej. arroyo de la Cañada) debido a la presencia de grandes extensiones de cultivos de regadío intensivos. Los afluentes de la margen derecha están mejor conservados ya que se encuentran dentro de los límites del Espacio Natural de Doñana (END) y además conectan con un humedal de especial interés (Lagunas de Ribetehilos).

Sin embargo, durante las últimas décadas la descarga de aguas subterráneas a los arroyos de la cuenca y a la marisma ha ido disminuyendo como consecuencia del descenso del nivel freático debido a la extracción intensiva de agua por la expansión de los cultivos en la zona alta de la cuenca. Como consecuencia, muchos de los arroyos, caños y cañadas vertientes a la marisma y La Rocina que antes eran permanentes ahora son temporales. Esto provoca un cambio en la distribución y duración de las fases secas y húmedas que afecta directamente a la accesibilidad de la vegetación al agua freática (Custodio et al. 2009). Lo anterior está afectando principalmente a las zonas bajas de la cuenca, que son las más dependientes de las aguas subterráneas.

La **cuenca de los Sotos** es con diferencia la más pequeña entre las que vierten a la marisma, cubriendo una extensión de 3.540 ha. Aproximadamente un tercio de la cuenca se encuentra dentro del Parque Nacional, otro tercio dentro del Parque Natural y otro tercio se divide en: 1) una zona de agricultura intensiva y 2) una zona que hasta 2015 también era agrícola pero que la Confederación Hidrográfica del Guadalquivir compró para fines de restauración ecológica (Finca "Cortijo de los Mimbrales"). Los arroyos principales en esta cuenca son tres y todos vierten directamente a la marisma: arroyo de Soto Grande, Soto Chico y Caño Mimbrales. Los tres arroyos son muy intermitentes y apenas mantienen una lámina de agua fina durante unos pocos meses en el periodo más húmedo. Según estudio previos (Higueras 2014) la fuente principal del agua son los excedentes de riego que los canales de drenaje introducen en las balsas de regulación de Mimbrales-Guayules construidas en el año 2002 en el marco del proyecto del Ministerio de Medio Ambiente Doñana 2005. Solo en la desembocadura a la marisma (los llamados "Sotos") los arroyos consiguen mantener durante más tiempo una lámina de agua superficial como resultado de que se conectan de nuevo con el nivel freático y porque también existe una capa de arcillas por debajo del lecho del cauce que impermeabiliza el agua y crea un humedal permanente durante todo el año. El arroyo de Soto Chico es el que más agua transporta de los tres porque ambas balsas desaguan hacia su cauce.

No obstante, el descenso generalizado del nivel freático en el acuífero de Doñana por el bombeo de agua para regadío también está afectando negativamente a los arroyos de esta cuenca, especialmente a su vegetación freatofítica. Otro problema asociado con la actividad agrícola en esta cuenca es el fuerte arrastre de sedimentos (con fertilizantes adsorbidos) desde los campos de cultivos que se produce durante los eventos de lluvias intensas, lo cual ha causado la formación de un delta arenoso en la desembocadura a la marisma (Higueras 2014).

La **cuenca del Guadiamar** cubre una extensión de 130.000 ha y su cauce principal es el río Guadiamar que nace en Sierra Morena, cerca del Municipio de Castillo de las Guardas (Sevilla), recorriendo 80 km y salvando un desnivel total de 320m hasta desembocar en el río Guadalquivir. La cuenca del Guadiamar presenta una clara división en tres ámbitos bien diferenciados: la sierra (tramo alto), la campiña (tramo medio) y la marisma (tramo bajo) (Borja et al. 2001). En la presente Tesis, los muestreos de campo en esta cuenca se llevaron a cabo únicamente en el tramo bajo. En este tramo las aguas discurren por la Marisma de Entremuros del Parque Natural, que es una zona prácticamente llana, canalizada entre dos diques artificiales y que desemboca en el Brazo de la Torre y posteriormente en el río Guadalquivir. La litología de este tramo bajo está formada por arenas y arcillas principalmente. Históricamente, el río Guadiamar ha sido el principal aporte de agua a la marisma de Doñana a través del Caño Guadiamar y el Caño Travieso. Sin embargo, las numerosas modificaciones en la hidrología de la zona y el accidente minero de Aznalcóllar en 1998, acabaron desconectando por completo la marisma de las aguas del Guadiamar. Casi dos décadas después del accidente minero las aguas del Guadiamar volvían a entrar a la marisma desde la Vuelta de la Arena (Entremuros) a través del Caño Travieso, como resultado del Proyecto de Restauración Hídrica Doñana 2005 (Proyecto Doñana 2005) (MIMAD 2001). Sin embargo, tanto los caudales como las aportaciones de agua a la marisma son muy irregulares y se producen principalmente durante eventos de avenidas con lluvias intensas.

En el pasado, la calidad del agua en el río Guadiamar ha sido baja debido a la contaminación por metales pesados derivados de la actividad minera en la cuenca y también por contaminación difusa de la agricultura intensiva y por vertidos no tratados de aguas residuales urbanas e industriales. Sin embargo, las medidas de descontaminación tras el accidente minero y la creación del Corredor Verde del Guadiamar (i.e. restauración ecológica de las márgenes) han supuesto una reducción muy significativa de los vertidos de aguas contaminadas por metales, de la actividad agrícola sobre las terrazas más bajas del río Guadiamar y de las extracciones de aguas subterráneas para regadío. No obstante, la agricultura sigue siendo una de la actividades más importantes en la cuenca y por consiguiente la contaminación difusa por nutrientes aún supone una amenaza para las aguas superficiales y subterráneas (Custodio et al. 2009).

### *Laguna Primera de Palos*

La laguna Primera de Palos (17 ha) se localiza dentro de la demarcación hidrográfica del Tinto, Odiel y Piedras, en los términos municipales de Palos de la Frontera y Moguer en la provincia de Huelva, a 35 km al noroeste del Parque Nacional de Doñana. Forma junto con las lagunas de la Jara, Mujer y de las Madres, el Paraje Natural de las Lagunas de Palos y de las Madres. Estas lagunas tiene un alto valor ecológico, especialmente teniendo en cuenta la fuerte antropización de los alrededores debido a la agricultura y a la industria petroquímica. A pesar de no formar parte de las cuencas vertientes a la marisma de Doñana, la laguna Primera de Palos se ha incluido en la presente Tesis con el fin de obtener valores isotópicos de referencia de un sistema acuático afectado casi exclusivamente por fertilizantes inorgánicos, derivados de los cultivos bajo plásticos de fresón y otros frutos rojos que ocupan la totalidad de la cuenca (280 ha).

La litología de la cuenca está compuesta principalmente por arenas y los sedimentos de la laguna están formados por limos, arcillas y una capa superficial formada materia orgánica en degradación. La cubeta de la laguna está cubierta de la macrófita *Ceratophyllum demersum*, y también se observan especies de helófitos en la zonas de las orillas (*Phragmites australis* y *Typha dominguensis*) (Fernández-Zamudio et al. 2005). La profundidad máxima de la laguna es aproximadamente 3 m. Sin embargo, las concentraciones de nutrientes disueltos suelen ser altas, sobre todo de nitratos durante el invierno, lo que indica que los nutrientes proceden de los fertilizantes usados para las prácticas agrícolas en la cuenca.


Marisma
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La Rocina
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## El Partido


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## Los Sotos


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## *Guadiamar*

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## *Laguna Primera de Palos*

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**Figura 9.** Pictures of the study area. 1) Flooded marsh (29/04/2015); 2) Dry marsh (29/04/2015); 3) Lucio Cardales (06/05/2015); 4) Ganado marismeño (24/10/2018; Foto de Juan Giralt); 5) Vado de Don Gil, arroyo de La Rocina (oct. 2015); 6) Arroyo de la Cañada (25/03/2015); 7) Puente de las Ortigas, arroyo de La Rocina (25/03/2015); Vado de Don Gil, arroyo de La Rocina (oct. 2015); 9) Arroyo del Partido (feb. 2015); 10) Arroyo del Partido aguas abajo de la depuradora de Almonte (feb. 2015); 11) Puente del Ajolí, arroyo del Partido (feb. 2015); 12) Vado de la Pasada del Chivo, arroyo del Partido (feb. 2015); 13) Soto Chico (18/02/2016); 14) Laguna de los Mimbrales (18/12/2015); 15) Laguna de los Mimbrales (26/04/2016); 16) Soto Grande (17/02/2015); 17) Laguna de la Dehesa de Abajo (22/03/2015, foto de Andy Green); 18) Presa de la laguna de la Dehesa de Abajo (16/12/2014); 19) Canal que conecta la laguna de la Dehesa de Abajo con Entremuros (22/12/2014); 20) Entremuros (aguas del río Guadiamar)(25/03/2015); 21) Laguna Primera de Palos (26/01/2016); 22) Arroyo que desemboca en la Laguna Primera de Palos (26/01/2016); 23) Invernaderos e industria petroquímica en los alrededores de la Laguna Primera de Palos (26/01/2016); 24) Laguna Primera de Palos (26/01/2016); 25) Excedentes de riego de los cultivos bajo plástico en los alrededores de la Finca de los Mimbrales (23/05/2017); 26) Efluente de la depuradora del Rocío (feb. 2015); 27) Canal de drenaje entre cultivos bajo plástico en los alrededores del Rocío (23/05/2017); 28) Actividad ganadera en la cuenca del Guadiamar (Dehesa de Abajo) (22/03/2015); foto de Andy Green).

## Metodología general

Para estudiar el estado y origen de la eutrofización en las aguas superficiales de la marisma del Parque Nacional de Doñana y los arroyos vertientes, en la presente Tesis doctoral se usaron algunos de los principales indicadores físico-químicos, biológicos e hidrológicos (concentración de nitrógeno y fósforo, clorofila-*a* del fitoplancton, conductividad, profundidad, caudal) que comunmente se incluyen dentro de los protocolos de seguimiento de la calidad de las aguas superficiales en España así como en el resto del ámbito europeo, según lo establecido por la Directiva Marco del Agua (DMA). Sin embargo, en un contexto en el que la contaminación de nutrientes proviene de múltiples fuentes, tanto difusas (ej. agricultura) como puntuales (ej. depuradoras urbanas o industriales), estos indicadores resultan insuficientes por sí solos cuando se trata de determinar origen de los nutrientes. La identificación de las principales fuentes difusas y puntuales, así como su contribución relativa, es fundamental para poder aplicar medidas de control efectivas que reduzcan la cantidad de nutrientes que llegan a los sistemas acuáticos. En este sentido, en la presente Tesis doctoral se incluyeron los análisis isotópicos (Caja 3) del nitrógeno, oxígeno e hidrógeno en plantas acuáticas emergentes (castañuela -*Bolboschoenus maritimus* y espadaña -*Typha domingensis*) ($\delta^{15}N$), nitratos disueltos ($\delta^{15}N$, $\delta^{18}O$) y agua ($\delta^{2}H$), y otros parámetros físico-químicos e hidrológicos del agua (ej. conductividad, profundidad de la columna de agua).

Todos los datos usados en la presente Tesis se recogieron en 56 puntos de muestreo diferentes durante múltiples muestreos de campo entre 2013 y 2016, principalmente durante el periodo más húmedo (octubre-junio), cuando la disponibilidad de agua fue mayor.

### *Caja 3. Isótopos estables y su aplicación*

Los isótopos estables son formas no radiactivas de átomos. Muchos elementos de la tabla periódica, entre ellos el H, N, O, C y S, poseen dos o más isótopos con el mismo número de protones pero se diferencian en el número de neutrones, y por lo tanto, en su masa. Se denominan "estables" porque no se descomponen con el tiempo a diferencia de los isótopos radiactivos. Los isótopos estables se encuentran ampliamente distribuidos en forma de diferentes moléculas por toda la hidrosfera, biosfera, litosfera y atmósfera. La característica más importante del análisis de isótopos estables es la capacidad de distinguir entre fuentes, procesos o niveles tróficos a partir de diferencias mínimas entre firmas isotópicas. La abundancia natural de los isótopos estables se expresa con la letra griega delta ($\delta$) que representa la desviación del ratio entre el isótopo pesado y el ligero de una muestra con respecto al ratio de un patrón estándar menos 1:

$$\delta = R_{muestra}/R_{estándar} - 1$$

donde R es el ratio de la muestra o del patrón (ej. $^{15}N/^{14}N$). Los patrones más comunes son el N atmosférico para $\delta^{15}N$, la caliza VPDB (Vienna PeeDee Belemnite) para el $\delta^{13}C$ y el SMOW (Estándar Mean Ocean Water, Vienna) para el $\delta^{17}O$ y $\delta^{18}O$. En general, el $\delta$ se multiplica por mil para amplificar las pequeñas diferencias entre la muestra y el patrón, expresándose en partes por mil (‰) (Fry, 2006).

Con las aproximaciones isotópicas se puede obtener información sobre el funcionamiento del ecosistema, los procesos biogeoquímicos y los tipos de fuentes contaminantes de nutrientes, lo cual no es posible si únicamente se usan parámetros tradicionales de cuantificación de solutos (Kendall et al. 1998, 2008; Kohzu et al. 2008).

Desde finales del siglo XX se recogen numerosos estudios sobre la trazabilidad de fuentes de nutrientes precursoras de la eutrofización, principalmente en relación al N de origen antrópico (Owens, 1989; Tucker et al.

1999; Costanzo 2001; Loomer et al. 2015). En áreas impactadas por la agricultura o las zonas urbanas, es común que la contaminación por N en aguas superficiales y subterráneas provenga de múltiples fuentes con firmas isotópicas ($\delta^{15}N$) diferentes. Sin embargo, hay ocasiones en las que los valores de $\delta^{15}N$ de distintas fuentes de N se pueden solapar (ej. aguas residuales urbanas vs. abonos orgánicos). Además, durante el transporte del N también se producen procesos de transformación (ej. desnitrificación, nitrificación, anammox) que llevan asociado un fraccionamiento isotópico, dando como resultado valores de $\delta^{15}N$ diferentes a los de la fuente. Por lo tanto, el uso exclusivo del $\delta^{15}N$ no siempre puede aportar una información definitiva sobre el origen de los compuestos nitrogenados en el agua. En este sentido se ha visto que el uso de las aproximaciones multi-isotópicas contribuye enormemente a diferenciar con más precisión las distintas fuentes del N. Por ejemplo, con respecto a los nitratos, es frecuente incluir también los valores de $\delta^{18}O_{NO3}$, pues las diferencias de $\delta^{18}O_{NO3}$ entre diferentes fuentes solapan mucho menos que en el caso del N. Los isótopos de O también se emplea para determinar si se están produciendo procesos de transformación ya que el fraccionamiento da lugar a cambios en los valores de $\delta^{18}O_{NO3}$ (Chang et al., 2002; Wassenaar, 1995). Aparte del O, existen otros isótopos estables, como por ejemplo, los isótopos de azufre (S) (Bates et al., 2002), de boro (B) (Barth, 2000), de estroncio (Sr) (Vitòria et al., 2004; Widory et al., 2004) y de litio (Li) (Qi et al., 1997), que pueden ayudar a identificar con mayor detalle las fuentes contaminantes de N en los ecosistemas mediante la detección de fertilizantes, detergentes y fármacos en el agua o la distinción entre nitratos de origen animal (i.e. abonos) o derivados de plantas de tratamiento de aguas residuales.


**Figura 10.** Muestreo de campo y procesado de muestras en laboratorio.

# Objetivos y estructura de la tesis doctoral

### *Objetivos*

Desde hace décadas, la integridad ecológica de Doñana ha estado amenazada por múltiples presiones antrópicas, entre las cuales destaca la eutrofización. Sin embargo, la escasa disponibilidad de datos históricos sobre calidad del agua y la baja resolución espacio-temporal de los mismos ha dificultado hasta el momento un análisis detallado del proceso de eutrofización en el que además se pueda determinar el origen específico de los nutrientes. Esto último es especialmente relevante, ya que la asignación de un peso relativo a cada una de las potenciales fuentes es necesaria para tomar medidas adecuadas y eficaces que aseguren la conservación de los ecosistemas acuáticos de Doñana.

En este contexto, el **objetivo general** de la presente Tesis es generar conocimiento sobre los patrones espacio-temporales en las concentraciones de nutrientes e identificar el posible origen de los mismos, para abordar las causas de la eutrofización, tanto en la marisma del Parque Nacional como en los arroyos de las cuencas vertientes. Los objetivos específicos se describen en el siguiente apartado.

### *Estructura*

La presente Tesis Doctoral consta de una **Introducción**, una **Discusión** y unas **Conclusiones generales**, y de tres **Capítulos**. Dos capítulos han sido publicados y el otro está enviado a revistas internacionales indexadas. Para cada uno de los capítulos se seleccionaron áreas de estudio específicas de Doñana y alrededores (Fig. 8). A continuación se detalla el contenido y **objetivos específicos** de cada uno de los capítulos.

En el **Capítulo 1** (*Ongoing anthropogenic eutrophication of the catchment area threatens the Doñana World Heritage Site (South-west Spain)*) estudio la variación espacio-temporal de las concentraciones de nutrientes (N y P) y clorofila-*a* en aguas superficiales de la marisma de Doñana y principales arroyos vertientes, durante el periodo 2013-2016. También realizo un análisis cuantitativo de la evolución de los cultivos bajo plástico desde 1995 hasta 2018 para evaluar el desarrollo de la agricultura en las cuencas y su impacto sobre la calidad de las aguas superficiales en los ecosistemas acuáticos que estudio en la presente Tesis.


En el **Capítulo 2** (*Stable isotopes in helophytes reflect anthropogenic nitrogen pollution in entry streams at the Doñana World Heritage Site*) estudio el posible origen de la contaminación antrópica por nitrógeno en la marisma de Doñana y en arroyos vertientes mediante la cuantificación de las concentraciones de N en las aguas superficiales y la composición isotópica del N ($\delta^{15}N$) en dos especies de helófitas ampliamente distribuidas en la zona de estudio (*Bolboschoenus maritimus* y *Typha domingensis*).


En el **Capítulo 3** (*Agricultural and urban delivered nitrate pollution input to Mediterranean temporary freshwaters*) analizo el posible origen de la contaminación por nitratos mediante la cuantificación de la composición isotópica del N y O ($\delta^{15}N$ y $\delta^{18}O$) en diferentes puntos de arroyos vertientes a la marisma de Doñana que están afectados en mayor o menor medida por la contaminación difusa (agricultura) y puntual (aguas residuales urbanas) en la cuenca.

Capítulo 1

# Ongoing anthropogenic eutrophication of the catchment area threatens the Doñana World Heritage Site (South-west Spain)

En revisión: Paredes, I., Ramírez, F., Aragonés, D., Bravo, M.A., Forero, M.G., Green, A.J.. Ongoing anthropogenic eutrophication of the catchment area threatens the Doñana World Heritage Site (South-west Spain).

# Abstract

In recent decades, reductions in nutrient inputs have led to improvements in water quality in many rivers and lakes in central and northern Europe, but long-term trends are less clear in southern Europe. We conducted the first comprehensive study of water quality in Doñana (SW Spain), one of the most important wetland complexes in Europe and the Mediterranean region. The core area of Doñana is a large shallow, seasonal marsh (UNESCO World Heritage Site) that floods during rainy, cool winter months, then dries out during the summer. The marsh is fed by three main streams whose catchments are impacted by greenhouses (irrigated with groundwater), poorly treated urban wastewaters and tourism. From 2013 to 2016, we monitored nutrient (N and P) and chlorophyll-*a* (chla) concentrations in surface waters of the marsh and three main tributary streams. We quantified changes in greenhouse cover since 1995 using satellite images. The Partido and Rocina catchments suffered a fivefold expansion of greenhouses from 1995 to 2016. Nutrient concentrations in streams were consistently higher than in the marsh, particularly in the Partido stream, and regularly reached concentrations equivalent to a "bad physico-chemical status" under the Water Framework Directive. Nutrient concentrations in spot samples within the marsh largely depended on a combination of evaporation and spatial processes. Patterns in chla concentrations were less consistent, and the Partido had lower concentrations than other streams likely due to generally higher stream flows. Anthropogenic nutrient pollution in entry streams is a serious problem in Doñana. Reinforcement of policies aimed at reducing nutrient inputs to Doñana are urgently required to guarantee meet the biodiversity conservation objectives for the protected area and WHS. Paradoxically the marsh is currently relied upon to purify the water entering from streams.

# Introduction

Wetlands constitute major biodiversity hotspots and provide multiple ecosystem services (Davidson et al., 2018; Finlayson et al., 2018). However, since the beginning of the 20$^{th}$ century, wetland loss and degradation has intensified due to increasing anthropogenic pressures (Davidson, 2014). Eutrophication is a major cause of wetland degradation worldwide (Ramsar Convention on Wetlands, 2018), mainly driven by agricultural, urban and industrial activities (Kingsford et al., 2016; Verhoeven et al., 2006), and causes multiple negative effects such as alteration of the biogeochemical cycling, toxic cyanobacterial blooms, anoxia and collapse of fish and submerged plant communities (Ansari et al., 2011; Sánchez-Carrillo et al., 2011). Eutrophication may also lead to negative socio-economic impacts, due to declining quality and increased disease risk of drinking water supplies, decline of fish stocks, or decreasing tourism and leisure activities (e.g. due to bad odors or loss of water transparency) (Schuyt, 2005). Eutrophication impacts are further enhanced due to synergy with climate change effects such as warming, soil erosion and droughts (Cramer et al., 2018; Geijzendorffer et al., 2019; Green et al., 2017). Thus, maintaining good water quality status of wetlands is essential for human well-being, biodiversity maintenance, and to enhance resilience to climate change (Green et al., 2017).

Implementation of broad-scale measures (e.g. European Union Directives) has led to major improvements of water quality through reductions of anthropogenic nutrient inputs in much of northern and central Europe (EEA, 2015). However, less information is available about southern Europe. Countries of the Mediterranean region have greater challenges due to lower precipitation and rapid increases in human population, particularly around coastal wetlands (MWO2, 2018). In the most important remaining Mediterranean wetlands, it is vital to increase knowledge on spatio-temporal nutrient inputs at the catchment scale, and plan effective actions for preventing and controlling eutrophication.

Although wetlands provide a vital ecosystem service by reducing nutrient loads and enhancing water quality (Johnston, 1991; Fisher and Acreman, 2004), high water quality should be ensured at the catchment level before surface water reaches wetlands, so as to guarantee their functioning and biodiversity value. This action may be accomplished by conserving natural vegetation in the catchment area, tertiary water treatment, or use of constructed wetlands. Green infrastructure, such as constructed wetlands and riparian buffer zones, has been used to reduce nutrient loadings into protected natural wetlands, such as the iconic Everglades National Park (Chimney and Goforth, 2006; Tonderski et al., 2017).

Doñana in SW Spain is an iconic wetland complex (Scheffer et al., 2015) recognized as one of the most biodiverse areas in Europe and the Mediterranean region (Green et al., 2018). However, the core area protected as a National Park and a UNESCO World Heritage Site (WHS) contains an extensive marshland threatened by impacts on water quantity and quality, especially due to intensive agriculture and municipal wastewaters in the catchment (Camacho-Muñoz et al., 2010; Green et al., 2017; Paredes et al., 2019). Nevertheless, there is a lack of detailed studies of nutrient status and eutrophication both inside and outside the boundaries of Doñana National Park.

In this study, we address this gap by studying the spatio-temporal variability of standard eutrophication indicators (nitrogen (N), phosphorus (P) and chlorophyll-*a* (chla) concentrations) across Doñana (both the marshland and the streams that supply it) during four consecutive years (2013 to 2016). Using accepted water quality standards, we assess whether poor water quality may be a threat to biodiversity in the study area. We evaluate long-term trends in intensive agriculture by quantifying the changes in surface area of greenhouses in the catchment between 1995 and 2018, since these contain berry farms relying on extraction of groundwater and intensive chemical use (Green et al., 2017). We hypothesized that: (1) streams show higher nutrient and chla concentrations than the marsh because they are located closer to pollution sources (agriculture and urban wastewaters); (2) streams located in sub-catchments most directly affected by greenhouses and urban effluents show higher nutrient and chla concentrations; (3) the marsh provides a purification service, reducing nutrient loads from input streams; (4) spatial patterns of nutrients and chla concentrations across the marsh and the streams consistently vary between the main seasons (i.e. between the cool, wet autumn/winter months and the warm, dry spring/summer months). To test these hypotheses, we analyzed spatial patterns in water quality across the study area, and considered how variation in nutrient and chla concentrations was related to variation of other physico-chemical variables (including depth variation and evaporation processes within the marsh). Our study provides insight into the eutrophication processes in Doñana, which may be common to other complex Mediterranean aquatic ecosystems affected by increasing nthropogenic presures.

# Materials and methods

## Study area

### *Doñana marsh*

The Doñana wetland complex occupies a seasonal brackish floodplain located in the estuary of the Guadalquivir River on the Atlantic coast in Southwestern Spain (37°0'N 6°37'W) (Fig.1). The natural marsh is of international importance for biodiversity conservation, covering a large area (270 km$^2$) of Doñana National Park (543 km$^2$), which was declared in 1969 and later designated as a Wetland of International Importance under the Ramsar Convention in 1982, EU-Special Protection Area for birds in 1988, and WHS in 1994 (Green et al., 2018). The entire wetland complex is included in a UNESCO Biosphere Reserve declared in 1980 (Fig.1). In the past, the natural marsh covered a much larger area, but about 80% of it was transformed into other land uses (mainly agriculture) during the 20th century (Zorrilla-Miras et al., 2014a).

Doñana is in a region with a subhumid Mediterranean climate with an Atlantic influence. Mean annual temperature is 17°C and the mean annual precipitation is 550 mm, with high interannual variation ranging between 170 and 1000 mm (see Fig.S1 and Díaz-Delgado et al., 2016). Precipitation is mainly concentrated between October and April with a hot, dry season from May to September. Mean annual precipitation during our study period (Sept. 2012 to August 2016) was 480.6 ± 90.3 mm, with 85.5% of total precipitation falling between October and April. Direct precipitation and surface flow from streams constitute the main water inputs into the marsh, which is largely isolated from the Guadalquivir River. Additional inputs to the marsh come from direct groundwater inputs and tidal inputs from the Guadalquivir estuary. There is high interannual variation in the extent and depth of flooding in the marsh (see Fig. S2 and Díaz-Delgado et al., 2016). Owing to flat, shallow topography, hydrodynamics and water distribution in the marsh are strongly affected by wind, which may produce changes in depth due to accumulation of water downwind (Ramos-Fuertes et al., 2014). In addition, water slowly moves by gravity in a southerly direction until flowing through sluices into the estuary of the Guadalquivir River. Evaporation meanwhile leads to a spatial salinity gradient with higher salinity towards the south, away from entry streams (Espinar and Serrano, 2009; Grillas et al., 1993) (Fig. S5).

Hydrological connectivity in the marsh depends on the flooding level and microtopography. In general, below a flooding level of about 1.3 masl, the marshes are highly fragmented with surface waters divided into different units (Fig. S2). The highest flooding levels are reached during winter months, and the marsh dries out completely in July-August (Díaz-Delgado et al., 2016). The mean annual maximum water column at the deepest point in the marsh is 0.51 m. Evaporation in the marsh is fairly constant between October and February (2 mm day$^{-1}$), then increases about 5% per day from March until reaching 8 to 10 mm day$^{-1}$ in June/July (Ramos-Fuertes, 2012).

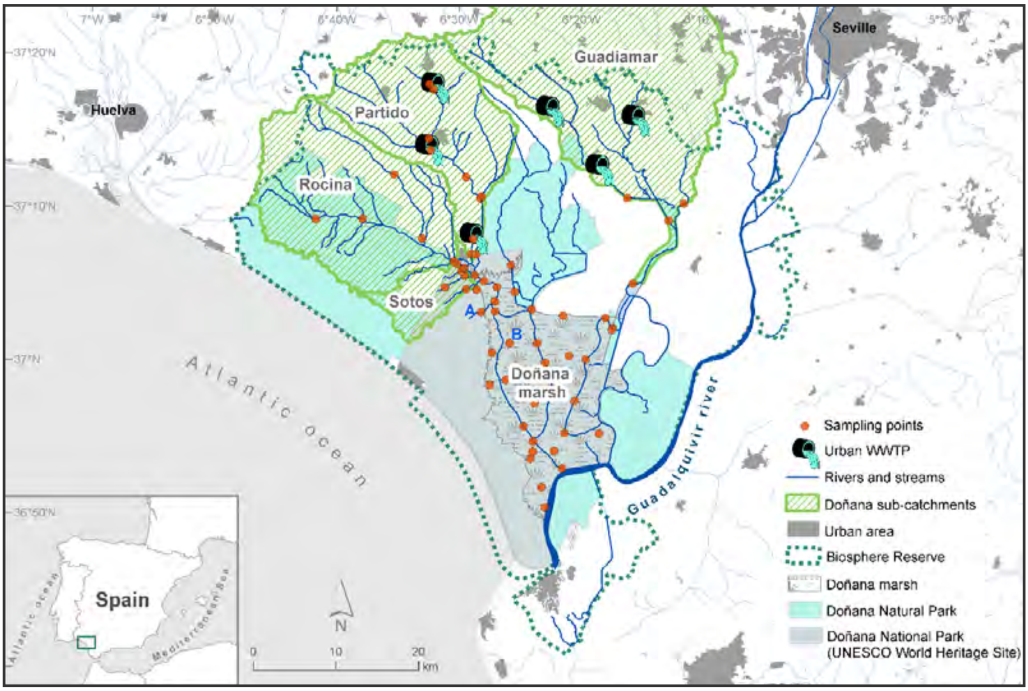


**Figure 1.** Study area, indicating the boundaries of protected areas and the sub-catchments that were studied. The boundary for the UNESCO World Heritage Site matches that of the National Park. Dots (in orange) represent the total number of sites used for surface water sampling in this study.

## *Entry streams*

The most important entry streams draining into the Doñana marsh are located within four sub-catchments (Guadiamar (1300 km$^2$), Rocina (416 km$^2$), Partido (291 km$^2$), Sotos (60 km$^2$)) (Fig.1). Apart from direct precipitation and runoff, all these entry streams are also partly fed by groundwater discharge (Arambarri et al., 1996; Guardiola-Albert et al. 2011). Streams of the Rocina, Partido and Sotos catchments enter the marsh in its north-west corner (Fig.1). The Rocina and Sotos catchments are impacted by similar agricultural activities without any influence of wastewater treatment plants, and owing to the small number of sampling points for Sotos, we combined these adjacent catchments (i.e. Rocina/Sotos) to facilitate statistical analyses. Since a restoration project completed in October 2014, water from the Guadiamar catchment flows again into the marsh through a channel at the north east corner (Fig. 1), after previously being disconnected by dykes for decades due to drainage of adjacent areas for agriculture. Since it was affected by a spill of mine waste in 1998, the Guadiamar catchment has been partly restored, with an increase in riparian vegetation (Ontiveros et al., 2013).

## Field sampling

We collected surface water samples over a broad network of points (56) across the Doñana marsh and entry streams (Fig.1) during four consecutive hydrological years from September to August of 2012/13, 2013/14, 2014/15 and 2015/16. From here on, these years are referred to as 2013, 2014, 2015 and 2016 respectively. The samples we analyzed were mainly collected between October and early June since surface water is absent in most streams and the marsh during high summer (late June to mid September) due to scarce precipitation and high temperatures, and because groundwater extraction (mainly for greenhouses) has severely reduced discharges into the streams (Guardiola-Albert and Jackson, 2011). Sampling time, location and number of water samples collected varied between months and years due to variation in the spatial distribution of surface water. During 2014, flooded areas were particularly scarce across the marsh due to low levels of precipitation (Fig. S2).

We collected 1-2L of surface water to measure N, P and chla concentrations. At the end of each field day, we filtered part of our samples through FILTER-LAB MFV5047 glass-fiber filters (0.45µm pore size) using a low-pressure vacuum pump. We stored all filtered samples in the freezer (-20°C) prior to analysis of dissolved nutrient concentrations. To determine hydrogen isotopic ratios ($\delta^2H$) in water, we filtered 2 ml of water through CHROMAFIL® Xtra PET-20/25 0.20µm and transferred it into a glass vial with a septum cap. We stored these samples at 5ºC prior to analysis. At the spot where the water samples were taken, we recorded *in situ* electrical conductivity (cond) using a WTW (Weilheim, Germany) Multi-340i handheld meter and water column depth using a measuring stick. Some parameters (e.g. chla or $\delta^2H$) were only measured in later years, thus leading to differences in sample sizes.

## Laboratory analyses

### *Nitrogen and phosphorus*

We used standard colorimetric methods to measure the concentration of three dissolved inorganic nitrogen (DIN) species (nitrate $NO_3$, nitrite $NO_2$ and ammonium $NH_4$) and phosphate $PO_4$ (ISO 13395:1996 for nitrate and nitrite; ISO 11732:2005 for ammonium; ISO 15681-2:2003 for phosphate) on a multi-channel SEAL Analytical AA3 AutoAnalyzer (Norderstedt, Germany). We used unfiltered water samples to analyze total nitrogen (TN) by digestion with potassium persulfate as described by Nydahl (1978) , and total phosphorus (TP) by digestion with a mixture of potassium persulphate and sulphuric acid following the colorimetric method (Murphy and Riley, 1962). We performed all the nutrient analyses at the Laboratory of Aquatic Ecology (LEA) at the Doñana Biological Station (EBD-CSIC; Seville, Spain).

### *Chlorophyll-a*

We determined chla concentrations using acetone extraction (UNESCO, 1966). We first filtered water samples through FILTER-LAB MFV5047 glass-fiber filters (0.45µm pore size) and then soaked the filters in acetone (90%) overnight at 4ºC in the dark. Then we filtered again all the acetone samples. Finally, we read absorbance values at 750, 663, 645 and 630 nm using a UV VIS spectrophotometer (AnalytiK Jena AG, model SPECORD ® 205), from which we calculated chla concentrations (the 750 nm reading was used to correct for turbidity).

### *Water isotopic analyses*

To obtain hydrogen isotopic values ($^2H/^1H$, $\delta^2H$, ‰), we analyzed the water samples by cavity ring-down spectroscopy (CRDS) using a L2130-i spectrometer (Picarro Inc., 480 Oakmean Parkway, Sunnyvale, California, 94085, USA; www.picarro.com) in the Stable Isotopes Laboratory (LIE-EBD) at the Doñana Biological Station (EBD-CSIC; Seville, Spain).

## Marsh flooding masks

Flooding masks were generated by the Remote Sensing and GIS Laboratory (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). These masks were used to illustrate the variation in flooding patterns during our sampling campaigns.

## Spatio-temporal evolution of greenhouses

In recent decades, greenhouses dedicated to strawberry, raspberry, blueberry and blackberry cultivation have substantially increased in the surroundings of Doñana, and now occupy more than half of the total cultivated area of the Rocina, Partido and Sotos catchments (WWF 2018). These crops depend on intensive groundwater extraction and agrochemical use, and to contaminate the entry streams with nutrients originating from fertilizers (Manzano et al. 2009). Hence we quantified their expansion within the Doñana catchment between 1995 and 2018 via remote sensing, applying an ensemble of target detection methods on radiometrically normalized Landsat images (Díaz-Delgado et al., 2016) to determine the annual greenhouse surface area in the Rocina, Partido and Sotos catchments.

From each hydrological year (24 in total) we selected one cloud-free Landsat image from autumn and another from spring (48 images in total). For each year, we grouped both autumn and spring images into a single 12-band image (B1: Blue, autumn; B2: Green, autumn; B3: Red, autumn; B4: NIR, autumn; B5: SWIR1, autumn; B6: SWIR2, autumn; B7: Blue, spring; B8: Green, spring; B9: Red, spring; B10: NIR, spring; B11: SWIR1, spring; B12: SWIR2, spring). For each image, we identified and classified different land covers such as greenhouses, urban areas, forested areas and other crops by simultaneously applying eight automatic target detection methods: Matched Filtering (MF) (Chen et al. 1987), Constrained Energy Minimization (CEM) (Chang et al. 2000), Adaptive Coherence Estimator (ACE) (Kraut et al. 2005), Spectral Angle Mapper (SAM) (Kruse et al. 1993), Orthogonal Subspace Projection (OSP) (Harsanyi and Chang et al.1994), Target-Constrained Interference-Minimized Filter (TCIMF) (Ren and Chang 1994), Mixture Tuned Target-Constrained Interference-Minimized Filter (MTTCIMF) (Jin et al. 2009), and Mixture Tuned Matched Filtering (MTMF) (Boardman, 1998). Afterwards, we used two filtering options (i.e. clumping and sieving) to clean up misdetected pixels and false positives in the category of "greenhouses". To check the quality of performance of the classification methods, we used the image corresponding to the 2013 hydrological year. We randomly distributed 1,200 points within the area classified as "greenhouse" and used an orthophoto from April 2013 (corresponding to the peak production season for greenhouse crops) to check whether these points fell in or out the real area covered by greenhouses. This work was carried out at the Remote Sensing Lab (LAST) at the Doñana Biological Station (EBD-CSIC, Seville). This work was carried out at the Remote Sensing and GIS Laboratory (LAST-EBD) at the Doñana Biological Station (EBD-CSIC; Seville, Spain).

## Data analyses

Based on the natural limits of the stream catchments and the marsh, we defined four habitat categories: (1) Partido, (2) Rocina/Sotos, (3) Guadiamar and (4) marsh. To visualize the spatio-temporal patterns of nutrients and chla across these different habitats, we mapped the concentrations of DIN, TN, $PO_4$, TP and chla from samples collected during December 2014 and 2015, February 2015 and 2016, and May of 2015 and 2016. These were the six most extensive of 19 sampling campaigns.

### *Principal component analysis (PCA)*

Principal Component Analysis (PCA) is widely used in water quality studies to identify patterns and reduce the dimensionality and complexity of multivariate datasets to a few, uncorrelated variables (i.e. Principal Components -PCs-) that explain a large percentage of the total variance in the dataset (Olsen et al., 2012) . We used PCA to determine whether there was a clear spatial pattern among our four habitats. Our dataset included 338 samples collected from 56 different sites across the four habitats during 19 different sampling periods between January 2013 and June 2016. Each observation contained information on nine different variables: TP, TN, $PO_4$, $NH_4$, $NO_3$, $NO_2$, cond, depth and $\delta^2H$.

We first standardized the variables to make the magnitude of the different variables comparable to each other (Gotelli and Ellison, 2004). Then we constructed a correlation matrix and extracted the eigenvalues and eigenvectors which inform about the significance of PCs and the importance of each original variable to a particular component. We only considered as significant those PCs with eigenvalues of 1 or higher since they retain the highest proportion of the variance (Le et al., 2017). We used the 'factoextra' (Kassambara and Mundt, 2017) and 'ggplot2' (Wickham, 2016) packages from CRAN-R software to perform the PCA and to plot the PCA output and the scree plots, respectively.

### *Two-way ANOVA and post-hoc pairwise comparisons*

To test for significant differences over time and space between our four habitats, we used two-way ANOVA with TP, TN, $PO_4$, $NH_4$, $NO_3$, $NO_2$ and chla as the dependent variables. We used visual ('Normal Q-Q' and 'Residuals vs. fitted values' plots) as well as statistical tools (global validation test of linear model assumptions: 'gvlma' package from CRAN-R) to validate model assumptions. We applied log-transformation to all variables to meet assumptions of normality and homoscedasticity. 'Habitat' was included as a categorical explanatory variable with four levels, and 'sampling period' as one of 16 levels. When there were significant effects of habitat, we determined pairwise differences with Tukey's "honestly significant difference" (HSD) post–hoc tests. Data used in these analyses were collected from 59 sampling sites between Jan 2013 and May 2016 (for nutrients) and from 55 sampling sites between Dec 2014 and May 2016 (for chla) (Fig. S3). Data from June, July and August were excluded because lack of surface water in summer severely reduced the number of available sampling sites. We performed the analyses using the 'stats' package in CRAN-R software.

### *Analyses of variation in water quality within the marsh*

To analyze variation in nutrient and chla concentrations within the extensive marsh itself, we used multiple linear regression models and hydrological predictor variables (cond, depth and $\delta^2$H). The dataset for these analyses included nutrient data from 26 sampling sites (Fig. S4) within four different periods (March-April 2013, December 2014, February 2015 and May 2015) when there was more extensive flooding across the marsh and we were able to sample a considerable number of localities. In the case of chla, we included data from 24 sampling sites (Fig. S4) during February 2015 and May 2015 (no data were available for earlier periods).

To run the models and obtain the regression parameters and partitioning of variance we used the functions 'lm' and 'aov', respectively, from the 'stats' package in CRAN-R. To validate model assumptions we used visual ('Normal Q-Q' and 'Residuals vs. fitted values' plots) as well as statistical tools (global validation test of linear model assumptions: 'gvlma' package from CRAN-R). We applied log or square root transformations to the variables when necessary to meet assumptions of normality and homoscedasticity. If even these transformations failed to meet assumptions, we then ranked the dependent variable and performed the models again (Seaman et *al.* 1994). For each response variable and sampling period, we retained the model with the smallest Akaike Information Criteria (AIC) value as the model with the best fit (from hereon "best model"). We obtained best models using the 'step' function (where "direction= both") from the 'stats' package in CRAN-R software 3.4.1. (https://www.R-project.org).

### *Comparing our data to water quality reference values*

To provide an assessment of the current surface water quality status in the Doñana marsh and entry streams, we used nutrient reference values suggested by the OECD (2007) for five different use classes. Class I represents an undisturbed, natural aquatic system, and may be considered equivalent to the "high status" classification of the Water Framework Directive (WFD). Classes I and II are the only ones supporting complete biodiversity functioning, whereas no fish are expected to survive in classes IV and V. We calculated the percentage of samples classified within each class for each nutrient parameter and habitat. We presented percentages with and without data from the summer period (June, July, August, September), given that the low water level and high evaporation rate during summer were expected to have a strong effect on nutrient values.

For chla concentrations we used reference values based on trophic classification (OECD, 1982), such that values <2.5 µg $L^{-1}$ can be considered oligotrophic, 2.5-8 µg $L^{-1}$ mesotrophic, 8-25 µg $L^{-1}$ eutrophic and >25 µg $L^{-1}$ hypereutrophic.

# Results

## Greenhouse expansion in the Doñana catchment

The surface area covered by greenhouses increased by 487% in 21 years from 939 ha in 1995 to a peak of 5510 ha in 2016 in the Partido and Rocina/Sotos catchments, but then the surface area declined to 3204 ha by 2018 (Fig. 2). During our study period for water quality (2013 to 2016), the mean area (±SD) of greenhouses was 1506 ± 338 ha in the Partido catchment and 3144 ± 501 ha in the Rocina/Sotos catchment. This represented 5.1% and 7.2% of the total subcatchment surface area respectively. There was relatively more expansion in the lower part of the Partido catchment and the upper part of the Rocina catchment (Fig. 2).

## Spatial heterogeneity in water quality parameters

### *General differences in water quality between habitats*

Within a PCA of the set of water quality variables, PC1 (eigenvalue PC1=3.84) explained 42.7% of the total variance and was highly correlated with $PO_4$, TP, $NO_3$, TN and $NO_2$. PC2 (eigenvalue PC2=1.70) explained 18.9% of the total variance and was highly correlated with conductivity and $\delta^2H$. The resulting biplot of PC1 vs. PC2 (Fig. 3) reveals major variation between habitats (Partido, Rocina/Sotos, Guadiamar streams and the marsh). Samples from the Partido and some samples from the Rocina/Sotos streams had higher PC1 values, reflecting higher nutrient concentrations. Samples from the marsh generally had higher PC2 values, reflecting higher conductivity and $\delta^2H$.

The marsh showed the highest conductivity values (mean ± s.d.=4330 ± 5737 µS $cm^{-1}$), followed by the Guadiamar (2114± 1908 µS $cm^{-1}$), the Partido

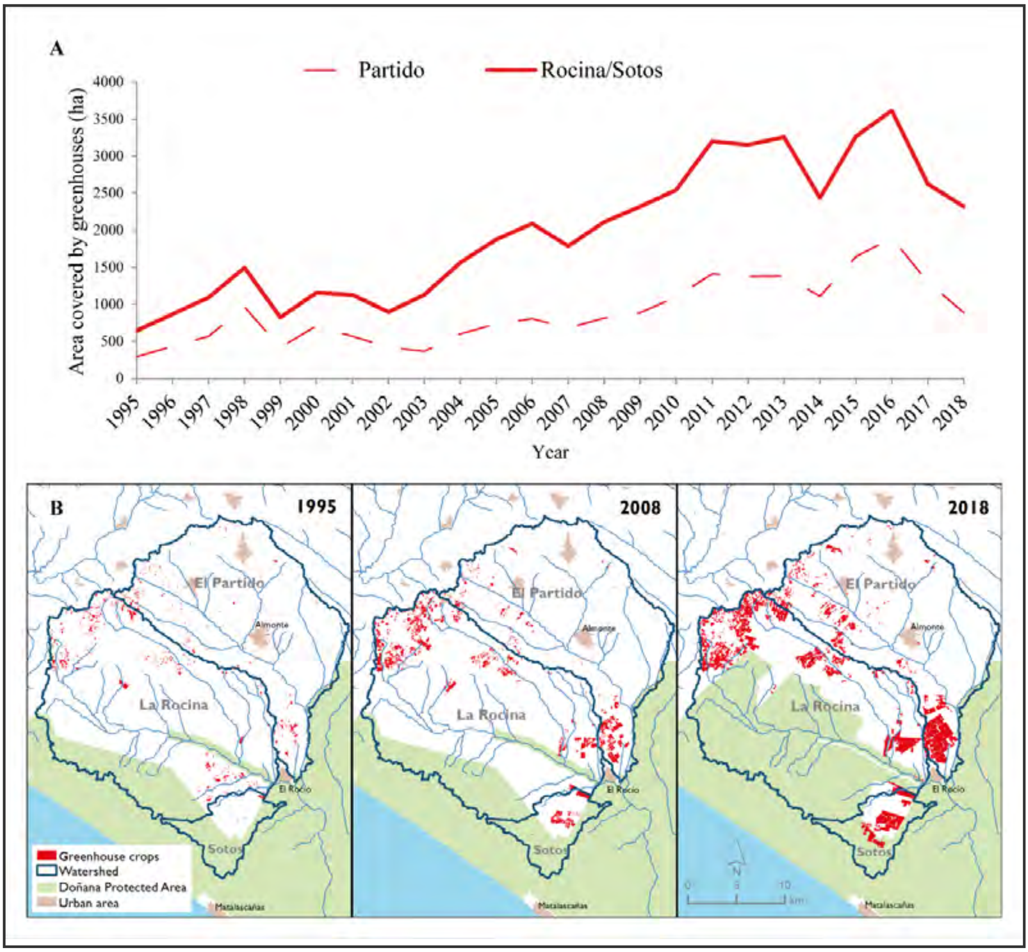


Figure 2. Surface area covered by greenhouses (mainly for berries) between 1995 and 2018 in the Partido and Rocina/Sotos catchments (A), and the spatial distribution of these greenhouses in 1995, 2008 and 2018 (B). There were no greenhouses in the Doñana marsh and almost none (17 ha in 2018) in the Guadiamar catchment.

(mean ± s.d.=1191± 766 µS $cm^{-1}$) and the Rocina/Sotos (mean ± s.d.= 544.2 ±195.8µS $cm^{-1}$). Mean water depth for each habitat ranged from 26.8 cm in the marsh to 67.1 cm in the Guadiamar. The maximum depth recorded was in the Partido stream in December 2014 (480 cm). Mean (± s.d.) $\delta^2H$ values for Partido (-26.9 ± 5.5‰), Rocina/Sotos (-26.5 ±7.3‰) and Guadiamar (-23.8 ±13.9‰) were very similar, and lower than the marsh (-1.5 ± 25.9‰,). The marsh had the largest range of values (-52‰ to 68.2‰) (see details in Table S1).

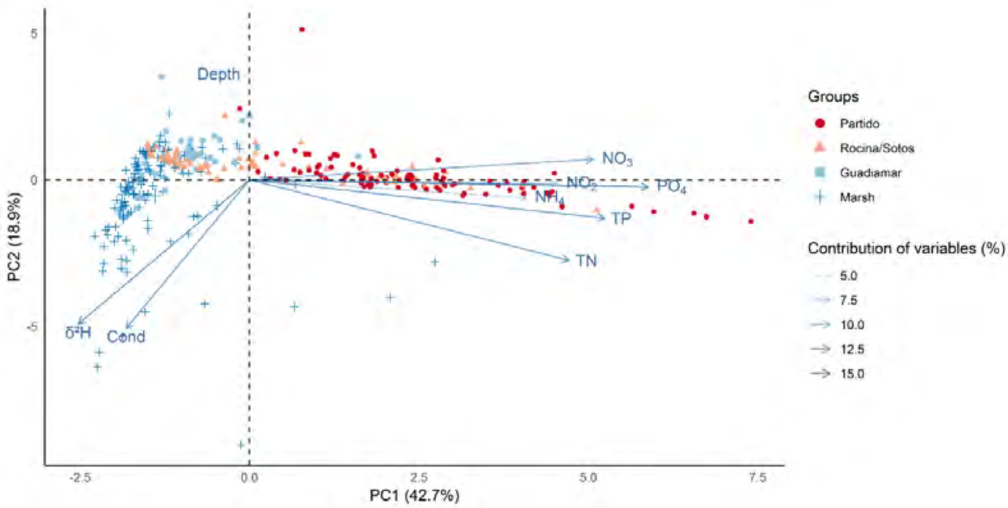


**Figure 3.** PCA of surface water quality from the Doñana marsh and three main entry streams.

### *Comparing nutrient and chla concentrations between habitats*

Maps showing the nutrient concentrations (dissolved inorganic nitrogen [DIN, the sum of $NH_4$, $NO_3$ and $NO_2$], TN, $PO_4$ and TP) in different habitats for six major sampling events (December 2014, February 2015, May 2015, December 2015, February 2016 and May 2016) confirmed that the streams had generally higher values compared to the marsh, a difference that was consistent between years and seasons (Fig. 4, 5, 6 and 7). In contrast, within a given habitat there were no consistent differences in the concentrations recorded between winter months (December or February) or spring (May). The Partido and the lower part of the Rocina/Sotos catchments were the areas with the highest nutrient concentrations. These spatial differences were more marked for dissolved inorganic nutrients than for Total N and Total P.

DIN concentrations during these six sampling events ranged from <0.1 to 9 mg N $L^{-1}$, and were much higher in streams (geometric mean= 1.16 mg N $L^{-1}$; 95% Confidence intervals (CIs) = 0.85 – 1.58; N= 132) than in the marsh (geometric mean= 0.04 mg N $L^{-1}$; 95% CIs= 0.03 – 0.05; N=92). Total N concentrations ranged from 0.4 to 12.5 mg N $L^{-1}$, with a geometric mean of 4.84

mg N $L^{-1}$ (95% CIs= 4.28 – 5.48) in streams compared to 2.20 mg N $L^{-1}$ (95% CIs= 1.96 – 2.47) in the marsh.

P-$PO_4$ concentrations ranged from <d.l. to 2.8 mg P $L^{-1}$, and were much higher in streams (geometric mean= 0.21 mg N $L^{-1}$; 95% CIs= 0.16 – 0.29) than in the marsh (geometric mean= 0.02 mg N $L^{-1}$; 95% CIs= 0.01 – 0.04). Total P concentrations ranged from 0.03 to 3 mg P $L^{-1}$ , with a geometric mean of 0.48 mg P $L^{-1}$ (95% CIs= 0.41 – 0.57) in streams and of 0.17 mg P $L^{-1}$ (95% CIs= 0.14 – 0.21) in the marsh. In the marsh, there were particularly high $PO_4$ and TP concentrations at one location adjacent to a large heronry in May 2015 (Fig. 5 and 7).

Differences between habitats were less consistent for chla, although the lower part of the Rocina and Partido catchments often had the highest concentrations (Fig. 8), and the concentrations in streams were sometimes clearly higher than in the marsh (e.g. in February 2015). Chla concentrations ranged from 0.5 to 88 µg $L^{-1}$ (n=172), and values in streams (geometric mean= 6.78 µg $L^{-1}$; 95% CIs= 5.66 – 8.13; N= 109) were generally higher than in the marsh (geometric mean= 4.27 µg $L^{-1}$; 95% CIs= 3.34 – 5.45; N=63).

Two-way ANOVAs confirmed that there were highly significant differences between 'habitats' ($P<0.0001$) for all dependent variables (nutrients and chla) (Table S2), where the Partido, Rocina/Sotos and Guadiamar showed consistently higher concentrations than the marsh as revealed by the model estimates (Fig. 9). There were also highly significant differences between sampling events (Table S2), although these differences were not consistent between seasons. Concentrations in the marsh were significantly lower than in all three streams for all nutrients except $NH_4$ and $PO_4$. The Partido had significantly higher concentrations than other streams for all nutrients except $NO_3$. There were no significant differences in nutrient concentrations between Rocina/Sotos and Guadiamar. Rocina/Sotos and Guadiamar both had significantly higher chla concentrations than the faster flowing Partido and the marsh (Fig. 9).

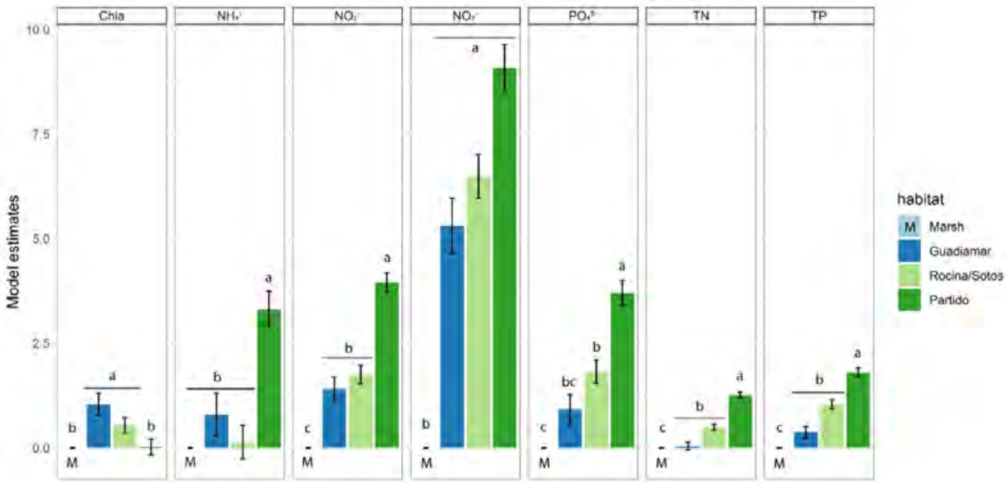


**Figure 9.** Comparison of the model estimates from two-way Analysis of Variance (ANOVA) showing the relative differences between the three stream habitats (Partido, Rocina/ Sotos and Guadiamar) and the marsh (M; model estimates are zero for this habitat in all cases). Higher model estimates indicate higher average concentration values. See Table S2 for further details.

### *Explaining spatial variation of nutrient and chla concentrations within the marsh*

We used multiple regression to explore the relationship between nutrient concentrations and conductivity, depth and isotopic variation $\delta^2H$ within the marsh. For each nutrient response variable, best models included at least one physicochemical variable for at least two of the sampling periods (Table 1), and the proportion of variation explained by the models was relatively high for TN, TP and $NO_2$. In the final models, conductivity and depth repeatedly showed significant negative partial effects on nutrient concentrations. $\delta^2H$ had a positive effect on nutrients when this was the only explanatory variable in a final model, but in contrast had a negative partial effect when the final model also included depth (Table 1).

Nutrient concentrations in the marsh were highest in those sampling sites that are closest to the entry points of the Partido and other contaminated streams (Fig. 4, 5, 6 and 7). This is further demonstrated when taking into account the additional data collected outside the sampling periods represented in Fig. 4, 5, 6 and 7. For example, the geometric mean of $NO_3^-$ is 0.22 mg N $L^{-1}$ (95% CIs= 0.10-0.49; N = 9) at point A in Fig. 1 (37°4'18"N, -6°27'17"W) situated at 6 km from the nearest upstream greenhouse area within the Rocina/Sotos habitat, compared to only 0.002 mg N $L^{-1}$ (95% CIs= 0.0004-0.01; N = 8) at point B (37°2'21"N, -6°24'52"W) 5 km further downstream within the marsh.

We used multiple regression to explore the relationship between chla concentrations in the marsh, with the above physicochemical variables as well as the six nutrient variables as predictors. Best models included at least one nitrogen variable together with conductivity for two sampling periods (Table 1). Conductivity had a significant positive partial effect on chla in May, yet an insignificant negative partial effect in February (Table 1).

**Table 1.** Best regression models of variation in water quality within the Doñana marsh in each sampling period. Symbols in brackets indicate positive (+) or negative (-) partial effects of predictor variables. Significance range for p-value: * <0.05; ** <0.01; *** <0.001.

| Response variables (ppm) | Predictor variables | | | |
|---|---|---|---|---|
| | **Mar/Apr 2013** | **Dec 2014** | **Feb 2015** | **May 2015** |
| **$NH_4$** | $\delta^2$H **(+)*** (adj.$R^2$= 0.16) | | | cond **(-)*** + depth **(-)*** (adj.$R^2$= 0.28) |
| **$NO_3$** | cond **(-) *** + depth **(-)** (adj.$R^2$= 0.23) | $\delta^2$H **(+)** (adj.$R^2$= 0.12) | $\delta^2$H **(-) *** + depth **(-)** (adj.$R^2$= 0.21) | |
| **$NO_2$** | cond **(-) *** + depth **(-)**** (adj.$R^2$= 0.44) | | | |
| **$PO_4$** | cond **(-)*** + depth **(-)**** (adj.$R^2$= 0.38) | cond **(-)** (adj.$R^2$= 0.21) | | $\delta^2$H **(-)** + depth **(-)** (adj.$R^2$= 0.06) |
| **TN** | $\delta^2$H **(-)*** + depth **(-) ***** (adj.$R^2$= 0.51) | cond **(-) ***** (adj.$R^2$= 0.59) | | $\delta^2$H **(+)**** (adj.$R^2$= 0.44) |
| **TP** | cond **(-)** + depth **(-)**** (adj.$R^2$= 0.40) | cond **(-)**** (adj.$R^2$= 0.43) | | |
| **Chla** | | | $NH_4$ **(-)** + cond **(-)** + $NO_2$ **(+)** (adj.$R^2$= 0.29) | $NO_2$ **(+)** + cond **(+)*** (adj.$R^2$= 0.30) |

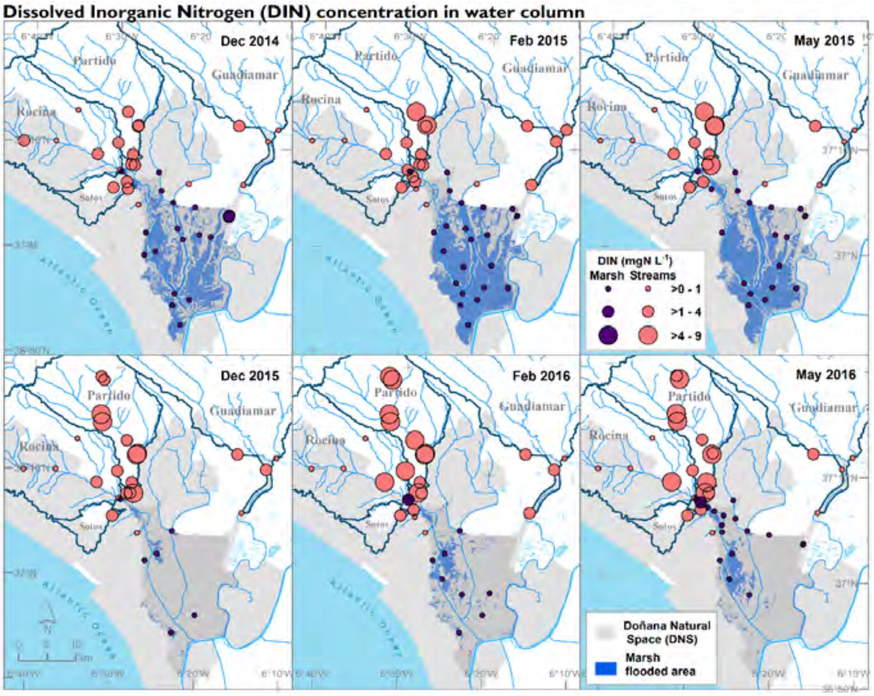


**Figure 4.** Concentration values of dissolved inorganic nitrogen (DIN) in 2015 and 2016 in the Doñana marsh and entry streams within the three sub-catchments "Rocina/Sotos", "Partido" and "Guadiamar". DIN is the sum of $NO_3$, $NO_2$ and $NH_4$ concentrations.

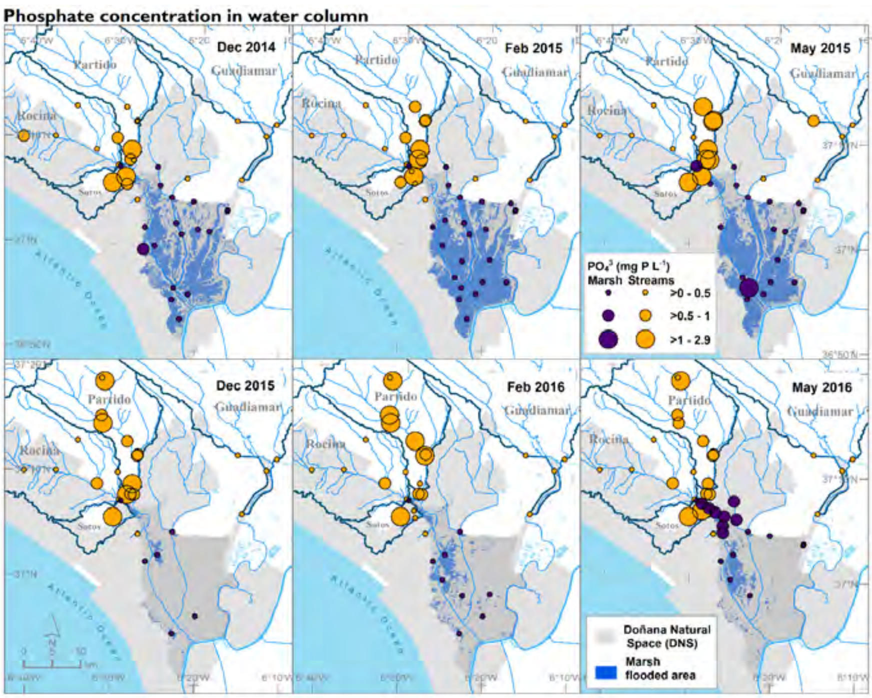


**Figure 5.** Concentration values of phosphate ($PO_4$) in 2015 and 2016 in the Doñana marsh and entry streams.

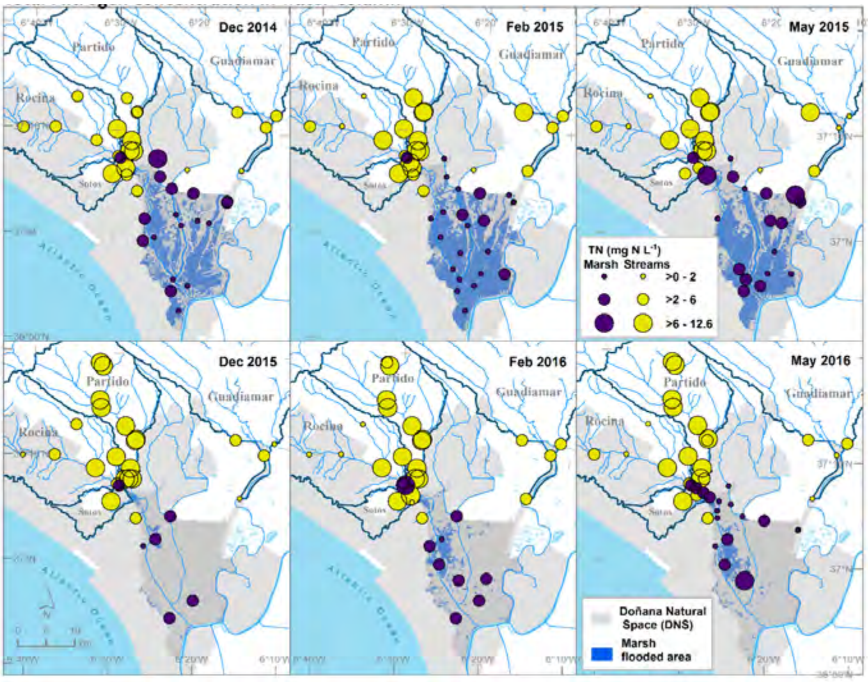


**Figure 6.** Concentration values of total nitrogen (TN) in 2015 and 2016 in the Doñana marsh and entry streams.

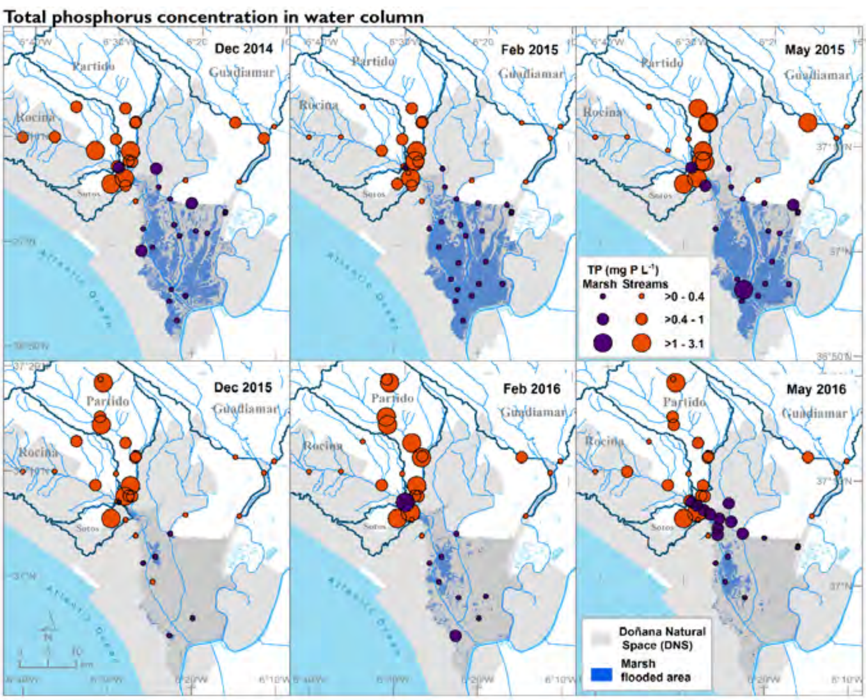


**Figure 7.** Concentration values of total phosphorus (TP) in 2015 and 2016 in the Doñana marsh and entry streams.

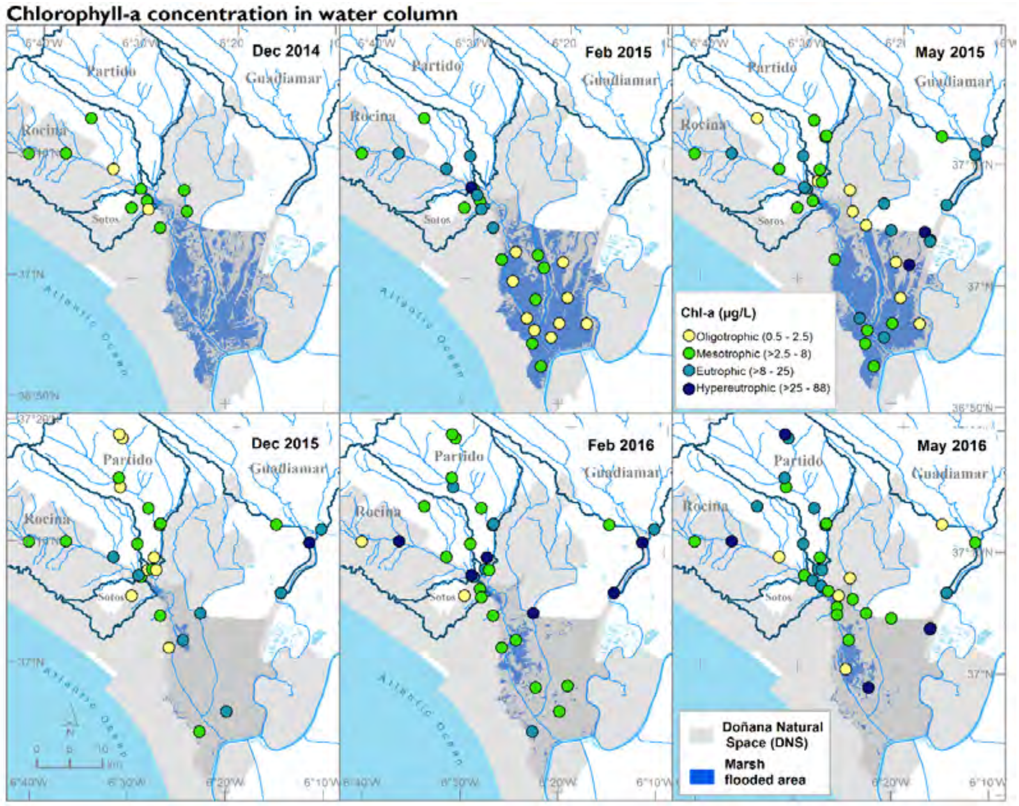


**Figure 8.** Concentration values of chlorophyll-*a* (chla) in 2015 and 2016 in the Doñana marsh and entry streams within the three sub-catchments "Rocina/Sotos", "Partido" and "Guadiamar". Thresholds used for the legend are based on the OECD (1982) classification.

## Surface water quality status

### *Water quality status based on nutrient concentrations*

Comparing all our nutrient data with the water quality classification of OECD (2007) (Table 2), we observed extensive differences between habitats in water quality. The Partido stream had the lowest quality, and showed the highest proportion of concentration values (>63.8%) classified within the three worst water quality categories. In particular, 93.4% of $PO_4$ and TP values, 71.4% of TN values and 69.2% of $NO_2$ values in the Partido were within the two worst classes (IV and V) not suitable for fish life.

Water quality was consistently higher in the marsh, followed by the Guadiamar stream. In the Rocina/Sotos between 14.1% and 48.7% of samples were in the worst two classes (IV and V) for four parameters (Table 2). In the marsh, for all parameters except TP, more than 80% of all samples were classified within the best two water quality categories (class I and II), and <9% in the worst two classes (IV and V). For TP, 16.8% of values were in the worst two classes, these being recorded close to stream entry points and in the western area of the marsh (Fig. 7). These results did not change when data from summer months were excluded (Table 2).

### *Water quality status based on chla concentrations*

Following the chla-based classification of trophic level from OECD (1982), 20.3% of all samples (63% of which were from streams; $n_{streams}$= 109; $n_{marsh}$= 63) were oligotrophic, 44.2% were mesotrophic, 26.8% were eutrophic and 8.7% were hypereutrophic. The majority (73%) of the 15 samples classified as hypereutrophic (chla >25 µg $L^{-1}$) were found in streams (Fig. 8). Similarly, 76% of 45 samples classified as eutrophic (chla >8 – 25 µg $L^{-1}$) were recorded in streams ($n_{streams}$= 34 vs. $n_{marsh}$=11). Most cases of hypereutrophy were recorded during February or May (Fig. 8).

**Table 2.** Percentage of spot samples (ntotal=337) for each habitat (Partido (n=91), Rocina/Sotos (n=78), Guadiamar (n=32) and Marsh (n=136) corresponding to five use classes defined by OECD (2007). Each class defines which water uses are supported for a given water quality (uses: ecosystem functioning, fish breeding/protection, drinking water supply, bathing/recreation, irrigation, industrial water use, power use, mineral extraction and transportation). Class I and II are the only ones considered compatible with biodiversity functioning. Class I may be considered equivalent to "high status" under the WFD, i.e. a virtually undisturbed, natural system. Class V is equivalent to the WFD's "bad status". No fish are expected to survive Classes IV and V. Figures in brackets are %s when excluding data from June to September (hot, dry months), so that ntotal=304, Partido (n=81), Rocina/Sotos (n= 72), Guadiamar (n= 29) and Marsh (n=122).

Good quality → Bad quality

| **Chemical parameter** | **habitat** | **CLASS I** | **CLASS II** | **CLASS III** | **CLASS IV** | **CLASS V** |
|---|---|---|---|---|---|---|
| | | Values shown as % *(in brackets excluding data from June to September)* | | | | |
| **Reference values (mg N L$^{-1}$)** | | **<=0.2** | **<=0.4** | **<=0.8** | **<=3.1** | **>3.1** |
| **$NH_4$** | Partido | **49.4 (49.4)** | 8.8 (8.6) | 8.8 (9.9) | 26.3 (26) | 6.7 (6.1) |
| | Rocina/Sotos | **84.6 (87.5)** | 10.2 (11.1) | 1.3 (0) | 3.9 (1.4) | 0 (0) |
| | Guadiamar | **90.6 (89.6)** | 9.4 (10.3) | 0 (0) | 0 (0) | 0 (0) |
| | Marsh | **97.1 (98.4)** | 2.9 (1.6) | 0 (0) | 0 (0) | 0 (0) |
| **Reference values (mg N L$^{-1}$)** | | **<=1** | **<=3** | **<=5.6** | **<=11.3** | **>11.3** |
| **$NO_3$** | Partido | 1.1 (1.2) | 35.1 (30.9) | **63.8 (67.9)** | 0 (0) | 0 (0) |
| | Rocina/Sotos | **66.6 (65.3)** | 9 (9.7) | 24.4 (25) | 0 (0) | 0 (0) |
| | Guadiamar | **56.2 (51.7)** | 37.5 (41.4) | 6.3 (6.7) | 0 (0) | 0 (0) |
| | Marsh | **97.8 (97.6)** | 2.2 (2.4) | 0 (0) | 0 (0) | 0 (0) |
| **Reference values (mg N L$^{-1}$)** | | **<=0.01** | **<=0.06** | **<=0.12** | **<=0.3** | **>0.3** |
| **$NO_2$** | Partido | 1.1 (1.2) | 15.4 (16) | 14.3 (14.8) | **37.4 (37)** | 31.8 (31) |
| | Rocina/Sotos | **43.6 (43)** | 28.2 (27.8) | 14.1 (13.9) | 11.5 (12.5) | 2.6 (2.8) |
| | Guadiamar | 25 (17.2) | **65.6 (72.4)** | 6.2 (6.9) | 3.2 (3.4) | 0 (0) |
| | Marsh | **81.6 (80.3)** | 17.6 (18.8) | 0.8 (0.9) | 0 (0) | 0 (0) |

| **Reference values (mg P L$^{-1}$)** | | **<=0.05** | **<=0.1** | **<=0.2** | **<=0.5** | **>0.5** |
|---|---|---|---|---|---|---|
| **$PO_4$** | Partido | 2.2 (2.5) | 0 (0) | 4.4 (3.7) | 9.9 (9.9) | **83.5 (83.9)** |
| | Rocina/Sotos | **39.7 (38.9)** | 9 (9.7) | 12.8 (13.9) | 14.1 (12.5) | 24.4 (25) |
| | Guadiamar | **40.6 (34.5)** | 21.9 (24.1) | 18.8 (20.7) | 15.6 (17.2) | 3.1 (3.4) |
| | Marsh | **78.7 (79.5)** | 6.6 (5.7) | 6.6 (6.6) | 5.1 (4.9) | 3.0 (3.3) |
| **Reference values (mg N L$^{-1}$)** | | **<=1.5** | **<=4** | **<=8** | **<=20** | **>20** |
| **TN** | Partido | 0 (0) | 2.2 (1.2) | 26.4 (27.2) | **71.4 (71.6)** | 0 (0) |
| | Rocina/Sotos | 9 (8.3) | **46.1 (47.2)** | 23.1 (20.9) | 21.8 (23.6) | 0 (0) |
| | Guadiamar | 18.8 (20.7) | **68.8 (65.5)** | 9.3 (10.3) | 3.1 (3.4) | 0 (0) |
| | Marsh | 24.3 (26.2) | **57.3 (60.7)** | 11 (10.6) | 4.4 (2.5) | 3 (0) |
| **Reference values (mg P L$^{-1}$)** | | **<=0.1** | **<=0.2** | **<=0.4** | **<=1** | **>1** |
| **TP** | Partido | 0 (0) | 1.1 (1.2) | 5.5 (4.9) | **51.6 (51.8)** | 41.8 (42) |
| | Rocina/Sotos | 7.7 (8.3) | 16.6 (16.7) | 27 (27.8) | **32 (30.5)** | 16.7 (16.7) |
| | Guadiamar | 3.1 (3.4) | **46.9 (48.3)** | 34.4 (31) | 12.5 (13.8) | 3.1 (3.4) |
| | Marsh | **33.8 (36.9)** | 23.5 (23.8) | 25.8 (27) | 12.5 (11.5) | 4.4 (0.8) |

**Percentage values (%) in (green) bold represent the highest values in each habitat for each chemical parameter.*

# Discussion

In this study we addressed the physico-chemical quality of surface waters and evidence for anthropogenic eutrophication in Doñana, the largest wetland complex in western Europe and one of the most important in the Mediterranean region (Green et al., 2018). We recorded persistent poor water quality in the major entry streams, despite the protection of the core area as a WHS since 1992, and the earlier protection of the entire catchment within a Biosphere reserve. Indeed, concentrations of nitrites, ammonia and other nutrients are often above toxic thresholds for aquatic life. This could be explained, in part, by the five-fold expansion of agriculture in greenhouses observed over two decades since 1995, the increasing human population in the region and the poor treatment of urban wastewaters. These anthropogenic impacts may also explain the spatial heterogeneity we observed in nutrient concentrations (i.e., differences between subcatchments), which was consistent in different seasons and years, despite major fluctuations in precipitation and surface water distribution. The more strictly protected marsh habitat has better water quality than the less protected streams, thus indicating its major ecosystem service as a "green filter" to purify contaminated waters from the catchment. However, the edge of the marsh is clearly impacted by anthropogenic nutrient inputs and is undergoing eutrophication (see also Paredes et al. 2019). This may compromise the biodiversity conservation objectives of this WHS, and suggests a need for policies aimed at reducing nutrient concentration in the catchment itself (Chimney and Goforth, 2006; Tonderski et al., 2017). All our initial hypotheses were supported, except that we did not identify consistent seasonal patterns in nutrient and chla concentrations. This is likely related to the complex hydrology of the study area, and the influence of high temporal variation in precipitation and irregular drought periods on flow rates and nutrient cycling.

Unfortunately, the poor water quality and negative trends in Doñana may reflect trends at a broader scale across southern Spain and much of the Mediterranean region. In many areas of Europe, water quality has improved in recent decades in major rivers with regard to BOD (Biochemical Oxygen Demand), nitrates, phosphates, and ammonium, although a quarter of European groundwaters have poor status, mainly due to nitrate concentrations (European Environment Agency, 2015). In contrast, Spain stands out at a global level as one of the countries most affected by anthropogenic phosphorus inputs to freshwaters, mainly due to agriculture (Mekonnen and Hoekstra, 2018). The south of Spain is also generally heavily affected by anthropogenic nitrogen pollution, but does not stand out at a global scale as much as it does for phosphorus (Mekonnen and Hoekstra, 2015).

Overall, Mediterranean wetlands have experienced rapid degradation and biodiversity loss in the past 50 years due to socio-economic pressures and human population growth (MWO2, 2018). Like much of the Mediterranean basin, Doñana is located in a region of high water stress caused by the synergies between climatic conditions (e.g. scarce precipitations and long dry periods) and exploitation of a high proportion of available water resources (Hofste et al. 2019). This inevitably puts pressure on natural freshwater ecosystems dependent on these same resources. Information about water quality in Mediterranean wetlands is currently limited, because little monitoring of pollutants other than nutrients is carried out, and few data are publicly available (Haener, 2008). The limited data available (e.g. UNEP, 2016; MEDPOL 2015) suggest a general decline in water quality across the Mediterranean Basin in recent decades, a disturbing trend which is predicted to continue (Veolia & IFPRI, 2015), and which matches our findings in this study.

## Drivers of eutrophication in Doñana: agriculture and urban development

Agricultural intensification and urban development have greatly increased in the surroundings of Doñana since the 1960s owing to rapid economic growth (Ales et al., 1992). In response to the imminent threat from land conversion, road construction and economic development, protection of Doñana began in 1963 when the World Wildlife Fund (WWF) and the Spanish government purchased and protected 6800 ha of what since has become a much larger National Park (Green et al., 2018). However, less attention has been paid to limiting development in the catchment area, where land conversion into intensive agrosystems has since occurred, with clear impacts on water quality and quantity (Zorrilla-Miras et al., 2014b). Our results for greenhouse expansion reflect broader trends across the Guadalquivir River Basin with a shift into more intensive, high value irrigated crops largely for export to other European countries (Expósito and Berbel, 2017; Rodríguez and De Stefano, 2012). In the Doñana catchment, this growth has partly been driven by the destruction of native forests and other vegetation, and partly by the conversion of vineyards and other crops with lower impacts on water quality and quantity (Junta de Andalucía, 2005a). In the case of the Guadiamar catchment, this trend has been partly reversed since 1998 (see below), and this is likely to explain the higher water quality we recorded in this stream.

Agricultural intensification, increasing car ownership and the expansion of tourism (Fernández-Delgado, 2017) has led to urban expansion and human population increase in the Doñana catchment since 1990, generating more urban wastewaters which are often poorly treated. On the one hand, as for many waste water treatment plants (WWTPs) in Andalusia, many of those around Doñana do not comply with the EU waste-water directive (https://uwwtd.eu/Spain/). On the other hand, the many agricultural workers in greenhouses produce waste which is not sent to WWTPs. Furthermore, the town and shrine of El Rocío on the very edge of the Doñana marsh has a WWTP designed for only c.5,000 people, yet the town receives over a million of visitors during the week-long annual pilgrimage in spring.

As a consequence of increasing anthropogenic nutrient loadings in the catchment, Doñana National Park (NP) was declared a sensitive area to eutrophication in May 1998 under the European Urban Waste-water Treatment Directive 91/271/CEE (EEC, 1991). Then in 2008, part of the marshland and catchment area were declared vulnerable to nitrate from agricultural origin by the Andalusian Government (Decree 36/2008), according to the European Nitrate Directive 91/676/EEC (EEC, 1991b). These and other relevant Directives such as the WFD (EC, 2000) have been insufficient to prevent further eutrophication in the catchment, as indicated by the long-term increase in phosphate concentration in a handful of sampling points reported by Espinar et al. (2015), as well as the expansion of greenhouses we documented in the Partido, Rocina and Sotos catchments (see also WWF, 2019a).

## Spatial variability of nutrient and chla concentrations

### *Heterogeneity within the catchment: explaining water quality differences between streams*

As predicted, we found that sampling points closer to greenhouses or WWTPs (Rocina/Sotos, Partido) showed higher nutrient concentrations than points located further away from any intensive anthropogenic activity, especially those within the marsh. These results were in agreement with those previously reported for nitrogen pollution in some of the same sampling points over a shorter period (Paredes et al. 2019). Samples collected within the Guadiamar catchment (not studied by Paredes et al. 2019) had intermediate nutrient levels.

The marked reduction in the flow rate of the Rocina/Soto streams in recent decades due to groundwater extraction for the greenhouses (Guardiola-Albert et al. 2011) may have contributed to high nutrient concentrations owing to reduced dilution of pollutants. Moreover, sampling points in the Partido are the most directly affected by point sources of pollution due to their shorter distance to upstream WWTP effluents and relatively high flow rates (see Table S3). The latter reduces the time for nutrient retention and transformation by *in-stream* biogeochemical processes (Peterson et al., 2001). The Guadiamar and the Rocina/Sotos habitats are mainly affected by diffuse sources (i.e. fertilizers and decentralized urban waste from agricultural workers spread around the area) since WWTPs are located further away from the sampling sites (i.e. Guadiamar catchment) or they are not present at all (Rocina/Sotos catchment). The influence of wastewaters in the Partido catchment has also been demonstrated by analyzing stable isotopes in emergent plants growing along the stream banks (Paredes et al. 2019). High concentrations of pharmaceuticals in the Partido stream and areas of marsh close to El Rocío village, some of which exceed toxic thresholds for aquatic invertebrates by tenfold, have also demonstrated the influence of urban wastewaters (Camacho-Muñoz et al., 2013, 2010).

Riparian vegetation also acts as buffer zones along the stream margins by reducing nutrient inputs into the streams, stimulating *in-stream* nutrient retention and attenuating flood runoff (Pinay et al., 2018; Weigelhofer et al., 2012; Wilkinson et al., 2019). In the Partido, riparian zones are considerably more degraded than in the Rocina/Sotos and Guadiamar catchments, thus increasing the vulnerability of surface waters to nutrient inputs by runoff (see SIOSE 2013 for detailed land use information). The Guadiamar catchment has benefitted from restoration since the 1998 mine spill which directly affected this area. Actions such as abandonment of surrounding agricultural areas in the surroundings and the creation of a "green corridor" with extensive Riparian vegetation (Ontiveros et al., 2013) have likely reduced nutrient inputs into the Guadiamar River.

In general, the nitrate concentrations recorded are not particularly high and would not alone indicate "bad status" of surface waters, even in the Partido stream (Table 2). This probably reflects high rates of denitrification within the groundwater before discharge into streams, as indicated by microbial activity, greenhouse gas emissions (Tortosa et al., 2011) and N and O isotopes in dissolved nitrate (Paredes et al. 2020). In contrast, nitrite concentrations are frequently well above levels lethal to fish life in the Partido and Rocina/Sotos streams, and ammonia concentrations are also high (a classic indication of waste-water influence). For example, the Freshwater Fish Directive 78/659/EEC (EEC, 1978) identified a guidance limit of 0.009 mg $L^{-1}$ N-$NO_2$ and 0.15 mg $L^{-1}$ N-$NH_4$ for cyprinid waters, thresholds greatly exceeded in the Doñana streams (Table 2).

Chla concentrations were generally higher in the streams than in the marsh, but the differences were much less marked than for nutrient concentrations. There was much spatial variation in chla concentration within and between streams, largely connected with varying depth. Towards the mouth of the Rocina, the stream widens and a dam creates a deeper pool where residence time is longer and phytoplankton can grow better and accumulate. This is not the case in the Partido for example, where chla concentration in the water column was particularly low. Measuring chla in benthic algae (i.e. periphyton) rather than in planktonic ones may have provided results that were more strongly related to nutrient concentrations in streams, since benthic algae often represent an important fraction of the primary productivity in shallow streams and rivers (Jarvie et al., 2003). In the Partido, lower chla concentrations may also be due to reduced amount of phytoplankton due to lower tolerance to continuous high $NH_4$, $NO_3$ or $NO_2$ concentrations in the water column (Domingues et al., 2011; Glibert et al., 2016).

***Temporal and spatial variation in water quality within the WHS***

In general, we found considerably lower nutrient concentrations within the marsh protected within the WHS, than in catchment streams. This difference may be partly explained by high nutrient removal capacity of the marsh due to biogeochemical processes such as plant uptake, P adsorption to sediments or nitrogen removal by denitrification (Fisher and Acreman, 2004), but also due to higher distance from the polluting sources in the catchment. Only a few sampling sites in the marsh showed high N and P concentrations, particularly in the north-west under the influence of catchment-derived nutrients entering from adjacent agricultural and urban areas (Fig. 4, 5, 6 and 7). Nevertheless, the marsh has experienced an long-term increase in phosphate concentration during the beginning of the 21st century which is thought to explain the invasion of the nitrogen-fixing alien floating fern *Azolla filiculoides* since 2001(Espinar et al., 2015). The expansion of this and other floating plants during eutrophication is likely to promote anoxia and reduce aquatic biodiversity in Doñana, as observed in many other wetlands (Green et al. 2017).

Moreover, particularly high $PO_4$ and TP concentrations were recorded in the marsh in May 2015 far from any entry area, in the proximity to a large heronry, suggesting the occurrence of *in situ* animal-derived nutrient loadings (i.e. guanotrophy) rather than catchment-derived nutrients. Guanotrophy has often been reported in freshwater systems as a direct consequence of waterbird excreta where birds concentrate for breeding or roosting (Hahn et al., 2007; Martín-Vélez et al., 2019). Indeed, guanotrophication is responsible for the decline of nesting oak trees in part of Doñana National Park, and expansion of heronries causes intense nutrient inputs into limited, specific areas of the Doñana marsh where colonies are located (Fedriani et al., 2017; Ramo et al., 2013).

Furthermore, the Doñana marsh has high concentrations of wild and domestic ungulates (Junta de Andalucía, 2015) which are controversial given evidence of negative impacts on insect communities, aquatic vegetation and nesting birds (Sharps et al., 2015; Verdú et al., 2018). Ungulates promote eutrophication through treading and their faeces (Declerck et al., 2006), and this may partly explain the relatively high phosphorous concentrations in the water column of some sites in the marsh. Nevertheless, our study suggests that any eutrophication problems in Doñana caused by bird or ungulate populations are of far less overall significance than the anthropogenic eutrophication of the entry streams.

According to our PCA and linear regression models, physico-chemical and hydrological variables (conductivity, depth and $\delta^2H$) play a key role in explaining the variation and nutrient concentrations between sites within the Doñana marsh. We found a negative effect of depth and a positive effect of $\delta^2H$ on nutrient concentrations. These results are consistent with water evaporation effects followed by subsequent decrease in depth, and increase in $\delta^2H$ and solute concentration (Fellman et al., 2011; Gat, 2010). Although we may therefore expect a positive effect of conductivity on nutrient concentrations due to evapoconcentration of solutes, we repeatedly found a negative effect. This is not a causal relationship, but instead likely reflects the existing conductivity gradient in the marsh, where the lowest conductivity values are found in the W and NW areas of the marsh due to groundwater discharges and stream inputs, respectively, and the highest conductivity values are found in the E and SE areas (see Fig. S5). This negative correlation between nutrients and conductivity is also consistent with a nutrient purification effect as surface water flows further into the marsh due to increasing distance from pollutant sources in the catchment.

## Eutrophication management: gaps, failures and future perspectives

### *Lack of adequate water quality indices*

We were forced to use water quality classes which were derived for wetlands in general, in a different country in the Mediterranean region (OECD 2007), because there is no reference system available that has been directly developed for shallow seasonal Mediterranean marshes such as those in Doñana. Similarly, although biotic indices based on macroinvertebrates have been developed for numerous catchments in Europe under the WFD, such indices are not available for Doñana (see Alcorlo et al., 2014 for information on macroinvertebrates in the marsh). The low diversity of invertebrates is obvious in degraded streams such as the Partido (author's casual observations), but was not quantified during this study.

Based on the OECD (2007) classification, the marsh showed considerably higher water quality than most entry streams. However, the status for phosphorus in the marsh is of concern, and only a third of samples were equivalent to a "high status" for Total P (Table 2). Although these results are a strong indication that eutrophication is ongoing, Serrano et al. (2017) argue that high Total P does not necessarily mean "poor" water quality status in naturally eutrophic shallow aquatic systems such as the Doñana marsh. Phosphate concentrations in the water column of shallow systems are not only the result of external inputs, and the chemical equilibrium between P- adsorption and release in the sediment plays a key role in the overall dissolved inorganic P availability in the water (Golterman, 2004). Thus, our results demonstrate the need for developing specific eutrophication indices for shallow, temporary Mediterranean systems which may better distinguish between external ("anthopogenic") and internal ("natural") P loadings.

### *Failures in the past*

Doñana is an iconic European wetland in which important conservation efforts have been made at national and international levels to protect the natural values against the impacts of anthropogenic land-use changes that have dominated the European landscape over the past century. Nevertheless, these measures have not been sufficient to protect the area from eutrophication. Concern has been expressed for decades about nutrient pollution in the marsh and its tributary streams due to changing land use and an increasing human population in the catchment (Serrano et al., 2006; Junta de Andalucía 1992; Novo 1994). Spanish administrations have historically focused on engineering based solutions, and projects to modify water quantity through earthworks have been favored ahead of projects focusing on improving water quality (Méndez et al., 2012). For example, in an extensive restoration project in Doñana that followed a toxic mine spill in 1998, various engineering works increased the supply of water into the marsh by restoring surface water inputs diverted during past transformations for agriculture (García-Novo et al., 2007; García-Novo & Marín 2006), despite concerns about the quality of some of those sources (Serrano et al., 2006) and without clear criteria for acceptable quality.

The eutrophication of the Doñana entry streams and those areas of marsh surrounding the stream mouths has accelerated in recent decades and now reached levels incompatible with biodiversity conservation. Our study of the growth of greenhouses demonstrates one of the most likely causes of this increase. As has also occurred in many other protected wetlands (Peterson et al., 2001; Pusch et al., 1998), in the case of Doñana a basin has been strictly protected whilst the catchment area has not, leading to increases in pollution sources and land-use changes within the catchment that ultimately translate into anthropogenic nutrient export into the protected wetlands, causing degradation.

How is it possible that such eutrophication has been permitted to occur in arguably Europe's most iconic wetland complex? One reason are the huge economic interests involved in part of what has become Europe's largest strawberry culture area in Huelva province. Another likely reason is that conservation interests since the foundation of the National Park have largely focused on "heroic megafauna" such as the Iberian lynx and the Spanish imperial eagle (Fernández-Delgado, 2017), which do not act as indicators of water quality. Remaining fish biomass has long been dominated by alien species such as the carp *Cyprinus carpio* and mosquitofish *Gambusia holbrooki*, and relatively little attention has been paid to aquatic fauna other than waterbirds, for which Doñana is particularly important and famous (Ramo et al., 2013; Rendón et al., 2008). However, waterbirds have high plasticity and are relatively insensitive to eutrophication (Amat and Green, 2010; Almeida et al. 2019). Nevertheless, those wintering waterbird species with negative population trends in Doñana, such as herbivorous ducks, are those most likely to be impacted by eutrophication in the marsh (Rendón et al., 2008). Direct monitoring of water quality has previously been neglected in Doñana, hence the importance of our study.

### *Conservation actions for the future*

Despite the existence of the Biosphere reserve, since its declaration the Doñana area has been managed under a conservation versus development paradigm in which the strictly protected National Park (including the marsh) has been managed for conservation purposes. In contrast, the surrounding area (including the entry streams) has been largely earmarked for economic development (Zorrilla-Miras et al., 2014), hence the expansion of greenhouses. A similar paradigm has led to protection of many shallow lakes in Andalusia without effective protection of their catchment areas (Rodríguez-Rodríguez et al. 2012).

To mitigate the excess of downstream anthropogenic nutrient transport into the Doñana WHS, a radical shift in land and water management is required towards a more holistic approach. Management should be based on multi-scale governance systems capable of meeting the basic demands of a variety of stakeholders and beneficiaries on local and larger scales without compromising biodiversity conservation (Defries and Nagendra, 2017). Water purification of anthropogenic inputs is necessary *before* water reaches the marshland, instead of relying on the marsh itself to provide these services. This requires specific actions such as restoring and increasing the area of riparian buffer zones along the affected streams, reversing agricultural encroachment up to the edge of streams, and other measures to mitigate soil erosion. Such riparian buffers can be highly effective (Weigelhofer et al., 2012; Teufl et al., 2013) and are particularly required in the Partido and Rocina/Soto catchments. Creation of a network of upstream constructed wetlands before the water enters the Doñana marsh, and the improvement of the existing wastewater collection and treatment would considerably reduce downstream nutrient transport. Furthermore, an expansion of water quality monitoring is crucial, particularly to identify point sources and to understand the impact of events such as runoff from agricultural lands during heavy rains, or pilgrimage celebrations. In addition, the extensive illegal expansion of greenhouses should be reversed, including closure of illegal wells, acquisition of water rights by the Government, or declaring the underlying aquifer as overexploited (WWF, 2019b). Given the synergy between eutrophication and climate change, it is essential that plans to reduce nutrient inputs to Doñana are ambitious, and this synergy should be recognized and accounted for by adopting a "safe operating system" approach to management of the entire Doñana catchment (Green et al. 2017).

# Conclusions

In conclusion, we found strong evidence that eutrophication is a major and ongoing environmental issue in Doñana, particularly in the streams flowing into the Doñana WHS and the areas of marsh around the mouth of streams. Nutrient concentrations were consistently lower within the marsh due to its “purification capacity”, and the greater distances to agricultural and urban sources of nutrients).

The current situation is partly the consequence of decades of polarized management, in which the strictly protected National Park (including the marsh) has been managed for conservation whereas the catchment area (including the streams) has been prioritized for economic development, leading to agricultural intensification (mainly greenhouse production for EU markets) and human population growth. Thus, the high nutrient concentrations entering the catchment, together with decreasing water quantity due to groundwater abstraction for agriculture, have seriously compromised surface water quality, and threaten the ecological value of this emblematic wetland. These conflicts are likely to be common in other Mediterranean wetlands. An urgent shift to more holistic, integrated management is necessary to improve water quality, and so ensure the long-term conservation of the Doñana wetland complex and its resilience against climate change.

## Acknoledgments and funding

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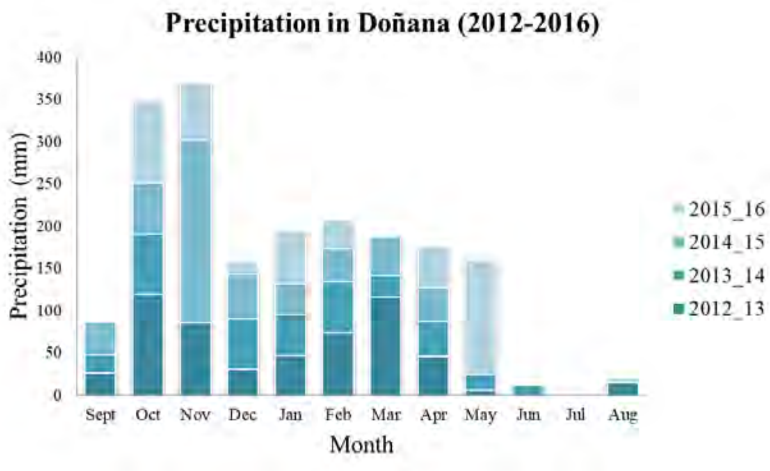



**Figure S1.** Monthly precipitation in Doñana National Park (DNP) over the four consecutive hydrological years included in this study. Each hydrological year extends from September until the next August. Data were collected from the Meteorological Station located at “El Palacio” within the DNP.

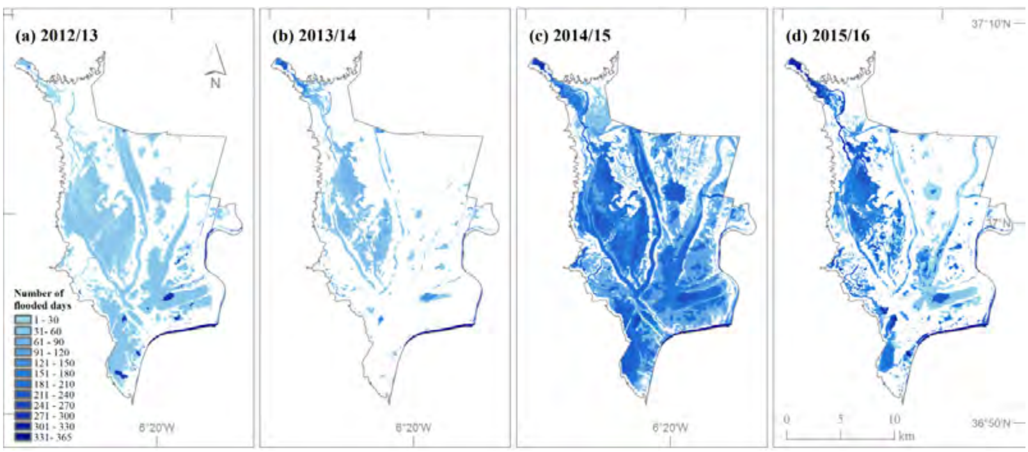



**Figure S2.** Estimated hydroperiod for the inundation cycles 2012/13 (total precipitation=566 mm), 2013/14 (359 mm), 2014/15 (528 mm) and 2015/16 (469 mm). Colour intensity indicates the number of days each pixel was detected as flooded according to inundation masks from Landsat images (see Díaz-Delgado et *al.* 2016 for methodological details).

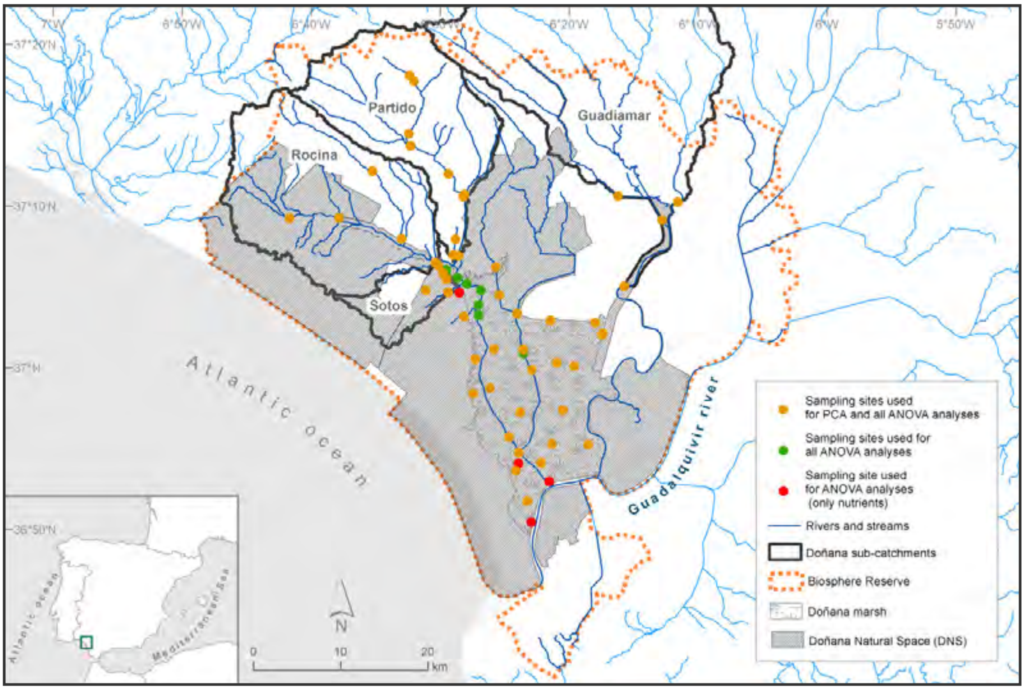



**Figure S3.** Location of sampling points used in the PCA analysis ($n_{sites}$=49; $n_{samples}$=338; orange dots), the two-way ANOVA analyses of nutrient concentrations ($n_{sites}$=59; $n_{samples}$=434; orange, green and red dots) and the ANOVA analyses of chla ($n_{sites}$=55; $n_{samples}$=264; orange and green dots). Data were collected between January 2013 and June 2016.

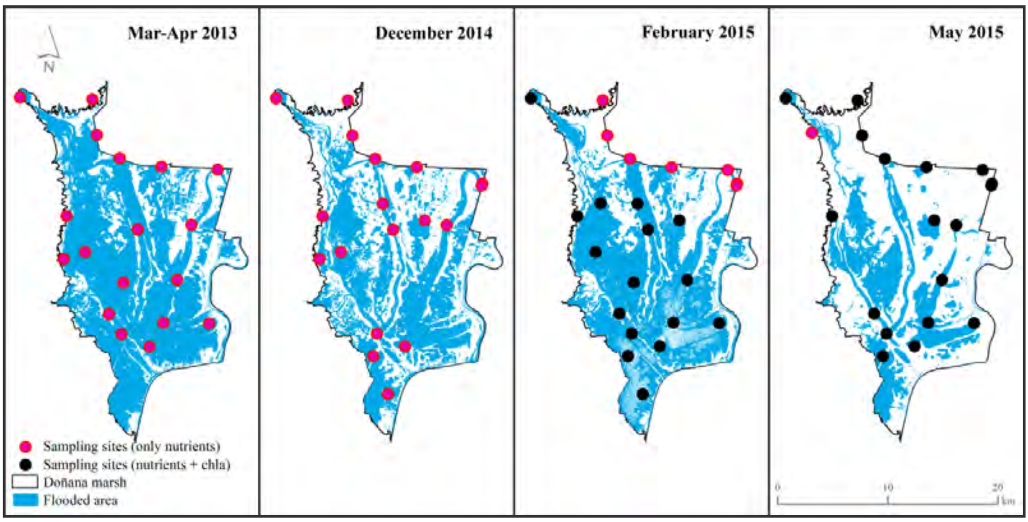



**Figure S4.** Location of sampling points within the Doñana marsh from which we obtained the nutrient (pink and black dots) and chla (only black dots) data used to perform the linear regression models (LM) over the four sampling periods with the highest flooding level during our entire study period. Inundation masks representing the flooded area correspond to the following dates (from left to right): 19/04/2013, 10/12/2014, 28/02/2015 and 11/05/2015.

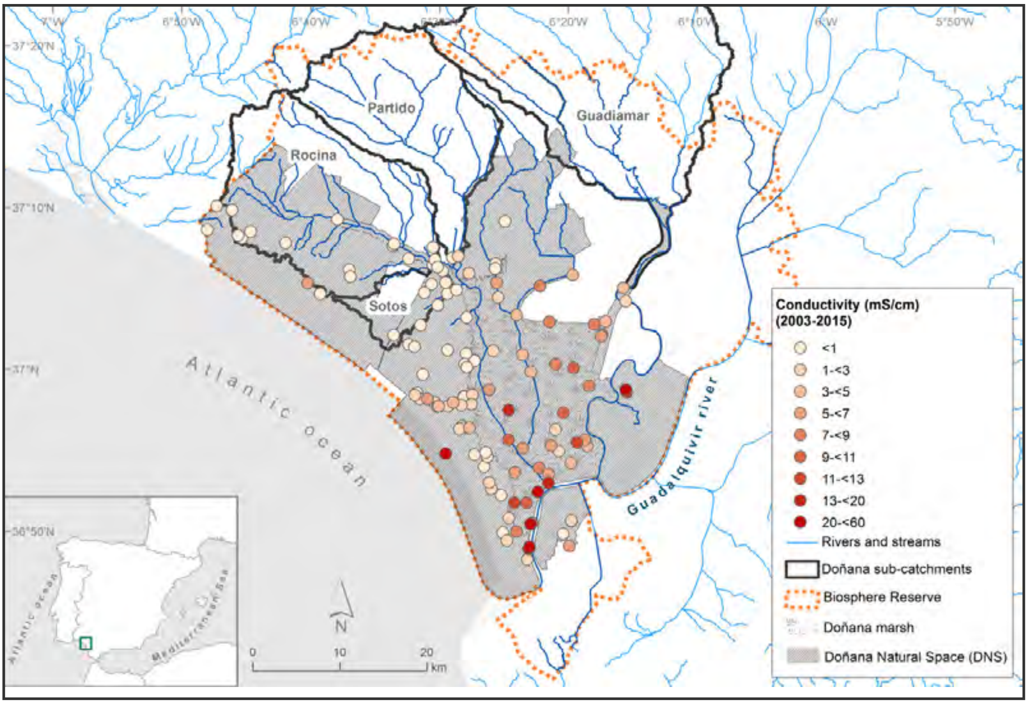



**Figure S5.** Conductivity (mS cm$^{-1}$) gradient across Doñana Natural Space (shaded area). Dots represent mean conductivity values of all samples collected at each point between 1st April and 30th June during the period 2003 to 2015. These data were collected by the Monitoring Team for Natural Resources and Processes of the Doñana Biological Station using a WTW (Weilheim, Germany) Multi-340i handheld meter for *in situ* measurements. Many of these points were not used in our study of nutrient analysis.

**Table S1.** Summary of conductivity, depth and stable isotope ($\delta^2H$) data collected between January 2013 and June 2016 in the marsh and the three streams.

| | Habitats | | | |
|---|---|---|---|---|
| **Variables** | **Partido (n= 92)** | **Rocina/Sotos (n= 79)** | **Guadiamar (n= 33)** | **Marsh (n= 137)** |
| **Cond (µS cm$^{-1}$)** | | | | |
| Min. | 267 | 222 | 325 | 156 |
| 1st Qu. | 945 | 393.5 | 719 | 1070 |
| Median | 1162 | 553 | 1439 | 2460 |
| Mean ± s.d. | 1191 ± 766.4 | 544.2 ± 195.8 | 2114 ±1908.2 | 4330 ± 5737.12 |
| 3rd Qu. | 1317 | 626 | 2690 | 5090 |
| Max. | 7860 | 1270 | 7820 | 32900 |
| **Depth (cm)** | | | | |
| Min. | 1 | 5 | 5 | 1 |
| 1st Qu. | 15 | 10 | 26 | 15 |
| Median | 23 | 20 | 60 | 28 |
| Mean ± s.d. | 36.2 ± 52.7 | 26.8 ± 23.8 | 67.6 ± 59.2 | 31± 22.1 |
| 3rd Qu. | 40 | 35 | 100 | 42 |
| Max. | 480 | 130 | 300 | 150 |
| **$\delta^2$H (‰)** | | | | |
| Min. | -50.1 | -55.9 | -49 | -52 |
| 1st Qu. | -28 | -29.3 | -35.6 | -21.5 |
| Median | -25.7 | -25.8 | -24.3 | -1.3 |
| Mean ± s.d. | -26.9 ± 5.5 | -26.5 ± 7.3 | -23.8 ± 13.9 | -1.5 ± 25.9 |
| 3rd Qu. | -23.9 | -23.1 | -14.9 | 13.1 |
| Max. | -17.9 | -3.4 | 7.5 | 68.2 |

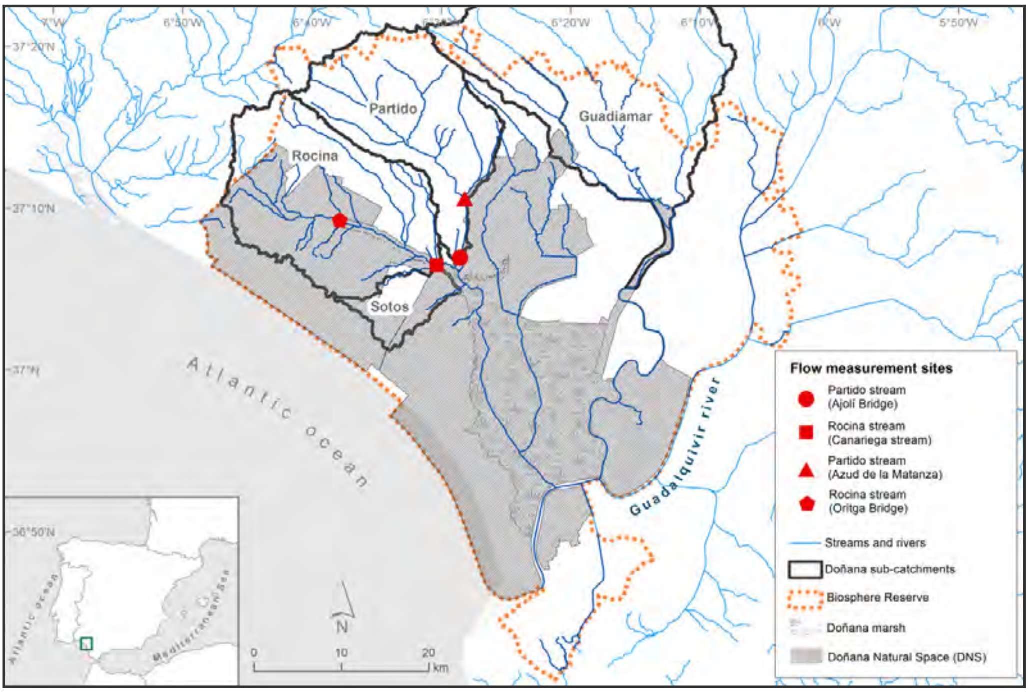
**Table S2.** Two-way analysis of variance (two-way ANOVA) with nutrient and chlorophyll-*a* (chla) concentrations as dependent variables (all log-transformed), and habitat as categorical predictor. Coefficients for habitat 'Marsh' are not shown because they are aliased, but they are effectively zero. 'Sampling period' refers to 16 different field campaigns carried out between January 2013 and May 2016. The degrees of freedom of the residuals were 411 for all nutrient effects and 250 for chla. This table corresponds to Fig. 9 in the manuscript.

| Variable | Effect | Level of effect | Estimate ± S.E. | Df | F | P | Model parameters | |
|---|---|---|---|---|---|---|---|---|
| | *Intercept* | | -3.92 ± 0.40 | 1 | 96.51 | <0.0001 | | |
| **Log PO4** | *Habitat* | Guadiamar | 0.92 ± 0.36 | 3 | 50.03 | <0.0001 | Adj. $R^2$ | 0.42 |
| | | Rocina/Sotos | 1.82 ± 0.28 | | | | F | 18.36 |
| | | Partido | 3.70 ± 0.30 | | | | P | <0.0001 |
| | *Sampling period* | | | 15 | 12.02 | <0.0001 | | |
| | *Intercept* | | -1.92 ± 0.15 | 1 | 155.65 | <0.0001 | | |
| **Log TP** | *Habitat* | Guadiamar | 0.38 ± 0.13 | 3 | 81.69 | <0.0001 | Adj. $R^2$ | 0.49 |
| | | Rocina/Sotos | 1.04 ± 0.10 | | | | F | 24.16 |
| | | Partido | 1.80 ± 0.11 | | | | P | <0.0001 |
| | *Sampling period* | | | 15 | 12.65 | <0.0001 | | |
| | *Intercept* | | -3.34 ± 0.57 | 1 | 34.00 | <0.0001 | | |
| **Log NH4** | *Habitat* | Guadiamar | 0.79 ± 0.51 | 3 | 25.44 | <0.0001 | Adj. $R^2$ | 0.20 |
| | | Rocina/Sotos | 0.14 ± 0.40 | | | | F | 7.18 |
| | | Partido | 3.31 ± 0.43 | | | | P | <0.0001 |
| | *Sampling period* | | | 15 | 3.53 | <0.0001 | | |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | *Intercept* | | -6.63 ± 0.73 | 1 | 82.50 | <0.0001 | | |
| **Log NO3** | *Habitat* | Guadiamar | 5.31 ± 0.66 | 3 | 97.11 | <0.0001 | Adj. $R^2$ | 0.53 |
| | | Rocina/Sotos | 6.49 ± 0.52 | | | | F | 28.70 |
| | | Partido | 9.08 ± 0.55 | | | | P | <0.0001 |
| | *Sampling period* | | | 15 | 15.02 | <0.0001 | | |
| | *Intercept* | | -5.58 ± 0.31 | 1 | 319.59 | <0.0001 | | |
| **Log NO2** | *Habitat* | Guadiamar | 1.4 ± 0.28 | 3 | 92.92 | <0.0001 | Adj. $R^2$ | 0.50 |
| | | Rocina/Sotos | 1.75 ± 0.22 | | | | F | 25.54 |
| | | Partido | 3.95 ± 0.23 | | | | P | <0.0001 |
| | *Sampling period* | | | 15 | 12.06 | <0.0001 | | |
| | *Intercept* | | 1.09 ± 0.10 | 1 | 117.31 | <0.0001 | | |
| **Log TN** | *Habitat* | Guadiamar | 0.04 ± 0.09 | 3 | 97.11 | <0.0001 | Adj. $R^2$ | 0.52 |
| | | Rocina/Sotos | 0.49 ± 0.07 | | | | F | 27.87 |
| | | Partido | 1.26 ± 0.07 | | | | P | <0.0001 |
| | *Sampling period* | | | 15 | 14.02 | <0.0001 | | |
| | *Intercept* | | -5.57 ± 0.18 | 1 | 968.27 | <0.0001 | | |
| **Log Chla** | *Habitat* | Guadiamar | 1.04 ± 0.26 | 3 | 8.87 | <0.0001 | Adj. $R^2$ | 0.14 |
| | | Rocina/Sotos | 0.54 ± 0.18 | | | | F | 4.54 |
| | | Partido | 0.02 ± 0.19 | | | | P | <0.0001 |
| | *Sampling period* | | | 10 | 3.24 | <0.0001 | | |

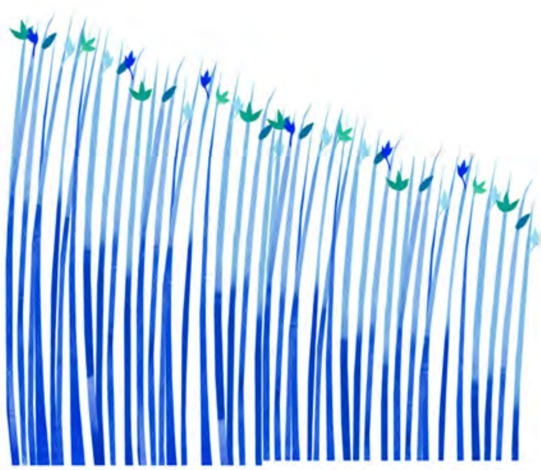
**Table S3.** Surface water flow ($m^3s^{-1}$) measured at different sites of the Rocina and Partido streams during February, April and May in 2016. We used a handheld Acoustic Doppler Velocimeter (Sontek FlowTracker). Final flow value is the average of several measurements along a transversal transect of the stream.

| | Surface water flow ($m^3s^{-1}$) [mean velocity ($m\ s^{-1}$)] | | | |
|---|---|---|---|---|
| ***Stream sites*** | ***$25^{th}$ Feb. 2016*** | ***$10^{th}$ April 2016*** | ***$20^{th}$, $^{†}21^{st}$ April 2016*** | ***$24^{th}$, $^{††}25^{th}$ May 2016*** |
| **Rocina stream** upstream (*Ortigas bridge*) | 0.0079 [0.1156] | 0.0048 [0.0732] | 0.1898 [0.3594] | 0.0141[0.1652] |
| **Rocina stream** downstream (*Canariega bridge*) | 0.0734 [0.2860] | 0.0325 [0.2030] | 0.2020† [0.5195] | 0.0564 [0.2849] |
| **Partido stream** upstream (*Azud de la Matanza*) | 0.0956 [0.4455] | 0.0472 [0.3299] | 0.6685† [0.3684] | 0.1089†† [0.4035] |
| **Partido stream** downstream (*Ajolí bridge*) | 0.0727 [0.0971] | 0.0902 [0.0356] | 0.7859† [0.2042] | 0.1770†† [0.2787] |



# Capítulo 2

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



Paredes, I., Ramírez, F., Forero, M.G., Green, A.J., 2019. Stable isotopes in helophytes reflect anthropogenic nitrogen pollution in entry streams at the Doñana World Heritage Site. Ecol. Indic. 97, 130–140. https://doi.org/10.1016/j.ecolind.2018.10.009

# 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 ($\delta^{15}N$) 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 $\delta^{15}N$ 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 $\delta^{15}N$ values is limited when key potential N sources are isotopically undistinguishable (e.g. fertilizers vs. atmospheric sources).

# 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 $\delta^{15}N$ 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 $\delta^{15}N$ values of residual nitrate (Mariotti et al., 1981; Minet et al., 2017). Therefore, the relative abundance of N isotopes ($\delta^{15}N$) 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 $\delta^{15}N$ 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 $\delta^{15}N$ values (15 to +20‰) depending on the available N sources, environmental conditions and physiological features (Kendall et al., 2008). For example, $\delta^{15}N$ 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 $\delta^{15}N$ 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 $\delta^{15}N$ 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 $\delta^{15}N$ 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).

# Materials and methods

## 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 $hm^3$ $year^{-1}$ and 20-140 $hm^3$ $year^{-1}$, 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)

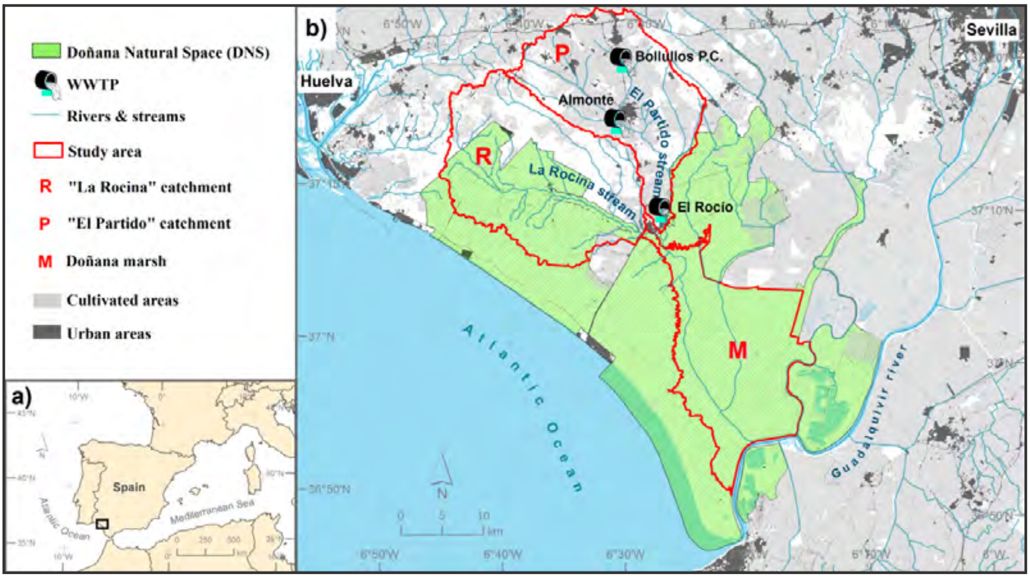



**Figure 1.** Location of (a) the Doñana wetlands in western Europe and (b) the limits of the Doñana Natural Space (DNS), the marsh (M) and the two catchment areas for the streams included in this study ("La Rocina" (R) and "El Partido" (P)). Two major anthropogenic pressures in this area are represented 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).

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.

## 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 $\delta^{15}N$ 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 $\delta^{15}N$ 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 37° 10' 23.25" N, 6° 53' 13.52" O).

## Laboratory analyses

### *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 1020°C 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 $\delta$-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).

### *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 $L^{-1}$ for N-$NO_3$ and N-$NO_2$, 0.04 µmol $L^{-1}$ for N-$NH_4$ and 40 µg $L^{-1}$ for TN.

## 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 ($\delta^{15}N$). We checked normality of the dependent variable ($\delta^{15}N$) 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 $\delta^{15}N$ 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 $\delta^{15}N$ 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.

## 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.

### 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).

## Results

### 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‰.

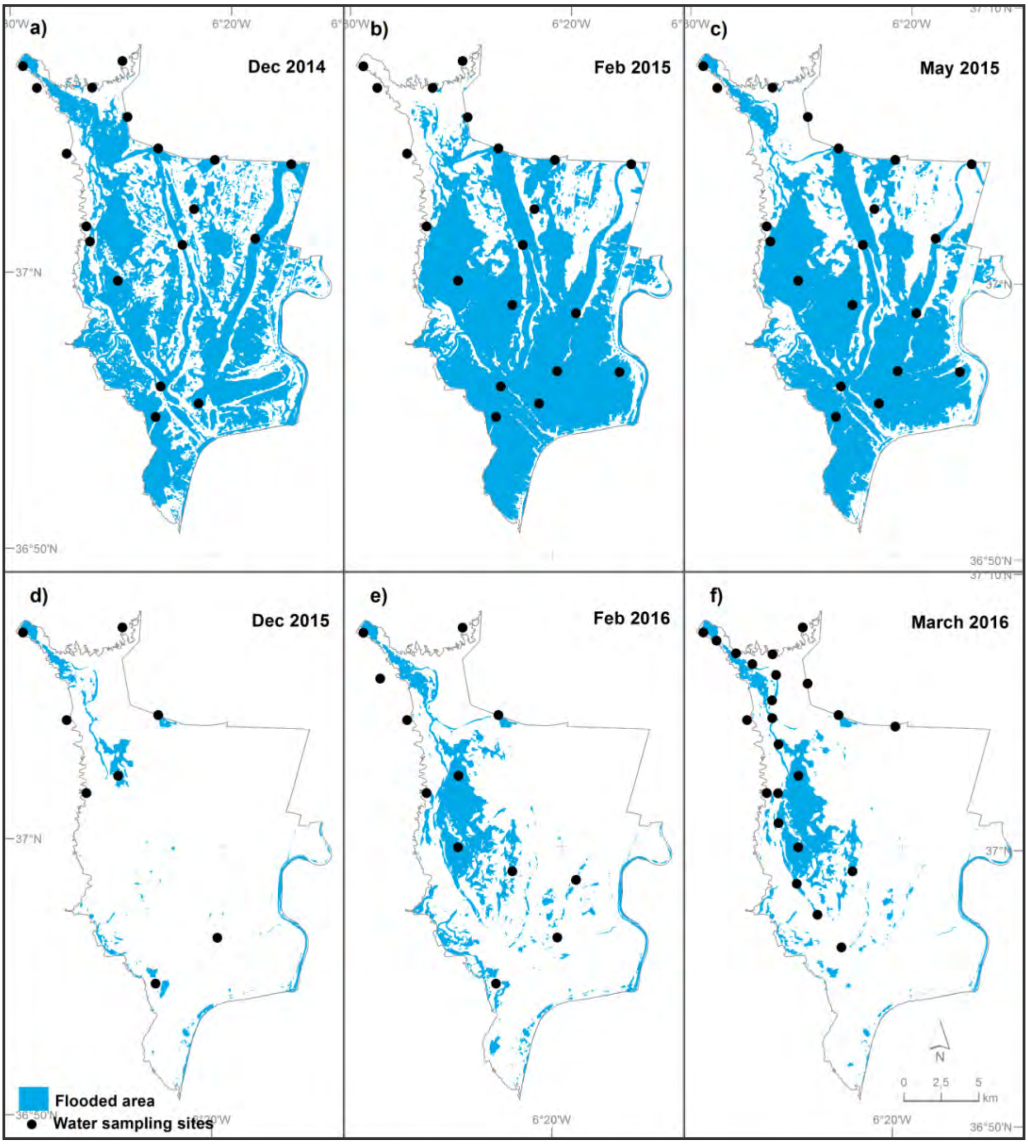



**Figure 2.** Extent of the flooded area in the Doñana marsh in two hydrological years (2015 and 2016). Inundation masks are based on Landsat 7 (ETM sensor) images acquired on 10 December 2014 (a), 19 May 2015 (c) and Landsat 8 (OLI) images from 20th February 2015 (b), 5th December 2015 (d), 23rd February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

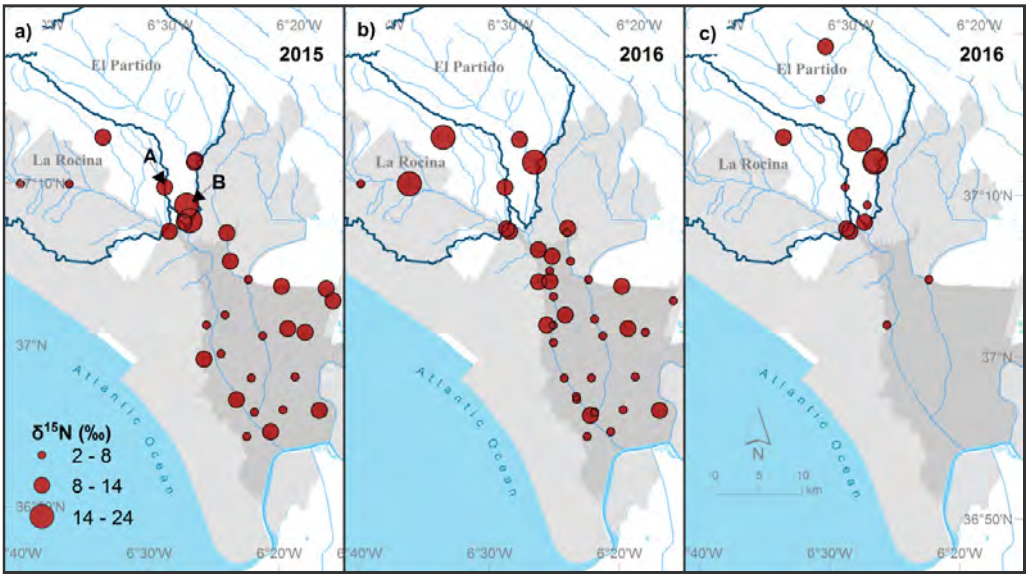



**Figure 3.** Variability of nitrogen stable isotopes obtained from helophytes. (a) B. *maritimus* collected in April-May 2015. (b) B. *maritimus* and (c) T. *domingensis* collected in April-May 2016. Dot size represents the isotopic values at each site ($\delta^{15}$N (‰)). In map (a) A and B indicate the points with the highest N concentration in relation to the measured $\delta^{15}$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.

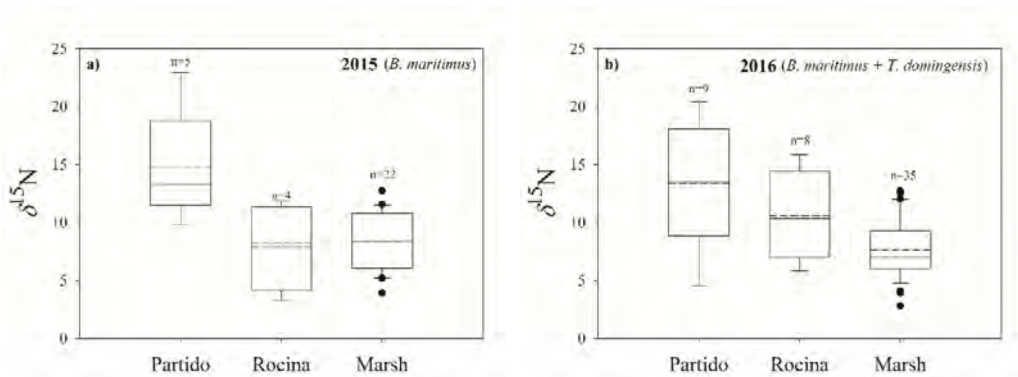



**Figure 4.** Box plots of (a) isotopic signatures ($\delta^{15}$N, (‰)) of B. *maritimus* in 2015 and (b) B. *maritimus* + T. *domingensis* in 2016 in each habitat ("El Partido" stream, "La Rocina" stream and the marsh). The solid horizontal line shows the median of $\delta^{15}$N values. The dashed horizontal line shows the mean of $\delta^{15}$N values (Partido2015= 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 25$^{th}$ and 75$^{th}$ percentiles, respectively. The whiskers are drawn out to the 10$^{th}$ and 90$^{th}$ percentiles (Cleveland method). Extreme values outside these percentiles are marked as outliers.

## 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.

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).

## 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 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 ($\alpha$=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 ˋSample 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 | $\chi^2$ | 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 |

**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.*

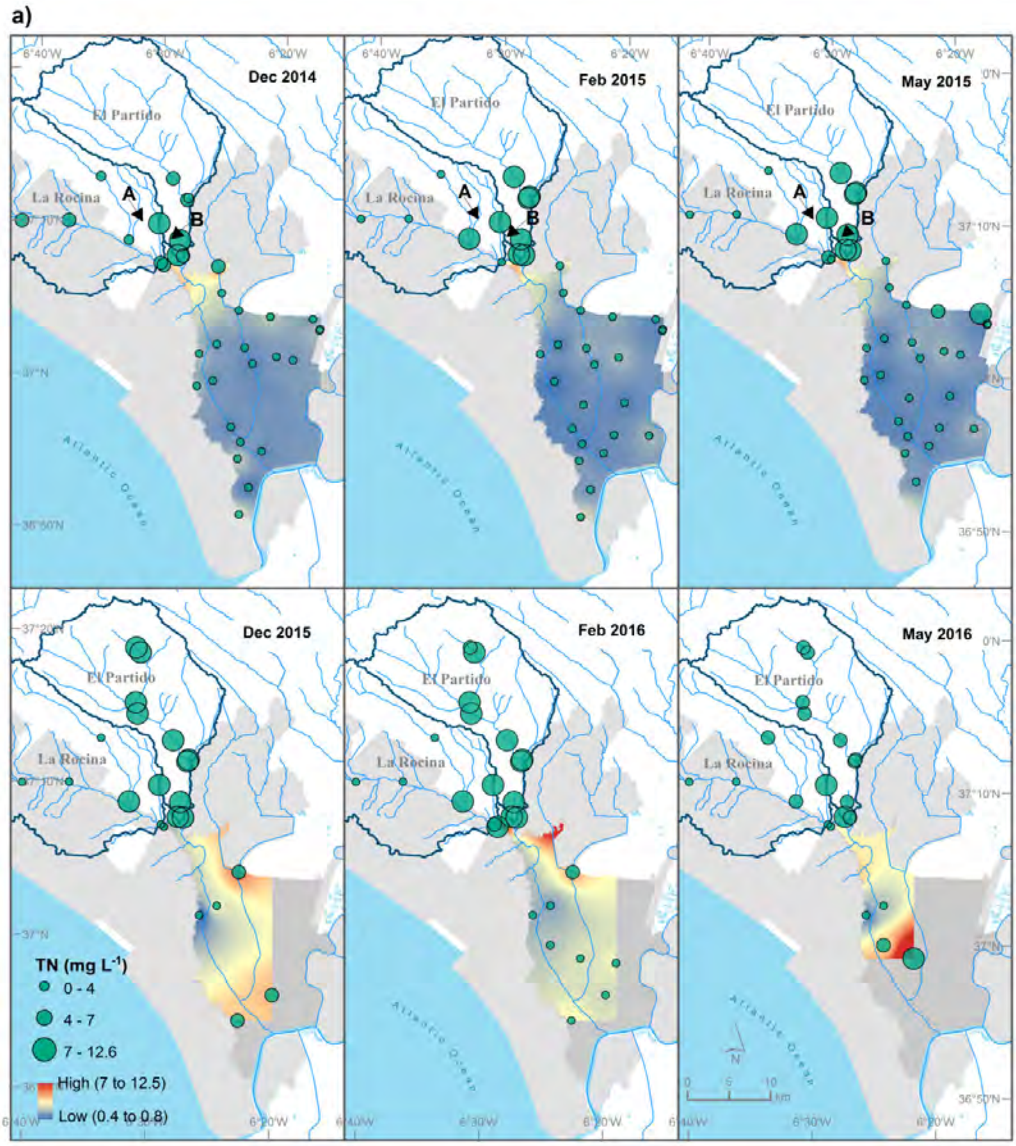
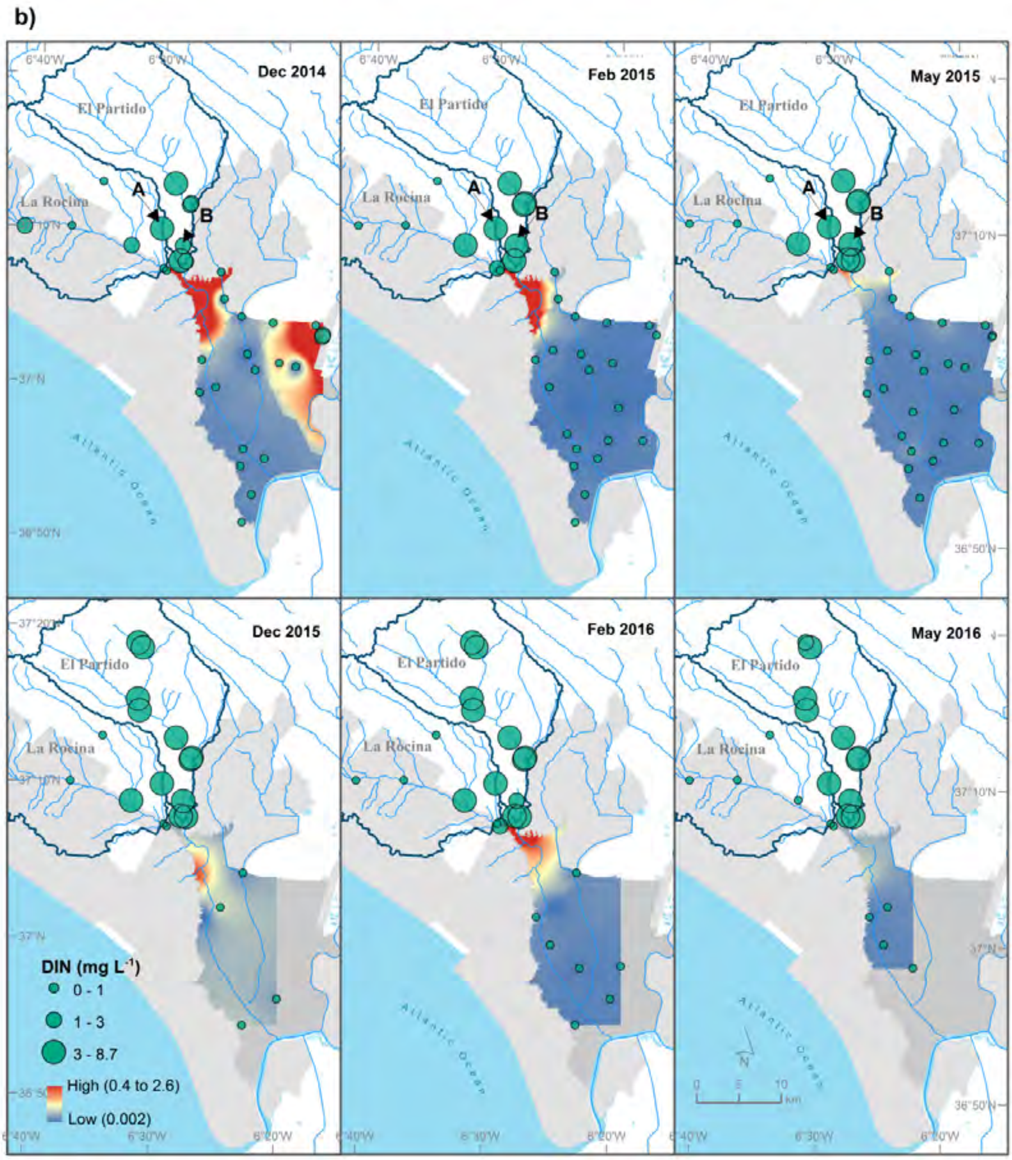



**Figure 5.** (a) Total N (TN) and (b) dissolved inorganic nitrogen (DIN) (mg N L-1) variability in two hydrological years (2015 and 2016) in the Doñana marsh and entry streams from the "La Rocina" and "El Partido" catchments. Dot size represents the average values of all samples collected in December, 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.


b)
Dec 2014
Feb 2015
May 2015
Dec 2015
Feb 2016
May 2016
El Partido
La Rocina
A
B
Atlantic Ocean
DIN (mg L-1)
0 - 1
1 - 3
3 - 8.7
High (0.4 to 2.6)
Low (0.002)
6°40'W
6°30'W
6°20'W
37°10'N
37°N
36°50'N
37°20'N
0 5 10 km
N

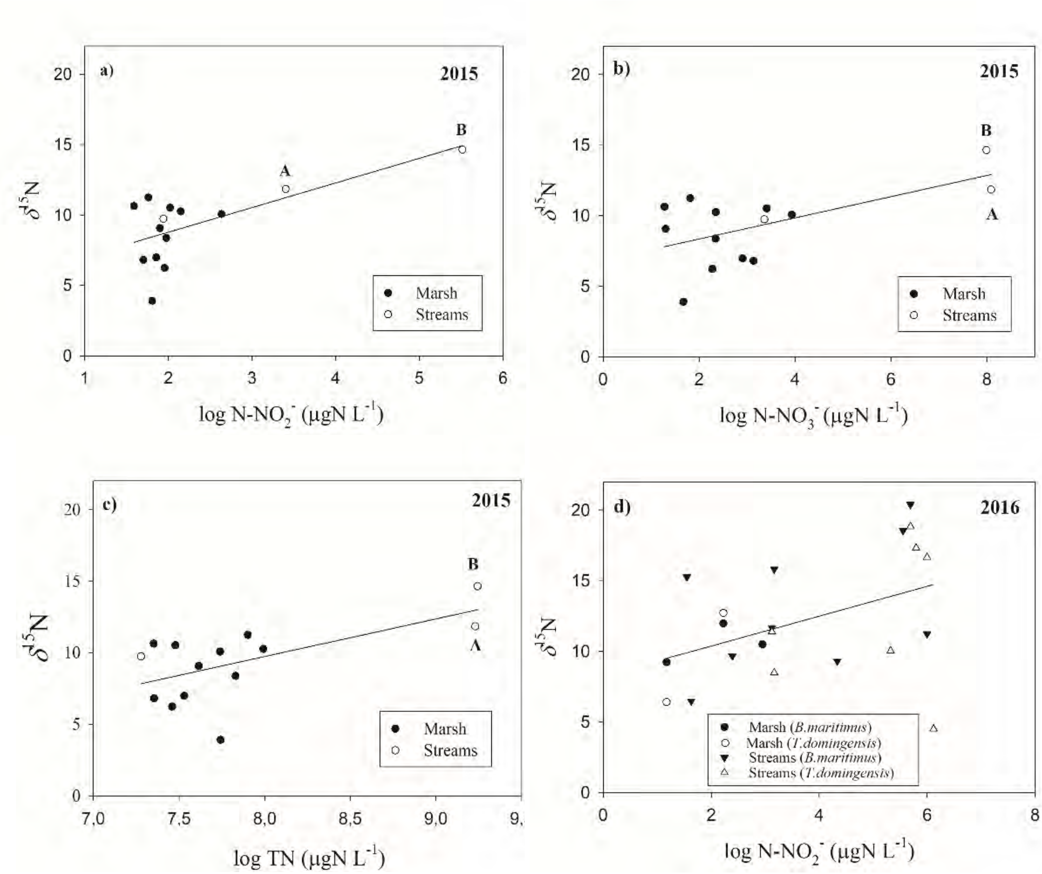



**Figure 6.** Relationship between $\delta^{15}$N (‰) of helophytes (*B. maritimus* and *T.domingensis*) and mean of surface water N concentrations ($NO_2$, $NO_3$ and TN in μg N $L^{-1}$) collected in 2015 (a, b, c) and 2016 (d) in the Doñana marsh and entry streams. In 2015 we only measured $\delta^{15}$N 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.

# 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 $\delta^{15}N$ 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 $\delta^{15}N$ 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 $\delta^{15}N$ 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).

## 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 $NH_4$ $L^{-1}$) 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).

## Helophyte $\delta^{15}N$ as an indicator of anthropogenic pressures

As with submerged aquatic plants, high $\delta^{15}N$ 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 $\delta^{15}N$ 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 $\delta^{15}N$ 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 $\delta^{15}N$ 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 $\delta^{15}N$ values we recorded in "La Rocina" watershed are in line with those recorded by Tortosa et *al.* (2011) in dissolved $NO_3$ ($\delta^{15}N$-$NO_3$), 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 $\delta^{15}N$ value recorded in our reference stream whose catchment is 100% strawberry fields.

However, $\delta^{15}N$ 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 $\delta^{15}N$ 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, $\delta^{15}N$ 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).

## 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.

# 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 $\delta^{15}N$ 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 $\delta^{15}N$ 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 $\delta^{15}N$ values (such as inorganic fertilizers or atmospheric N deposition) or high $\delta^{15}N$ 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 $\delta^{15}N$ 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 $\delta^{15}N$ 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.

## Acknowledgments

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# Supplementary material


**Figure S1.** Municipalities within "La Rocina" and "El Partido" catchments.

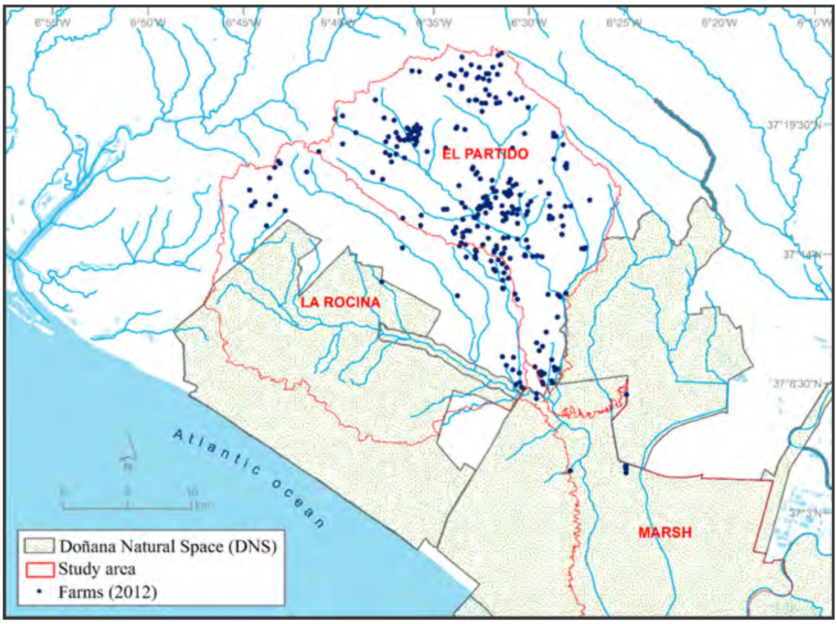


**Figure S2.** Location of farms within the study area in 2012. Each dot represents one herd of cows, sheep, pigs, horses or goats. Source: Spanish Ministry of Agriculture. Food and Environment. http://www.magrama.gob.es/ (2012).

**Table S1.** Numbers of farms and head of livestock in 2009 in the municipalities within "La Rocina" and "El Partido" catchments (see Fig. S1). Source: Institute of Statistics and Cartography of Andalusia. "Censo Agrario 2009 del INE". http://www.juntadeandalucia.es/institutodeestadisticaycartografia/iea/consultasActividad.jsp?CodOper=104&sub=38120

| Municipality | Area (ha) | No. Farms | Head of livestock (Census 2009) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Bovine | Ovine | Goat | Equine | Porcine | Fowl (per mil) | Rabbit | Hives |
| *Almonte* | 85920 | 410 | 2172 | 3820 | 61 | 1502 | 100 | 35 | 120 | 1962 |
| *Rociana del Condado* | 7200 | 80 | 0 | 692 | 10 | 163 | 4 | 195 | 4 | 30 |
| *Lucena del Puerto* | 6930 | 26 | 155 | 490 | 429 | 70 | 0 | 51 | 0 | 0 |
| *Bonares* | 6520 | 31 | 0 | 2 | 12 | 165 | 0 | 50 | 0 | 50 |
| *Bollullos Par del Condado* | 4930 | 107 | 90 | 0 | 100 | 221 | 752 | 367 | 0 | 0 |

Capítulo 3

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

Paredes, I., Otero, N., Soler, A., Green, A.J., Soto, D.X., 2020. Agricultural and urban delivered nitrate pollution input to Mediterranean temporary freshwaters. Agric. Ecosyst. Environ. 294, 106859. https://doi.org/10.1016/j.agee.2020.106859

# Abstract

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

# Introduction

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

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

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

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

A simultaneous dual nitrate isotope approach ($\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$) offers the advantage of a more precise distinction between sources and processes, since $\delta^{18}O_{NO3}$ (i.e. the ratio $^{18}O/^{16}O$) shows a greater resolution for the origin of certain sources that overlap for $\delta^{15}N_{NO3}$ (Craine et al., 2015). For example, while synthetic fertilizers and nitrate atmospheric deposition show overlapping $\delta^{15}N_{NO3}$, the $\delta^{18}O_{NO3}$ values of synthetic fertilizers (around +23 ‰) (Michalski et al., 2015) are considerably higher than those of atmospheric deposition (ranging from +60 ‰ to +98 ‰) (Kendall et al., 2008). Furthermore, overlapping of $\delta^{15}N_{NO3}$ values may also occur when there are changes in $\delta^{15}N_{NO3}$ for one of the sources due to transformation processes (e.g. nitrification, denitrification, mineralization, ammonia volatilization or assimilation) (Kendall et al., 2008). Hence, nitrate removal by denitrification or assimilation may produce $^{15}N_{NO3}$ enrichment in the residual nitrate of an originally $^{15}N_{NO3}$-depleted source (e.g. synthetic fertilizers), which can make it undistinguishable from another $^{15}N_{NO3}$ enriched, untransformed source (e.g. human wastewaters) (Kendall, 1998). Such fractionating processes also produce $^{18}O_{NO3}$ enrichment, resulting in comparatively higher $\delta^{18}O_{NO3}$ values in the residual nitrate than in the $^{15}N_{NO3}$ enriched, untransformed sources (Mariotti et al., 1988; Granger et al., 2004; Søvik and Mørkved, 2008). Thus, nitrate isotopic composition in most aquatic systems is the result of simultaneous transformations and nitrate source mixing which are often undistinguishable from each other without the application of multi-isotopic approaches such as the dual nitrate isotope approach (Kendall et al., 2008, Otero et al., 2009, Yue et al., 2017). The latter has been used to study nitrate transport and transformations in numerous watersheds worldwide. However, there is a lack of isotope studies in arid and semiarid areas subjected to warm temperatures, strong rainfall variation and water scarcity, such as the Doñana wetland (Custodio et al., 2009; Tortosa et al., 2011, Wong et al., 2018). Given climate change and the ongoing nutrient inputs from anthropogenic activities, vulnerability to eutrophication is expected to continue increasing in Mediterranean wetlands (Green et al. 2017). In this context, it is critical to improve our understanding of natural nitrate removal processes (e.g. denitrification) which can reduce eutrophication.

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

# Materials and methods

## 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

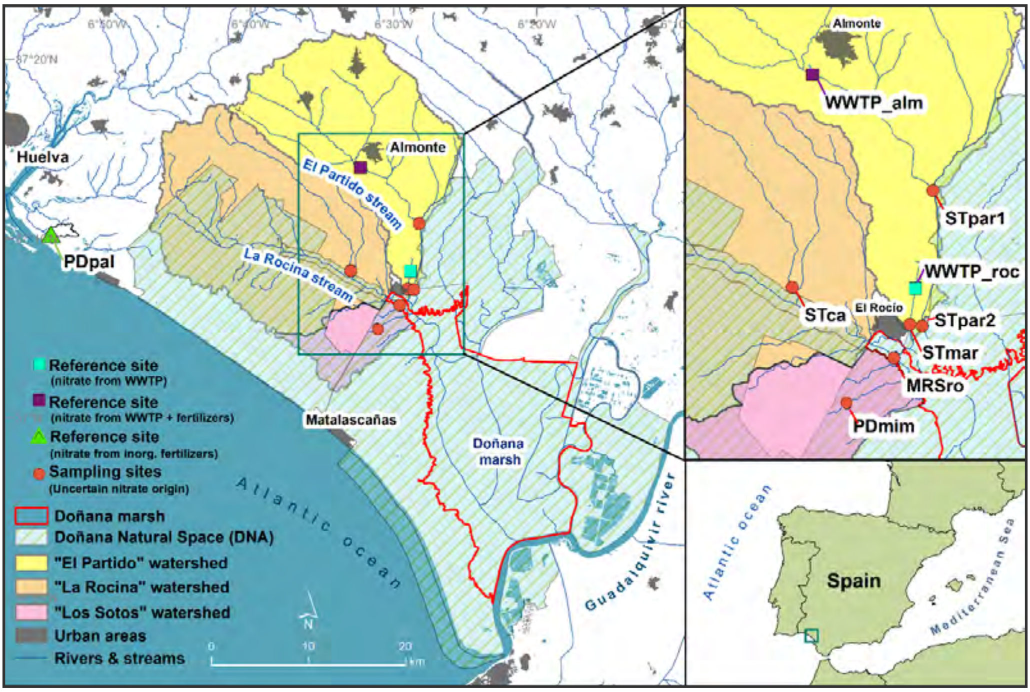


**Figure 1.** Location of the sampling points selected for this study. Red dots represent locations where the contribution and type of nitrate sources are uncertain. Purple squares indicate reference sites for nitrate related to urban wastewaters (WWTP_alm and WWTP_roc) and the green triangle a reference 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.

climate, as well as anthropogenic pressure such as groundwater abstraction for agriculture (Manzano et al. 2005; 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 (Table 1). 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.

## Sample collection

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

Although nitrate sources were generally uncertain, three sampling sites (PDpal, WWTP_roc and WWTP_alm) were assumed to receive nitrate predominantly from one specific source, this being the criterion we used to consider them as "reference sites" (Fig. 1). PDpal receives surface and groundwater affected by chemical fertilizers used in the surrounding intensive greenhouse strawberry production, especially between October and June when only chemical fertilizers are applied (mainly ammonium

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

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

* *Reference site for nitrate pollution derived from synthetic fertilizers.*

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

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

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

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

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

## Stable isotope and nitrate concentration analyses

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

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

## Identification of nitrate sources

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

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

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

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

## Denitrification processes

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

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

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

where εP/S is the isotopic fractionation, δs and δs,0 are the isotopic composition of the residual (s) and initial (s,0) nitrate, and *f* is the remaining nitrate fraction. Both $\varepsilon^{15}N_{NO3}$ and $\varepsilon^{18}O_{NO3}$ can be modeled.

Denitrification percentages were estimated using an average isotopic fractionation of $\varepsilon^{15}N_{NO3}/N_2$ = -15‰ (Böttcher et al., 1990) and a $\varepsilon^{18}O_{NO3}/N_2$ $/\varepsilon^{15}N_{NO3}/N_2$ ratio of 0.7. Different denitrification models were calculated based on the initial isotope values ($\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$) of the original nitrate source:

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

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

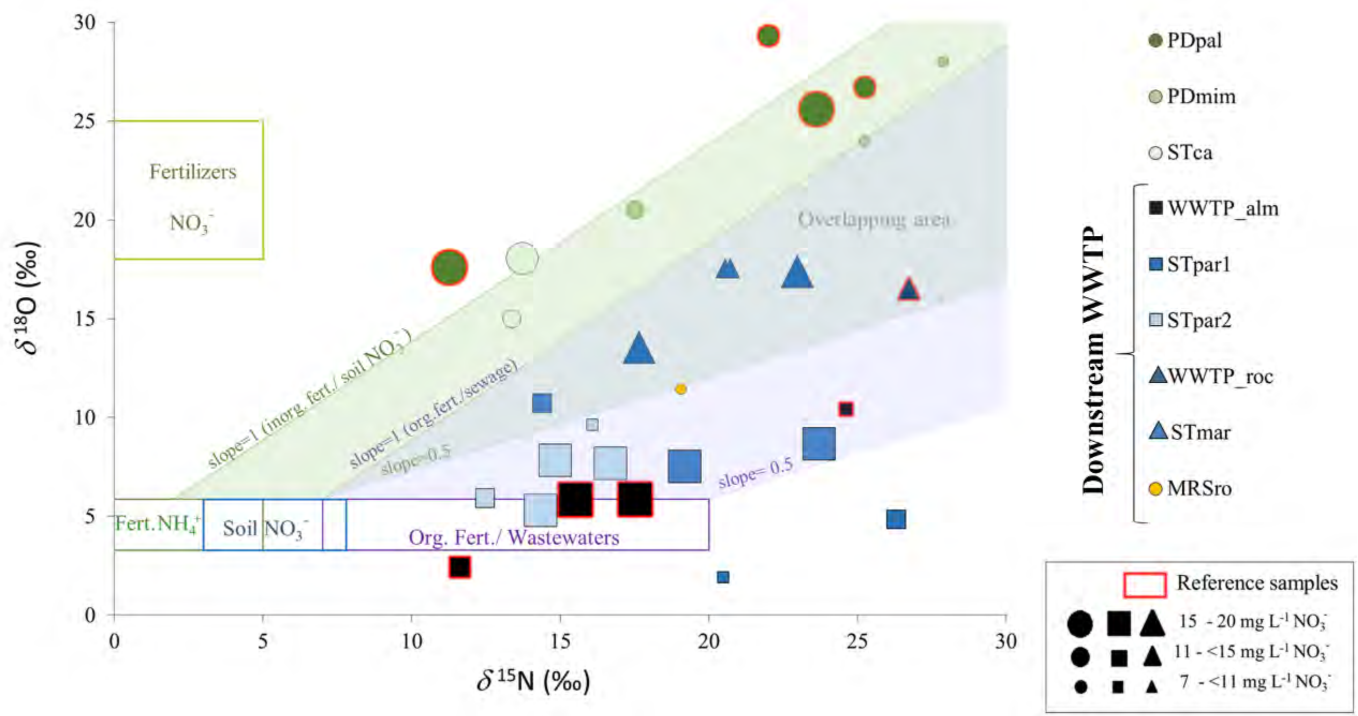


**Figure 2.** $\delta^{15}N$ vs. $\delta^{18}O$ values of the samples together with published reference data (in boxes, Table SP3, Sup. Mat.) from chemical fertilizers (Vitòria et al., 2004), wastewaters and/or organic fertilizers (Widory et al., 2004). Shaded areas between slopes 0.5 and 1 represent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

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

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

## Results and discussion

### Isotopic values and nitrate concentrations

Isotopic values ($\delta^{15}N_{NO3}$, $\delta^{18}O_{NO3}$) and nitrate concentrations measured between February and June (2015 and 2016) in surface waters of the Doñana watershed were highly variable (Table 2). Nitrate concentrations varied between 7.3 and 19.2 mg $NO_3$ $L^{-1}$ with a median of 14.4 mg $NO_3$ $L^{-1}$, being generally higher in winter than in spring-summer. Isotopic values for $\delta^{15}N_{NO3}$ were higher during spring, ranging between +11.3 ‰ and +27.9 ‰ with a median of +19.1 ‰. Isotopic values for $\delta^{18}O_{NO3}$ ranged between +1.9 ‰ and +29.3 ‰ with a median of +11.4 ‰. However, no clear temporal trend was observed for $\delta^{18}O_{NO3}$ during the study period (Fig. 3).

Nitrite concentrations varied between 0.1 and 3.4 mg $NO_2$ $L^{-1}$ with a median of 0.8 mg $NO_2$ $L^{-1}$. Ammonium concentrations varied between 0.001 and 8.6 mg $NH_4$ $L^{-1}$ with a median of 2.2 mg $NH_4$ $L^{-1}$ (Table SP4 Sup. Mat.).

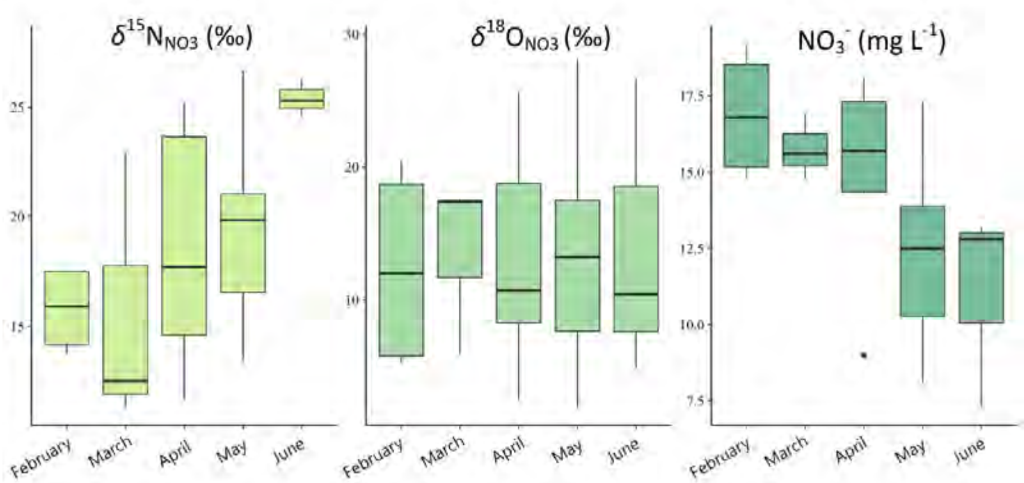


**Figure 3.** Temporal variation of isotopic values ($\delta^{15}N$ and $\delta^{18}O$) and $NO_3^-$ concentrations. Each boxplot contains pooled data for all sites and both years (2015 and 2016) by month (February to June).

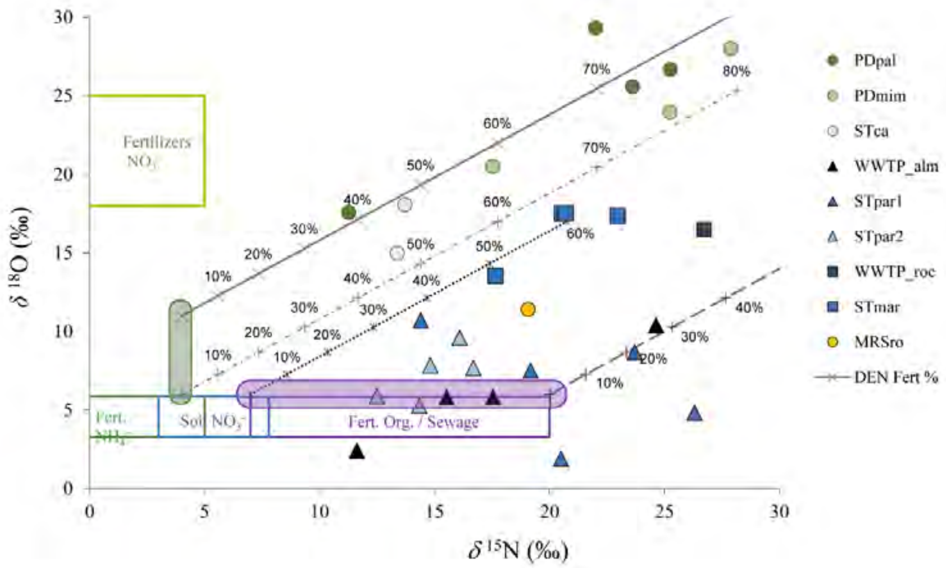


**Figure 4.** $\delta^{15}N$ vs. $\delta^{18}O$ values of the samples together with the modelled denitrification trends. The shadowed areas represent the initial values used in the models.

**Table 2.** $\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$ values and $NO_3^-$ concentrations measured in surface water samples collected at nine different sampling sites between February and June in 2015 and 2016. Sampling points are located in the Doñana marsh catchment area except for PDpal. Reference sites (†) are those where the main N source was known.

| Site | n | Main N source | Date | $\delta^{15}N_{NO3}$ (‰) | $\delta^{18}O_{NO3}$ (‰) | $NO_3^-$ ($mgNO_3$ $L^{-1}$) |
|---|---|---|---|---|---|---|
| **WWTP_roc†** | 1 | *Urban wastewaters* | 06/05/2015 | +26.7 | +16.5 | 12.1 |
| **WWTP_alm†** | 4 | *Urban wastewaters** | 18/02/2016 | +17.5 | +5.9 | 18.3 |
| | | | 20/04/2016 | +11.6 | +2.4 | 14.4 |
| | | | 24/05/2016 | +15.5 | +5.8 | 15.8 |
| | | | 22/06/2016 | +24.6 | +10.4 | 7.3 |
| **PDpal†** | 4 | *Chemical fertilizers* | 15/03/2016 | +11.3 | +17.6 | 16.9 |
| | | | 20/04/2016 | +23.6 | +25.6 | 15.7 |
| | | | 24/05/2016 | +22.0 | +29.3 | 11.9 |
| | | | 22/06/2016 | +25.3 | +26.7 | 13.2 |
| **PDmim** | 3 | *Uncertain* | 11/05/2015 | +27.9 | +28.0 | 9.0 |
| | | | 18/02/2016 | +17.5 | +20.5 | 14.8 |
| | | | 26/04/2016 | +25.2 | +24.0 | 9.0 |
| **STca** | 2 | *Uncertain* | 06/05/2015 | +13.4 | +15.0 | 13.3 |
| | | | 25/02/2016 | +13.7 | +18.1 | 19.2 |
| **STmar** | 4 | *Uncertain* | 06/05/2015 | +20.5 | +17.5 | 13.2 |
| | | | 16/03/2016 | +23.0 | +17.4 | 15.6 |
| | | | 10/04/2016 | +17.7 | +13.5 | 17.3 |
| | | | 25/05/2016 | +20.7 | +17.5 | 12.9 |
| **STpar2** | 5 | *Uncertain* | 06/05/2015 | +16.1 | +9.6 | 10.2 |
| | | | 25/02/2016 | +14.3 | +5.3 | 15.3 |
| | | | 16/03/2016 | +12.5 | +5.9 | 14.8 |
| | | | 10/04/2016 | +14.8 | +7.8 | 17.3 |
| | | | 25/05/2016 | +16.7 | +7.7 | 15.6 |
| **STpar1** | 5 | *Uncertain* | 06/05/2015 | +20.5 | +1.9 | 10.3 |
| | | | 10/04/2016 | +23.7 | +8.7 | 18.1 |
| | | | 21/04/2016 | +14.4 | +10.7 | 14.3 |
| | | | 25/05/2016 | +19.2 | +7.5 | 17.3 |
| | | | 21/06/2016 | +26.3 | +4.8 | 12.8 |
| **MRSro** | 1 | *Uncertain* | 24/05/2016 | +19.1 | +11.4 | 8.1 |

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

## 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.).

### *Measured vs. reference nitrate isotopic values*

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

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

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

### *Denitrification*

Denitrification was likely to cause nitrate isotopic increase in the study sites, so we estimated percentages of denitrification using an average isotopic fractionation value $\varepsilon^{15}N\text{-}NO_3/N_2$ = -15‰ (Böttcher et al., 1990). Although this should be considered only as a rough estimate, both ponds (PDpal and PDmim) showed the highest estimates of denitrification percentages among all sites (40% to 80%), especially in samples collected during spring/summer (Fig. 4). Estimated denitrification percentages for the rest of the sampling sites (STca, WWTP_alm, STpar1, STpar2, WWTP_roc and STmar) showed generally lower values than for the ponds (10-60%). Although our results showed that all nitrate concentrations kept below the maximum recommended for surface waters (50 mg $L^{-1}$ $NO_3^-$), we could expect that real nitrate inputs may be considerably higher since a large proportion is removed by denitrification, according to the estimated denitrification percentages. Thus, water quality monitoring programs measuring only nitrate concentrations in surface water are most likely underestimating the real amount of nitrate exported from the watershed into the streams and ponds. Although we did not study where the nitrate reduction processes take place within our study sites, we assume that it simultaneously occurs: (1) in the water column and sediments of streams and ponds (Tortosa et al. 2011), (2) in the riparian groundwater zone prior to reaching the surface waters (Sebilo et al., 2003; Griffiths et al., 2016), and/or (3) in the deeper groundwater system affected by nitrate leaching from the intensive use of fertilizers (Kim et al., 2015; Otero et al., 2009). In addition, denitrification may often occur within WWTPs as observed in the sample collected at WWTP_roc (directly from the effluent pipe) where both $\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$ values were increased compared to the reference values for WWTP nitrate sources (Fig. 2). Overall, the degree to which denitrification may take part in each compartment would depend on the particular site characteristics and the environmental conditions. Thus, we further evaluate the relative contribution of each source into the surface waters by including different scenarios of denitrification in these Mediterranean wetlands and streams. This information may be particularly relevant for management of surface waters with similar characteristics to the study area, since denitrification constitutes a key process in the attenuation of nitrate.

### *Source mixture tracking and quantification*

Mixing of multiple nitrate sources (e.g. fertilizers, wastewaters, precipitation), is a common process in watersheds and has a direct effect on nitrate isotopic composition (Kendall et al. 2008). Mixing can also occur between transformed and untransformed nitrate from either the same source or different sources. Although denitrification seems to be the most important process explaining nitrate isotopic variation within our sites, we cannot rule out the possibility that mixing also played a key role in the observed nitrate isotopic values. For example, isotopic values of samples collected in STmar fell completely within the denitrification overlapping area (Fig. 2), which could suggest that the nitrate source could be either organic or inorganic, or a mix of the two. Indeed, this site not only receives water from El Rocío WWTP effluent but also from the upstream drainage area where a high percentage of land is dedicated to intensive greenhouse crops (Fig. SP1, Sup. Mat.). Moreover, areas affected by one main nitrate source, such as synthetic fertilizers from agricultural practices in La Rocina, Los Sotos and Laguna de Palos watersheds (without any WWTP influence), could exhibit mixing of waters of the same nitrate source but with different level of isotopic fractionation (e.g. non-denitrified surface waters mixing with denitrified groundwaters). Therefore, we followed a Bayesian approach in order to quantify the relative contribution of the nitrate sources (i.e. soil and fertilizer $NH_4^+$, nitrate-based fertilizers, organic fertilizer and wastewaters) at a given site under different denitrification scenarios. Some of the samples fell outside the mixing polygon formed by the potential nitrate sources in the first scenario that considers a 25% level of denitrification (Fig. 5), which indicates that the other two scenarios at 50% and 75% level are more likely.

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

Furthermore, direct relationships between the proportional contributions of each sources and land use cover were evaluated (Fig. 6). The contribution of soil and fertilizer ammonium positively correlated with the percent cover of agricultural crops (without including greenhouses) at a 75% level of denitrification ($R^2$=0.37), but not significantly ($p$>0.05). Greenhouse cover percentage was also correlated with nitrate-based fertilizer contributions in all scenarios ($R^2$=0.46-0.53, $p$<0.05). There is clearly a direct link between the agricultural practices in the watershed and their contamination inputs to the riverine nitrate. For the other sources, we also found a relationship with land uses or with the drainage area for all scenarios (all cases were significant, $p$<0.05). There was a negative correlation between the drainage area and the contribution of atmospheric deposition, which indicates a potential dilution effect with groundwater water sources in the watershed. At last, but not least, a positive relation of wastewater sources and urban cover suggested a direct link with the WWTPs from the area. Said that, these connections with land use practices were evaluated at the same level of denitrification, which is likely not the case.

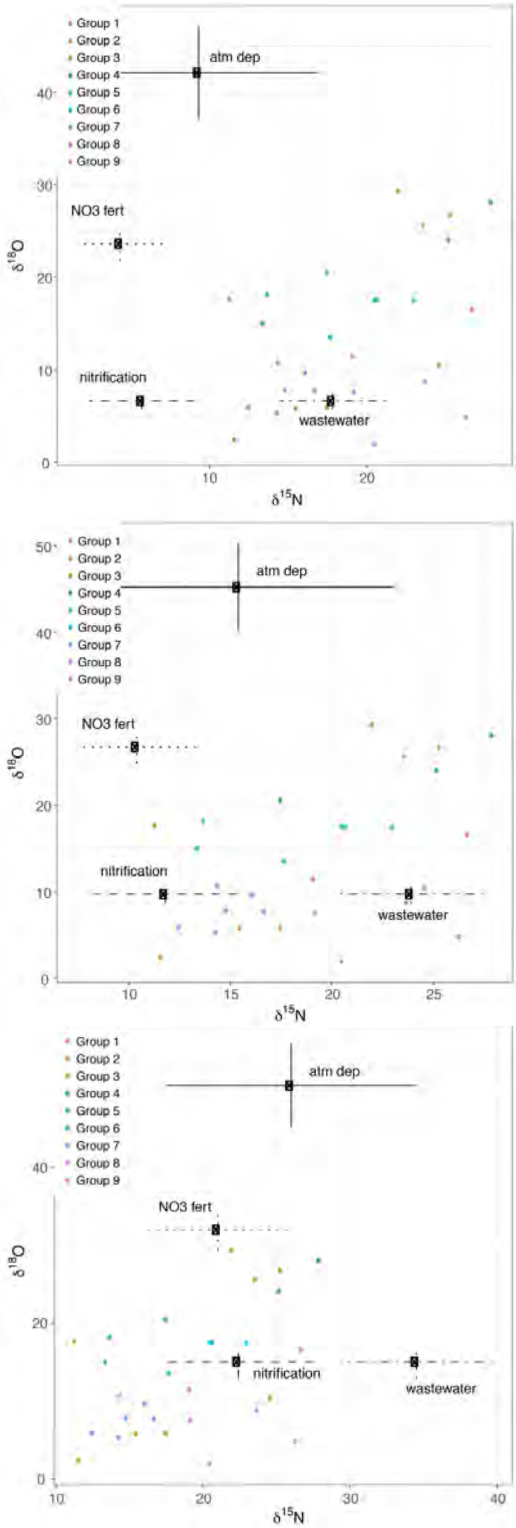


**Figure 5.** Bi-plots of the $\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$ values of primary sources (error bars) and riverine samples (dots) for each site (group) from the Doñana watershed. Source isotope values are corrected for the fractionation associated with denitrification at 25% (top), 50% (medium) and 75% (bottom) levels.

**Table 3**. Proportional contributions of primary sources (soil and fertilizer $NH_4^+$, fertilizer $NO_3^-$, wastewater, and atmospheric deposition) to dissolved nitrate in the Doñana marsh catchment area estimated using a dual isotope Bayesian mixing model. Median (and SD) contribution values are shown for each source.

| | Site | n | Soil and fert $NH_4$ | SD | $NO_3$ fert | SD | Wastewater | SD | Atm Dep | SD |
|---|---|---|---|---|---|---|---|---|---|---|
| 25% denitrified | *WWTP_roc†* | 1 | 0.024 | 0.037 | 0.015 | 0.028 | 0.801 | 0.061 | 0.14 | 0.048 |
| | *WWTP_alm†* | 4 | 0.008 | 0.036 | 0.005 | 0.015 | 0.948 | 0.044 | 0.026 | 0.02 |
| | *PDpal†* | 4 | 0.019 | 0.046 | 0.014 | 0.05 | 0.514 | 0.06 | 0.427 | 0.055 |
| | *PDmim* | 3 | 0.019 | 0.045 | 0.013 | 0.051 | 0.571 | 0.066 | 0.369 | 0.063 |
| | *STca* | 2 | 0.031 | 0.104 | 0.02 | 0.097 | 0.612 | 0.106 | 0.281 | 0.079 |
| | *STmar* | 4 | 0.019 | 0.05 | 0.013 | 0.046 | 0.728 | 0.06 | 0.213 | 0.048 |
| | *STpar2* | 5 | 0.019 | 0.079 | 0.009 | 0.028 | 0.892 | 0.081 | 0.054 | 0.029 |
| | *STpar1* | 5 | 0.006 | 0.022 | 0.003 | 0.01 | 0.967 | 0.029 | 0.016 | 0.014 |
| | *MRSro* | 1 | 0.016 | 0.073 | 0.01 | 0.039 | 0.848 | 0.096 | 0.088 | 0.056 |
| 50% denitrified | *WWTP_roc†* | 1 | 0.15 | 0.11 | 0.04 | 0.05 | 0.7 | 0.104 | 0.08 | 0.04 |
| | *WWTP_alm†* | 4 | 0.09 | 0.13 | 0.01 | 0.03 | 0.86 | 0.131 | 0.02 | 0.02 |
| | *PDpal†* | 4 | 0.11 | 0.11 | 0.04 | 0.15 | 0.44 | 0.105 | 0.35 | 0.1 |
| | *PDmim* | 3 | 0.09 | 0.11 | 0.04 | 0.12 | 0.53 | 0.105 | 0.29 | 0.09 |
| | *STca* | 2 | 0.3 | 0.22 | 0.06 | 0.15 | 0.37 | 0.164 | 0.17 | 0.09 |
| | *STmar* | 4 | 0.14 | 0.15 | 0.04 | 0.09 | 0.63 | 0.128 | 0.14 | 0.07 |
| | *STpar2* | 5 | 0.33 | 0.21 | 0.02 | 0.04 | 0.61 | 0.192 | 0.03 | 0.02 |
| | *STpar1* | 5 | 0.04 | 0.06 | 0.01 | 0.01 | 0.93 | 0.066 | 0.01 | 0.01 |
| | *MRSro* | 1 | 0.12 | 0.18 | 0.03 | 0.06 | 0.74 | 0.176 | 0.05 | 0.05 |
| 75% denitrified | *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 |

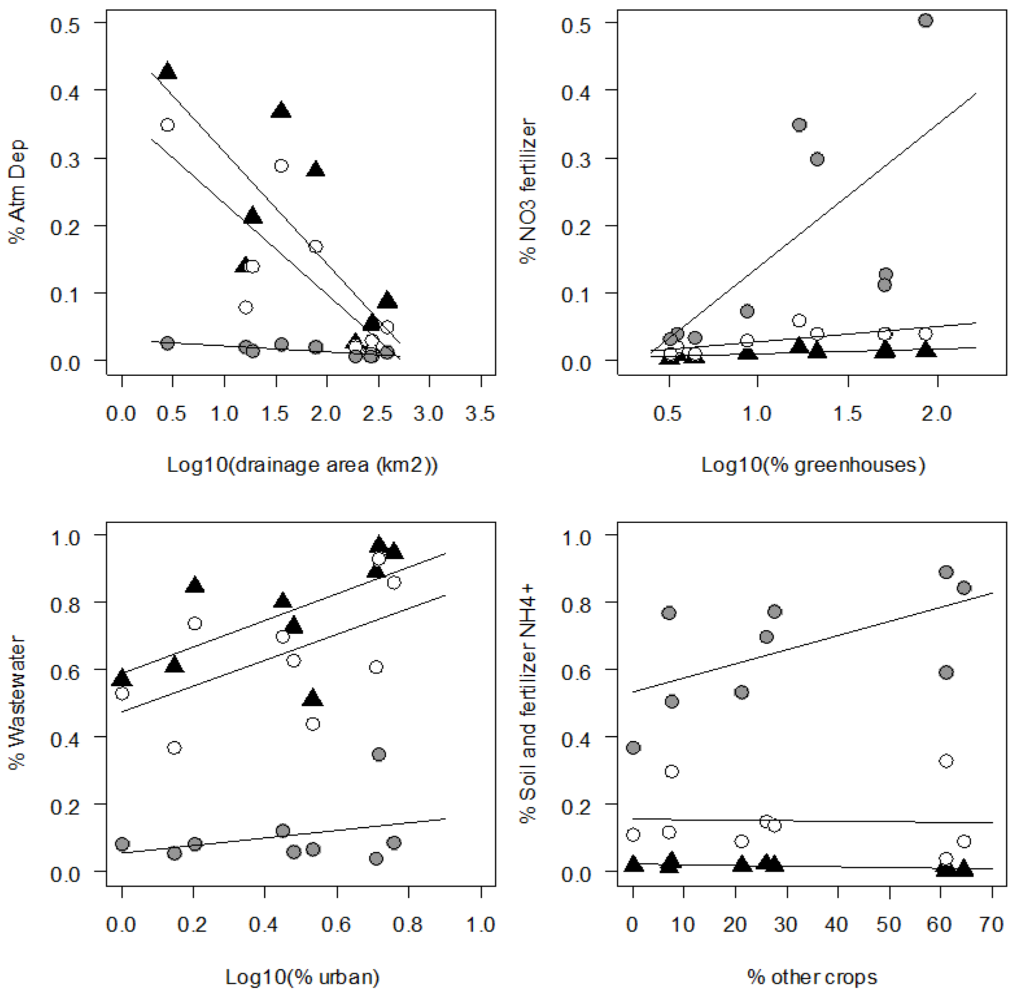


**Figure 6.** Relationships between the estimated proportional contributions of each source at 25% (black triangles), 50% (white circles), and 75% (grey circles) level of denitrification and potential land use parameters (from Table 1).

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

### *Other processes affecting nitrate isotopic composition*

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

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

(2) *Seasonal variations* in climatic conditions (temperature and precipitation) and anthropogenic activities (e.g. agricultural cycles) can strongly influence nitrate inputs into watersheds. Although our sampling was not systematic, we assume that seasonality is an important factor affecting

nitrate transformation and mixing processes in our study area. Our results revealed that most sites showed increasing coupled $\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$ values from winter to spring (Fig. SP2 Sup. Mat.). Similarly, when combining all sites from the watershed, $\delta^{15}N_{NO3}$ isotopic values increased from winter to late spring (February to June) whereas nitrate concentrations decreased (Fig. 3). These results could be partly related to higher denitrifying microbial activity due to gradual temperature increase (Chen et al. 2009). Furthermore, in the Mediterranean area precipitations are often concentrated within a short period of time. Intense precipitations over a short period can cause considerable watershed runoff and rapid transport of nitrate from the agricultural areas, or inputs of atmospherically-derived nitrate, into the receiving streams or ponds, which may result in abrupt changes of $\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$ values (Divers et al., 2014; Soto et al., 2019). In the Doñana watershed the amount of nitrate loading into the stream and the predominant nitrate sources after a particular heavy rain event would be linked to the ongoing specific agricultural activities at that moment, for example, fertigation of berries from December until June or application of manure for agricultural land conditioning during summer months. In contrast, during prolonged periods of scarce precipitations (late spring, summer and early fall) base-flow in streams remains low, or even ceases completely in some cases, whereas WWTP effluents are continuous throughout the year, thus we expect that nitrate isotopic composition downstream of WWTPs would mainly reflect urban wastewaters (Lin et al. 2019). During dry periods the relative contribution of groundwater to the streams may also be important (Custodio et al., 2009). Denitrification may occur in groundwaters due to the infiltration of nitrates from the agricultural practices (Rodríguez and Stefano, 2012), hence stream water would probably reflect a high proportion of $^{15}N_{NO3}$ and $^{18}O_{NO3}$ enriched groundwater. Dry periods are predicted to increase in the Mediterranean region during coming decades lowering the water table (Guardiola et al. 2011; Cramer et al., 2018), and nitrate removing processes such as denitrification could be negatively affected (Manis et al., 2014) increasing the sensitivity of the system to eutrophication.

# Conclusions

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

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**Table SP1**. Reference $\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$ values of nitrate used to compare the isotopic values of our samples. † The expected $\delta^{18}O_{NO3}$ derived from nitrification of $NH_4^+$ (either from soil, manure or fertilizer) was calculated following Eq. 2.

| Nitrate source | $\delta^{15}N_{NO3}$ (‰) | $\delta^{18}O_{NO3}$ (‰) |
|---|---|---|
| Synthetic fertilizer ($NO_3^-$) | -5 to +5 | +18 to +25 |
| Synthetic fertilizer ($NH_4^+$) | -5 to +5 | +3 to +5.9† |
| Soil $NO_3^-$ | +3 to +7.8 | +3 to +5.9† |
| Organic fertilizer/sewage | +7 to +20 | +3 to +5.9† |

**Table SP2.** Results of $\delta^2H_{H2O}$ and $\delta^{18}O_{H2O}$ values (‰) measured in the water samples collected in nine different sampling sites between Feb-Jun in 2015 and 2016 in the Doñana watershed.

| Code | Sampling date | $\delta^2H_{H2O}$ (‰) | $\delta^{18}O_{H2O}$ (‰) |
|---|---|---|---|
| WWTP_roc | 06/05/2015 | -24.4 | -4.2 |
| WWTP_alm | 18/02/2016 | -25.6 | -4.8 |
| WWTP_alm | 20/04/2016 | -26.9 | -5.4 |
| WWTP_alm | 24/05/2016 | -29.5 | -5.2 |
| WWTP_alm | 22/06/2016 | -24.7 | -4.5 |
| PDpal | 15/03/2016 | -19.7 | -3.5 |
| PDpal | 20/04/2016 | -30.3 | -5.6 |
| PDpal | 24/05/2016 | -19.6 | -2.7 |
| PDpal | 22/06/2016 | -18.1 | -3.0 |
| PDmim | 11/05/2015 | -26.3 | -4.6 |
| PDmim | 18/02/2016 | -32.7 | -6.56 |
| PDmim | 26/04/2016 | -17.7 | -3.7 |
| STca | 06/05/2015 | -32.6 | -6.0 |
| STca | 25/02/2016 | -30.0 | -5.9 |
| STmar | 06/05/2015 | -26.1 | -4.4 |
| STmar | 16/03/2016 | -24.6 | -4.8 |
| STmar | 10/04/2016 | -24.7 | -4.8 |
| STmar | 25/05/2016 | -26.7 | -5.1 |
| STpar2 | 06/05/2015 | -24.1 | -3.8 |
| STpar2 | 25/02/2016 | -27.4 | -5.1 |
| STpar2 | 16/03/2016 | -24.6 | -4.6 |
| STpar2 | 10/04/2016 | -19.3 | -3.8 |
| STpar2 | 25/05/2016 | -25.1 | -4.7 |
| STpar1 | 06/05/2015 | -24.1 | -3.8 |
| STpar1 | 10/04/2016 | -22.1 | -4.2 |
| STpar1 | 21/04/2016 | -21.9 | -5.0 |
| STpar1 | 25/05/2016 | -26.7 | -4.9 |
| STpar1 | 21/06/2016 | -21.8 | -4.0 |
| MRSro | 24/05/2016 | na | na |

**Table SP3.** Precipitation (mm) and evapotranspiration (mm) corresponding to each sampling event. PDpal is not included in this table because precipitation and evapotranspiration data might not be accurate as this site is located further away from the Almonte Meteorological Station than the other sampling sites.

| Code | Sampling date | Precipitation (mm) | Accumulated precipitation previous 5 days (mm) | Evapotranspiration (mm) | Average evapotranspiration in the previous 5 days (mm) | SD |
|---|---|---|---|---|---|---|
| WWTP_roc | 06/05/2015 | 0.0 | 0.4 | 5.08 | 4.48 | 1.09 |
| WWTP_alm | 18/02/2016 | **11.6** | 0.8 | 1.41 | 2.29 | 0.55 |
| WWTP_alm | 20/04/2016 | **15.4** | **28.2** | 2.75 | 2.85 | 0.96 |
| WWTP_alm | 24/05/2016 | 0.0 | 0.0 | 5.06 | 5.51 | 0.17 |
| WWTP_alm | 22/06/2016 | 0.0 | 0.0 | 8.62 | 7.49 | 1.02 |
| STmar | 06/05/2015 | 0.0 | 0.4 | 5.08 | 4.48 | 1.09 |
| STmar | 16/03/2016 | 0.4 | 0.8 | 3.20 | 2.61 | 0.25 |
| STmar | 10/04/2016 | 0.0 | 0.0 | 2.74 | 4.19 | 0.22 |
| STmar | 25/05/2016 | 0.0 | 0.0 | 4.85 | 5.48 | 0.25 |
| PDmim | 11/05/2015 | 0.0 | 0.0 | 5.76 | 5.18 | 0.71 |
| PDmim | 18/02/2016 | **11.6** | 0.8 | 1.41 | 2.29 | 0.55 |
| PDmim | 26/04/2016 | 0.0 | 0.6 | 3.89 | 3.62 | 0.49 |
| STca | 06/05/2015 | 0.0 | 0.4 | 5.08 | 4.48 | 1.09 |
| STca | 25/02/2016 | 0.0 | 5.4 | 1.98 | 2.14 | 0.20 |
| STpar2 | 06/05/2015 | 0.0 | 0.4 | 5.08 | 4.48 | 1.09 |
| STpar2 | 25/02/2016 | 0.0 | 5.4 | 1.98 | 2.14 | 0.20 |
| STpar2 | 16/03/2016 | 0.4 | 0.8 | 3.20 | 2.61 | 0.25 |
| STpar2 | 10/04/2016 | 0.0 | 0.0 | 2.74 | 4.19 | 0.22 |
| STpar2 | 25/05/2016 | 0.0 | 0.0 | 4.85 | 5.48 | 0.25 |
| STpar1 | 06/05/2015 | 0.0 | 0.4 | 5.08 | 4.48 | 1.09 |
| STpar1 | 10/04/2016 | 0.0 | 0.0 | 2.74 | 4.19 | 0.22 |
| STpar1 | 21/04/2016 | 0.2 | **35.2** | 3.00 | 2.81 | 0.96 |
| STpar1 | 25/05/2016 | 0.0 | 0.0 | 4.85 | 5.48 | 0.25 |
| STpar1 | 21/06/2016 | 0.0 | 0.0 | 8.34 | 6.92 | 1.19 |
| MRSro | 24/05/2016 | 0.0 | 0.0 | 5.06 | 5.51 | 0.17 |

**Table SP4.** Ammonium ($NH_4^+$) and nitrite ($NO_2^-$) concentrations (mg $L^{-1}$).

| Code | Sampling date | $NH_4^+$ (mg $L^{-1}$) | $NO_2^-$ (mg $L^{-1}$) |
|---|---|---|---|
| WWTP_roc | 06/05/2015 | 0.05 | 1.5 |
| WWTP_alm | 18/02/2016 | 0.6 | 1 |
| WWTP_alm | 20/04/2016 | 0.8 | 0.4 |
| WWTP_alm | 24/05/2016 | 1.5 | 0.4 |
| WWTP_alm | 22/06/2016 | 8.6 | 0.7 |
| PDpal | 15/03/2016 | 0.01 | 1.8 |
| PDpal | 20/04/2016 | 0.7 | 0.9 |
| PDpal | 24/05/2016 | 1.6 | 2.9 |
| PDpal | 22/06/2016 | 1.2 | 0.5 |
| PDmim | 11/05/2015 | 0.5 | 1.5 |
| PDmim | 18/02/2016 | 0.1 | 0.6 |
| PDmim | 26/04/2016 | 0.5 | 3.4 |
| STca | 06/05/2015 | 0.3 | 0.7 |
| STca | 25/02/2016 | 0.2 | 0.5 |
| STmar | 06/05/2015 | 0.3 | 0.8 |
| STmar | 16/03/2016 | 2.2 | 2.5 |
| STmar | 10/04/2016 | 2.1 | 1.5 |
| STmar | 25/05/2016 | 5.5 | 2.3 |
| STpar2 | 06/05/2015 | 4.1 | 2.4 |
| STpar2 | 25/02/2016 | 0.1 | 0.2 |
| STpar2 | 16/03/2016 | 0.04 | 1.5 |
| STpar2 | 10/04/2016 | 0.01 | 0.3 |
| STpar2 | 25/05/2016 | 0.03 | 0.1 |
| STpar1 | 06/05/2015 | 7.6 | 1.3 |
| STpar1 | 10/04/2016 | 0.04 | 0.1 |
| STpar1 | 21/04/2016 | 0.02 | 0.4 |
| STpar1 | 25/05/2016 | 0.1 | 0.4 |
| STpar1 | 21/06/2016 | 0.2 | 1.2 |
| MRSro | 24/05/2016 | 0.2 | 0.5 |

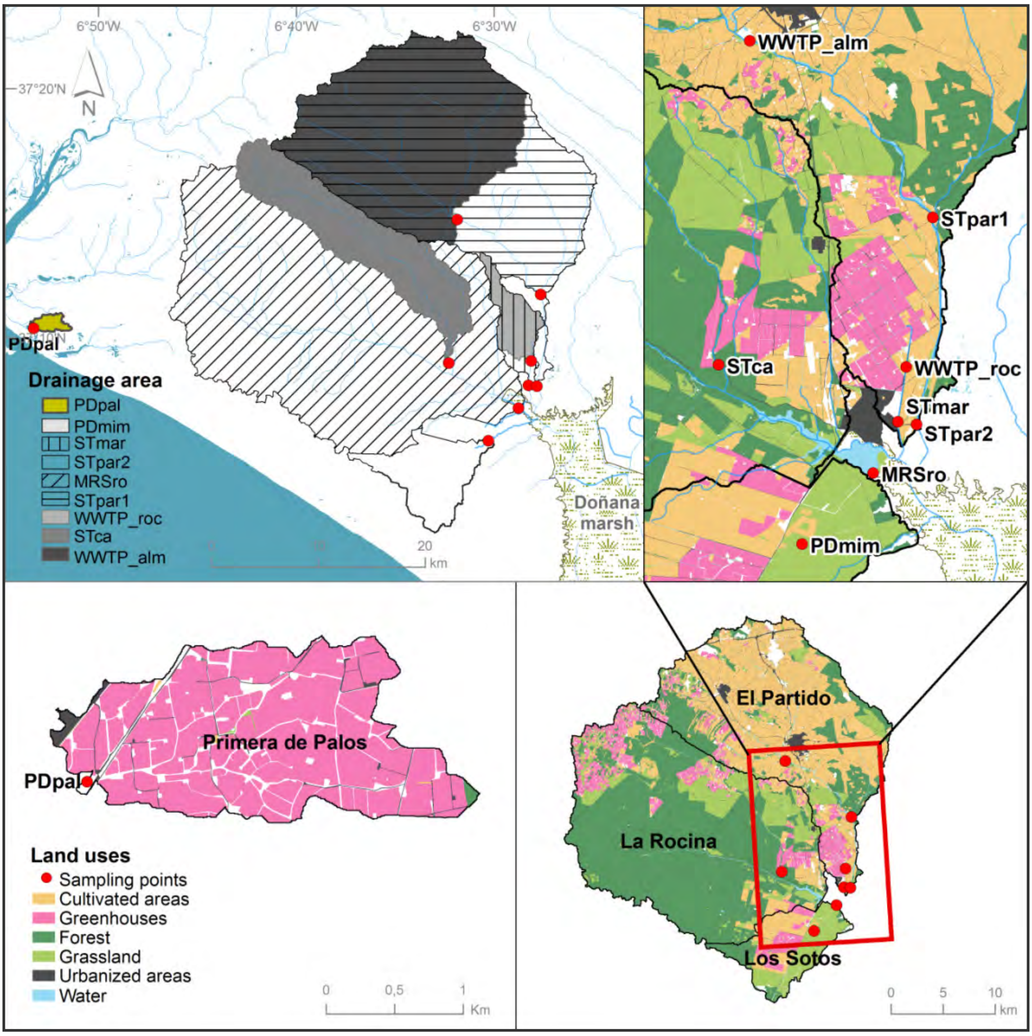


**Figure SP1.** Distribution of different land uses within each sampling site's watershed (red points correspond to the sampling sites). Categories were created based on land use data (2017) from the Spanish Land Parcel Information System (SIGPAC): cultivated areas (yellow), greenhouse crops (pink), forest (dark green), grassland (light green), urban areas (grey) and water (blue). We used the Digital Surface Model (DSM) of 5m resolution to obtain the specific catchment areas for each point. Sources:

Land use data: https://descargasrediam.cica.es/repo/s/RUR

DSM: http://centrodedescargas.cnig.es/CentroDescargas/catalogo.do?Serie=LIDAR

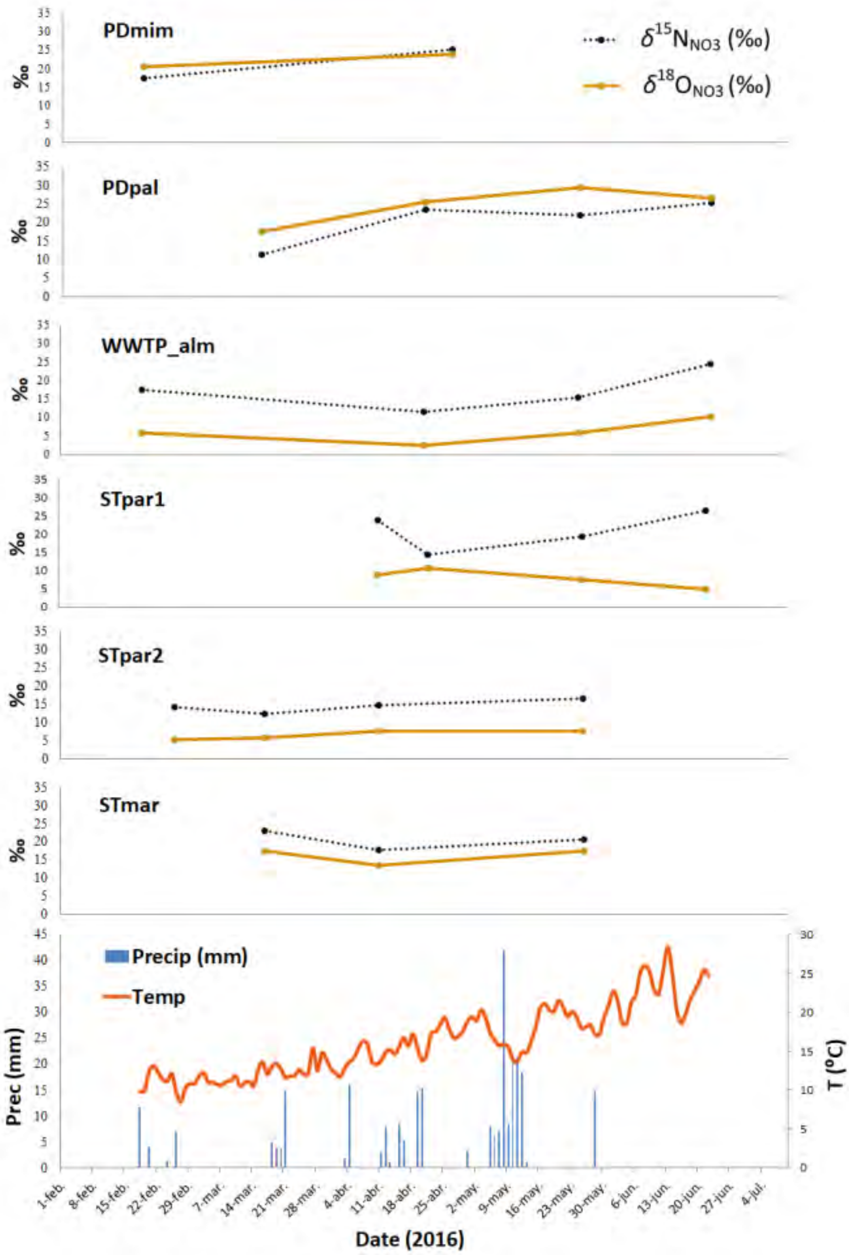


**Figure SP2.** Seasonal variation of $\delta^{15}N_{NO3}$ (dotted line) and $\delta^{18}O_{NO3}$ (solid line) in different sampling sites in 2016. The dark orange line represents daily average temperature (°C) and blue bars total daily precipitation (mm).

# Discusión general

## Pasado, presente y futuro de la eutrofización en Doñana

Teniendo en cuenta los conocimientos previos y aquellos generados en esta tesis, podemos afirmar que existe una tendencia al alza en el proceso de eutrofización en la marisma de Doñana y sus arroyos vertientes (Novo 1994; Arambarri et al. 1996; Serrano et al. 2006; Espinar et al. 2015). Sin embargo, la escasa disponibilidad de datos históricos sobre la calidad de las aguas superficiales y la baja resolución espacio-temporal de los mismos dificultan la posibilidad de realizar un análisis detallado del proceso de eutrofización. Además, hasta la fecha se dispone de muy poca información sobre el origen específico de los nutrientes. En este contexto, la presente tesis contribuye a aumentar el conocimiento sobre los patrones espacio-temporales en las concentraciones de nutrientes y a identificar el origen de la eutrofización, tanto en la marisma del Parque Nacional como en los arroyos de las cuencas vertientes. Desde un punto de vista de la gestión, esto último es esencial a la hora de establecer medidas de gestión adecuadas.

Respecto a la situación actual de la eutrofización, los resultados de esta tesis confirman que la marisma presenta valores de concentraciones de nutrientes consistentemente más bajos que en las aguas superficiales de los arroyos entrantes. Esto no quiere decir que en la marisma no exista un problema de eutrofización (Espinar et al. 2015), sino que el estado del mismo es menor, aunque no menos preocupante, que en los arroyos. En ciertos arroyos se observó un alto porcentaje de muestras con concentraciones de nutrientes que superaban niveles umbrales a partir de los cuales muchas especies acuáticas podrían verse negativamente afectadas (**Capítulo 1**). Aunque en menor medida, estos niveles umbrales también se llegaron a superar en las zonas de entrada de los arroyos a la marisma. Con los resultados isotópicos de los **Capítulos 2 y 3** confirmamos además que el nitrógeno en los arroyos y las entradas a la marisma tiene un origen principalmente antrópico (fertilizantes y aguas residuales urbanas), mientras que en el interior la marisma parece tener un origen más natural, proveniente del propio ecosistema, aunque sería necesario un estudio más detallado para confirmarlo.

Sobre la evolución futura del proceso de eutrofización en la marisma de Doñana y sus cuencas vertientes, cabe esperar que siga en aumento si no se toman medidas adecuadas y urgentes para reducir el impacto de las presiones humanas y la competencia por los recursos hídricos. Además, en un contexto de cambio climático, donde las tendencias apuntan a una disminución de las precipitaciones y aumento de las temperaturas en la región mediterránea, se espera que el efecto sinérgico con las presiones antrópicas continúe acentuando los procesos locales de eutrofización (Green et al. 2017). En este sentido, la reducción y control de la eutrofización en este espacio natural requeriría, por un lado de la implementación de un programa de monitoreo sostenible a largo plazo acompañado de una adecuada gestión ambiental y de los recursos hídricos que además tenga en cuenta los impactos potenciales del cambio climático. En el último apartado de esta discusión se proponen una serie de **medidas específicas** que podrían contribuir notablemente a reducir el proceso de eutrofización y mejorar la calidad de las aguas superficiales en la marisma de Doñana y sus cuencas.

## La importancia de lo antrópico, lo hidrológico y lo biogeoquímico para entender el problema de la eutrofización en Doñana

Para poder proponer medidas adecuadas que den solución al problema de la eutrofización en Doñana es necesario contar, entre otras cosas, con un **conocimiento más profundo sobre los principales factores** que modulan la carga y transformación de los nutrientes en los ecosistemas acuáticos. En esta tesis agrupamos los principales factores potenciales en los siguientes tres grupos: **antrópicos, hidrológicos y biogeoquímicos**.

### *Antrópico*

Durante el último siglo, el intenso desarrollo de la agricultura, las aguas residuales urbanas pobremente tratadas, las innumerables modificaciones en la hidrología y la inadecuada gestión ambiental y de los recursos hídricos han sido los principales motores del aumento de la contaminación difusa y puntual por nutrientes en las aguas superficiales y subterráneas de Doñana y su entorno (Custodio et al. 2009; Serrano et al. 2006; Zorrilla-Miras et al. 2014; Paredes et al. 2019, 2020). En el **Capítulo 1** nuestros resultados muestran una clara diferencia espacial en las concentraciones de nutrientes entre los arroyos y la marisma, siendo más altas y heterogéneas en los arroyos. Esta segregación espacial refleja la fuerte polarización en cuanto a protección ambiental entre la marisma del Parque Nacional (elevado grado de protección) y las cuencas vertientes (arroyos, bajo grado de protección). Desde 1969 en adelante y de manera progresiva, se crearon el Parque Nacional inicialmente, y el resto de figuras de protección posteriores (ej. Reserva de la Biosfera, Parque Natural, Espacio Natural de Doñana) con el objetivo de frenar la degradación ecológica causada por la intensificación de la agricultura y otras actividades humanas. Dentro de la zona núcleo de mayor protección (Parque Nacional) las actividades socioeconómicas quedaron limitadas principalmente a la ganadería y, en menor medida, al turismo de naturaleza. Mientras, la mayor parte de las actividades humanas (agricultura, turismo, zonas urbanas) se iban concentrando en las zonas colindantes al Parque Nacional, donde las restricciones eran menores (Zorrillas-Miras et al. 2014). Desde 2013, las cuencas vertiente a la marisma de Doñana forman parte de la zona de transición de la Reserva de la Biosfera Doñana (UNESCO). El objetivo de esta figura de protección es "*armonizar la conservación de la diversidad biológica y cultural y el desarrollo económico y social a través de la relación de las personas con la naturaleza*". El desarrollo de un modelo socioeconómico sostenible en Doñana, que fomente prácticas locales apropiadas, es fundamental para asegurar la conservación de los espacios naturales, no solo los más protegidos, sino también todos aquellos que forman parte del entorno de Doñana. Sin embargo, la Reserva de la

Biosfera no parece estar teniendo un impacto evidente sobre el desarrollo de una agricultura más sostenible en las cuencas, al menos a corto plazo, pues según nuestros resultados sobre la evolución de cultivos bajo plástico (**Capítulo 1**), la agricultura ha seguido expandiéndose en estos últimos años, y con ella la contaminación difusa por nutrientes. A esto hay que añadirle el aumento de la población en la región en las últimas décadas (INE 2019) y el consecuente aumento de aguas residuales, tanto tratadas en estaciones depuradoras como vertidas directamente (asentamientos sin sistemas de depuración), cuyo impacto podría reducirse si se aplicasen los tratamiento adecuados (ej. terciario) o se regulasen los vertidos ilegales mediante fosas sépticas u otros sistemas de depuración de aguas. En este sentido, determinar en detalle el origen de la contaminación y su variación espacio-temporal es un factor clave a la hora de proponer soluciones al problema de la eutrofización en Doñana. Como se ha demostrado en esta tesis, la aplicación de determinados marcadores como los isótopos estables (**Capítulos 2 y 3**), pueden arrojar bastante luz a la hora de distinguir entre las distintas fuentes contaminantes, contribuyendo así a incrementar la información sobre los aspectos cualitativos del agua.

### *Hidrológico*

En los sistemas acuáticos mediterráneos la hidrología está muy condicionada por las fuertes variaciones estacionales e interanuales tanto en las precipitaciones como en la carga y descarga de las aguas subterráneas (Alvarez-Cobelas et al. 2005; Díaz-Delgado et al. 2016; Costigan et al. 2017). Del mismo modo, la carga, retención y transformación de los nutrientes en estos sistemas depende en gran medida de la dinámica hidrológica y la conectividad del sistema (Sánchez-Carrillo et al. 2001; Bernal et al. 2013). En Doñana, además de las variaciones naturales mencionadas, la extracción de agua para regadío en las cuencas tiene un impacto muy importante en la hidrología de la marisma y los arroyos, haciendo que los ciclos de sequía sean cada vez más prolongados y pronunciados (Custodio et al. 2009; Guardiola et al. 2011; Ramos-Fuertes 2012; Green et al. 2018).

En el **Capítulo 1** observamos una mayor heterogeneidad en las concentraciones de nutrientes en los arroyos con respecto a la marisma. Una de las posibles causas que puede explicar estas diferencias es la propia heterogeneidad en la hidrogeología de los diferentes arroyos en relación a la marisma. Entre las cuencas estudiadas en esta tesis, en la cuenca del Partido el mayor porcentaje de su superficie esté dedicada a la agricultura (**Capítulo 3**) y los bosques de ribera estén muy degradados (Withers et al. 2007, Bernal et al. 2013), haciendo que el arrastre de nutrientes por **escorrentía superficial** sea más relevante que en otras cuencas, especialmente durante los eventos de lluvias torrenciales. Por el contrario, los arroyos de la Rocina y el río Guadiamar mostraron concentraciones de nutrientes más bajos, probablemente favorecidas por contar con bosques de ribera en mejor estado de conservación (i.e. Corredor Verde del Guadiamar y Zona de Protección de la Rocina), que contribuyen a una mayor retención, transformación y eliminación de los nutrientes (Pinay et al. 2018).

Las concentraciones de nutrientes en las aguas superficiales no solo dependen de la cantidad total de nutrientes importada desde las zonas terrestres, sino también de la **capacidad de dilución de los ecosistemas acuáticos receptores** (Elósegui et al. 1995; Bernal et al. 2013). Los resultados de caudales puntuales que se recogen en el **Capítulo 1** concuerdan con las escasas medidas fiables de caudal registradas durante las últimas décadas, donde también se muestran valores superiores en el Partido frente a la Rocina (Custodio et al. 2009). En este sentido, cabría esperar una mayor capacidad de dilución en el arroyo del Partido frente a la Rocina. Sin embargo, las concentraciones de nutrientes observadas son en general superiores en el Partido en relación a la Rocina. Una posible explicación es que los arroyos de la cuenca del Partido reciben, a diferencia de en la Rocina, aportes continuos de agua y nutrientes de estaciones depuradoras. Esto puede implicar además, que durante las épocas más secas, la proporción del caudal derivada de los aportes de las depuradoras pueden llegar a ser incluso dominantes frente a los escasos o nulos caudales naturales, y que por ello las concentraciones de nutrientes sean relativamente más altas aguas abajo de las depuradoras (Marti et al. 2004; Brooks et al. 2006).

En relación a la hidrología y la carga de nutrientes en la marisma, en el **Capítulo 1** encontramos que las variables relacionadas con procesos de evaporación (i.e. $\delta^2H$) y nivel del agua (i.e. profundidad de la columna de agua) mostraron un efecto positivo y negativo, respectivamente, con las concentraciones de nutrientes en la columna de agua. Estos resultados concuerdan con otros trabajos en los que los procesos de evaporación se relacionan con una disminución en la profundidad de la columna de agua y un incremento en los valores de $\delta^2H$ y concentración de solutos (Fellman et al., 2011; Gat, 2010). Sin embargo, a pesar de que también esperábamos que la conductividad tuviera un efecto positivo con la concentración de nutrientes como consecuencia de los procesos de evaporación, nos encontramos que el efecto era negativo. Como explicamos en el **Capítulo 1**, creemos que este resultado no es casual, sino que podría estar relacionado con que a medida que el agua se mueve desde las entradas de los arroyos hacia el sureste de la marisma, la concentración de nutrientes en la columna de agua disminuye (el agua se va "purificando" por el efecto filtrador de la marisma) mientras que la conductividad aumenta a medida que se va evaporando.

### *Biogeoquímico*

Los procesos biogeoquímicos juegan un papel fundamental en el transporte, transformación y retención de los nutrientes en los ecosistemas acuáticos. En el contexto de Doñana estos procesos son de vital importancia porque pueden ayudar a amortiguar el impacto de la eutrofización, siempre y cuando la carga de nutrientes no exceda la capacidad del sistema (Marti et al. 2004). Los resultados isotópicos en el **Capítulo 3** muestran que la **desnitrificación** parece ser un proceso predominante en los puntos de arroyos y lagunas muestreados. Estimamos que este proceso podría estar eliminando hasta un 60% del nitrato disuelto en algunos puntos de los arroyos y hasta un 80% en las lagunas. Si tenemos en cuenta que además los mayores porcentajes se observaron en las zonas dedicadas principalmente a la agricultura intensiva, estos resultados estarían indicando que procesos naturales como la desnitrificación podrían estar subestimando la cantidad de nitratos real que reciben los arroyos a causa de la contaminación difusa por la agricultura. Aunque en esta tesis no se aborda, en un futuro sería importante determinar si la desnitrificación tiene lugar dentro de los propios sistemas acuáticos y/o fuera, y qué importancia relativa tendrían cada una de esas zonas en la eliminación de nitratos.

Estudios previos mostraron que existe actividad desnitrificante en los sedimentos del arroyo de la Rocina (Tortosa et al. 2011) y que probablemente la desnitrificación también podría estar ocurriendo en las aguas subterráneas de la zona de ribera (Sebilo et al., 2003; Griffiths et al., 2016) o incluso en las aguas subterráneas a mayor profundidad que reciben nitratos provenientes del uso intensivo de fertilizantes en la agricultura (Kim et al., 2015; Otero et al., 2009). Además de la desnitrificación, sería necesario que estudios futuros incluyeran el análisis de otros procesos biogeoquímicos relevantes (ej. nitrificación, amonificación, adsorción/liberación de fósforo). También sería imprescindible **incrementar la resolución espacio-temporal** de los datos para poder comprender mejor la variabilidad de los procesos biogeoquímicos, dado que el sistema estudiado, y en general los sistemas acuáticos intermitentes, se caracterizan por una extrema dinámica hidrológica e importantes diferencias estacionales (Hernández & Mitsch 2007; Costigan et al. 2017; Frei et al. 2020).

Refiriéndonos a las diferencias estacionales cabría esperar que, durante los periodos más cálidos y secos, cuando aumentan la actividad microbiana y producción primaria, hubiera una mayor reducción de nutrientes disueltos en la columna de agua en comparación a los periodos más fríos y húmedos (White et al. 1991; Pinay et al. 2007). Sin embargo, nuestros resultados no mostraron ningún patrón estacional claro en cuanto a la concentración de nutrientes (**Capítulo 1**). En relación a la marisma, las concentraciones de nutrientes disueltos apenas variaron estacionalmente y los valores se mantuvieron en niveles inferiores a los arroyos. Estos resultados sugieren que la marisma, al igual que la mayoría de humedales, posee una capacidad autorreguladora importante. Una de las posibles explicaciones sea la alta densidad de plantas macrófitas, principalmente emergentes, que crecen anualmente durante el comienzo de la primavera por toda la extensión de la marisma (Espinar, 2004, Reina et al. 2006). Las macrófitas son capaces de retirar grandes cantidades de nutrientes, tanto del agua como de los sedimentos, para convertirlos en biomasa (Levi et al. 2015). A esto habría que sumarle el efecto los procesos biogeoquímicos que ocurren en los sedimentos (ej. desnitrificación, adsorción de fósforo) que tienen tanta o incluso más importancia que las macrófitas a la hora de eliminar los nutrientes de la columna de agua (Golterman, 1995; Golterman, 2004). El por qué no vemos precisamente una reducción importante en la concentración de los nutrientes durante la época de crecimiento de las macrófitas podría ser porque coincide con la época en la que los procesos de evaporación son más pronunciados (a partir de marzo) y hacen que la columna de agua se reduzca y los solutos (ej. nutrientes) se concentren (Ramos-Fuertes 2012). En relación a los arroyos observamos una mayor heterogeneidad en las concentraciones de nutrientes con respecto a la marisma (**Capítulo 1**). Esto podría estar indicando que debido a las diferencias hidrológicas entre los arroyos, la tasa de eliminación de nutrientes mediante procesos biogeoquímicos sea muy variable ya que estos están muy influenciados por las condiciones hidrológicas en cada momento (Bernal et al. 2013; Gómez et al. 2017).

## Valoración de las aproximaciones isotópicas en el estudio de la contaminación por nitrógeno en aguas superficiales de Doñana

En esta tesis hemos usado los isótopos estables del N y O como indicadores ecológicos para diagnosticar e interpretar el problema de la contaminación por N en las aguas superficiales de la marisma de Doñana y arroyos vertientes. A pesar de ser una herramienta ampliamente validada a nivel mundial (Kendall et al. 2010), nunca antes se había aplicado de forma sistemática y a escala de cuenca para estudiar la calidad de las aguas superficiales de Doñana y su entorno. Los únicos estudios previos similares están centrados en el acuífero de Doñana, donde los isótopos estables se utilizaron para obtener información sobre la hidrología, los procesos de transformación y la contaminación por nitratos en las aguas subterráneas (Manzano et al. 2009; Higueras, 2014).

En esta tesis, hemos podido obtener información espacial muy valiosa sobre la variabilidad de la composición isotópica del N ($\delta^{15}N$) en plantas helófitas con relativamente poco esfuerzo de muestreo y bajo coste (**Capítulo 2**). Además, en humedales temporales como Doñana, donde los periodos de sequía son cada vez más prolongados, usar helófitas como indicadores es una ventaja adicional porque no se requiere de agua superficial en el momento del muestreo. Aunque en nuestro estudio solo hemos usado dos especies de helófitas (*Bolboschoenus maritimus* y *Typha domingensis*), sería interesante determinar qué otras especies de plantas acuáticas abundantes en la zona podrían ser más o menos adecuadas como indicadoras de cambios en la contaminación antrópica a corto, medio y largo plazo, ya que según estudios previos la composición isotópica del N en una planta ($\delta^{15}N$) puede variar en función de la fuente de N y su concentración, de la especie de planta y del mecanismo de asimilación de N (ej. a través de raíces u hojas) (Bannon & Roman, 2008; Serrano, 2015; Kohzu et al. 2008). En este sentido es importante que la información isotópica vaya acompañada de información adicional sobre el sistema, como las concentraciones de N y usos del suelo (**Capítulo 2**), y también sobre la propia planta (ej. fraccionamiento isotópico). Sin embargo, debido al solapamiento existente entre los valores de $\delta^{15}N$ provenientes de las diferentes fuentes potenciales y procesos biogeoquímicos en el sistema, no fue posible determinar con exactitud el origen del N medido en las helófitas. La medición adicional de los isótopos de oxígeno ($\delta^{18}O$) permite distinguir con mayor resolución de qué fuentes de N y procesos de transformación (ej. desnitrificación) se deriva la firma isotópica de un determinado indicador (ej. nitratos disueltos). En este sentido, los análisis isotópicos de N y O en nitratos disueltos del **Capítulo 3** aportan una información muy valiosa al estudio de la eutrofización en el entorno de Doñana, pues confirman que los arroyos y lagunas estudiadas reciben contaminación por nitrógeno de múltiples fuentes antrópicas (ej. fertilizantes inorgánicos, aguas residuales), y que los procesos de desnitrificación parecen estar teniendo un papel clave en la eliminación natural de los nitratos. Estos resultados corroboran la utilidad de las aproximaciones multisotópicas a la hora de determinar con mayor precisión el origen de los nutrientes en un sistema acuático (Gooddy et al. 2016; Biddau et al. 2019). Además, tanto nuestros resultados como estudios previos sugieren que esta herramienta es muy útil a la hora de determinar de qué fuentes proviene la contaminación en aquellos momentos del año donde se producen los mayores "pulsos" de aporte de N a los arroyos y la marisma, como pueden ser lluvias torrenciales, vertidos puntuales, excedentes de riego durante la época de producción agrícola o descargas de aguas subterráneas contaminadas durante momentos de bajo caudal. Desde el punto de vista de la gestión, la información tanto del estudio isotópico con helófitas como con nitratos puede resultar de gran utilidad a la hora de diseñar programas de seguimiento de la calidad de las aguas superficiales en el entorno de Doñana y dentro de la marisma.

## Perspectivas futuras y recomendaciones

*"El calentamiento del sistema climático es inequívoco, existiendo una clara influencia humana en su evolución. Muchos aspectos del cambio climático y los impactos asociados continuarán durante siglos, incluso si se detienen totalmente las emisiones antropogénicas de gases de efecto invernadero"* (Fig. 11; IPCC 2016)

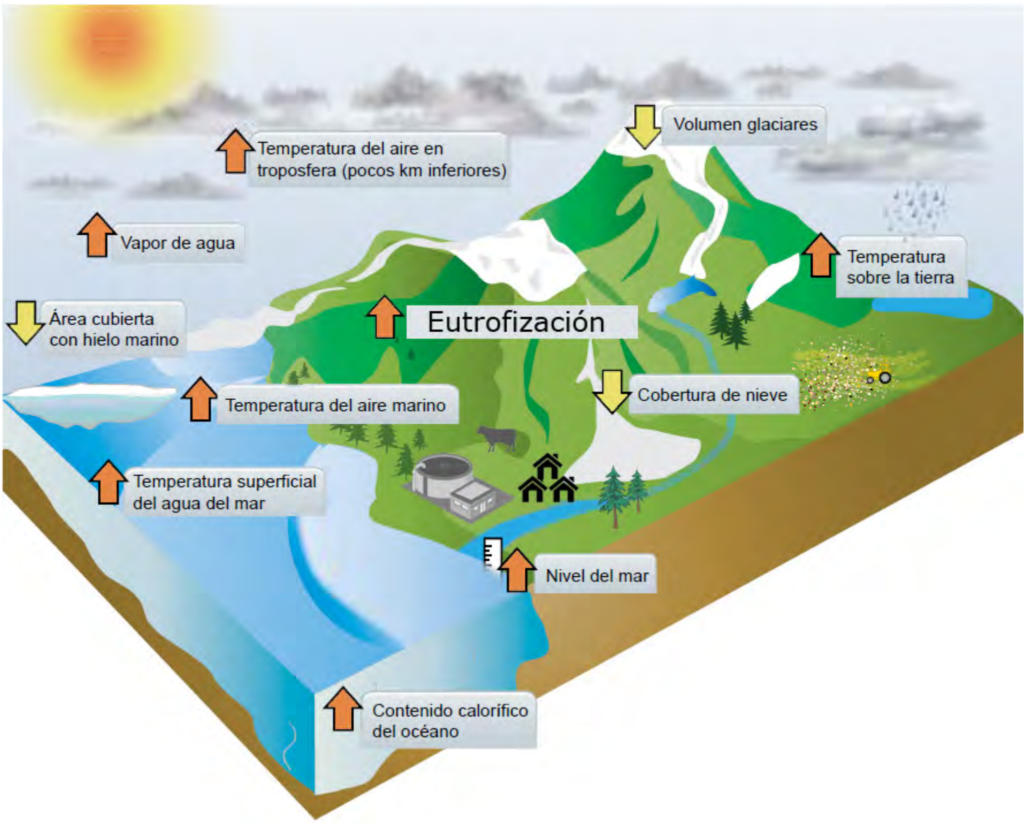


**Figura 11.** Efectos del calentamiento global sobre la Tierra (las flechas indican el sentido del cambio) (Figura adaptada de IPCC 2016).

Estamos viviendo una crisis climática en la que muchos de los cambios observados desde mediados del siglo XX no tienen precedentes en décadas ni milenios (IPCC 2016). En la cuenca del Mediterráneo se prevé que el cambio climático tenga un impacto negativo cada vez mayor sobre los humedales y sus servicios ecosistémicos, debido principalmente al aumento de las temperaturas, descenso de las precipitaciones, mayor frecuencia de eventos climáticos extremos (sequías e inundaciones) y aumento del nivel del mar (MWO2, 2018). En la Península Ibérica concretamente, el efecto del cambio climático ya ha provocado en las últimas décadas un descenso generalizado de los caudales de los ríos y una disminución en la tasa de recarga de los acuíferos (Estrela et al. 2012). Las sinergias que se crean entre estos vectores del cambio climático y las actividades humanas agudizan aún más los impactos negativos del incremento de la eutrofización en los ecosistemas acuáticos, afectando severamente tanto a la calidad como la cantidad de agua y la biodiversidad asociada (Ansari et al. 2011).

En este contexto, la elaboración de estrategias de adaptación al cambio climático en humedales mediterráneos son clave para hacer frente a los desafíos climáticos a corto, medio y largo plazo, tanto para preservar el buen estado ecológico y la calidad de las aguas así como por su papel amortiguador frente al cambio climático (ej. almacenan el exceso de lluvia evitando inundaciones, funcionan como importantes almacenes de carbono; Mitsch et al. 2013) y por sus servicios ecosistémicos y el valor ecológico que sustenta (ej. en el caso de Doñana, como zona de cría o invernada para un enorme variedad de aves acuáticas). De hecho, en el Acuerdo de París de 2016 se determinó que a partir de 2026 los humedales serán incluidos en la contabilidad de emisiones y absorciones de gases de efecto invernadero a escala nacional y europea, por lo que su gestión tendrá un papel clave en el cumplimiento de los objetivos de reducción de emisiones a nivel global (Reglamento (UE) 2018/841 del Parlamento Europeo y del Consejo, de 30 de mayo de 2018). En muchos humedales mediterráneos ya se han implantado con éxito diferentes estrategias de adaptación, tanto de tipo estructural (ej. restauración funcional de ecosistemas, mejorar la conectividad hidrológica) como preventivas (incrementar el conocimiento, sistemas de alerta temprana, ordenación del territorio). Podemos encontrar numerosos ejemplos de actuaciones a nivel europeo en la Plataforma Europea de Adaptación al Cambio Climático "Climate-ADAPT" (https://climate-adapt.eea.europa.eu/about) y a nivel nacional dentro del marco del Plan Nacional de Adaptación al Cambio Climático (PNACC).

En relación al problema principal que tratamos en esta tesis, **la contaminación por nutrientes en la marisma de Doñana y sus cuencas vertientes**, se espera que el cambio climático en la región mediterránea (disminución de las precipitaciones y el aumento de las temperaturas) y la cada vez mayor competencia por los recursos hídricos en la región (principalmente entre la agricultura, el turismo, la demanda en los núcleos urbanos y los ecosistemas acuáticos), acentúen progresivamente los procesos locales de eutrofización y sus efectos negativos (Moss et al. 2011, Green et al. 2017). Es por ello que se deben adoptar medidas urgentes que ayuden a mitigar la eutrofización a corto y largo plazo, y a incrementar la resiliencia de los ecosistemas acuáticos de Doñana frente a escenarios climáticos y socioeconómicos futuros.

Para hacer frente a la eutrofización en el sistema estudiado, el objetivo prioritario a alcanzar a corto plazo debería ser la reducción de la carga de nutrientes en las aguas superficiales. En esta tesis hacemos especial hincapié en la importancia de que dicha reducción se lleve a cabo *antes* de que las aguas superficiales lleguen a la marisma del Parque Nacional, ya que el objetivo por el que se protege a nivel nacional e internacional es conservar su alto valor ecológico y su biodiversidad, y no como "filtro verde" para procesar el exceso de nutrientes derivados de la actividad humana en las cuencas.

A continuación se presenta un listado de **medidas específicas** que necesitarían implementarse de una manera efectiva y urgente si queremos poner solución al problema de la eutrofización en la marisma de Doñana y sus cuencas a corto y largo plazo (Fig. 12). La integración de los análisis isotópicos en el seguimiento de la calidad de las aguas superficiales en Doñana es una medida que surge originalmente a raíz de la presente Tesis, mientras que las demás medidas surgen de propuestas ya planteadas anteriormente por diferentes agentes implicados directa o indirectamente en la gestión del agua y conservación de Doñana (WWF 2016, 2019a). Muchas de las anteriores se enmarcan dentro del Plan de Ordenación de los Recursos

Naturales (PORN) y Plan Rector de Uso y Gestión (PRUG) del Espacio Natural de Doñana, así como en el Plan Andaluz de Humedales (PAH, 2002). En cada una de las medidas propuestas explicamos por qué pueden contribuir a solucionar este problema y qué actuaciones concretas serían adecuadas. Estas medidas, total o parcialmente, podrían ser extrapolables a situaciones o ecosistemas similares al estudiado, incorporando las particularidades de los mismos.

### *Integrar los isótopos estables en el seguimiento de la calidad del agua*

Los isótopos estables pueden ser una herramienta muy valiosa y complementaria a otros parámetros tanto en programas de seguimiento rutinario de la calidad del agua como en proyectos puntuales de investigación (Kendall et al. 2010; **Capítulos 2 y 3**). Con las aproximaciones isotópicas se puede obtener información sobre el funcionamiento del ecosistema, los procesos biogeoquímicos y los tipos de fuentes contaminantes de nutrientes, lo cual no es posible si únicamente se usan parámetros tradicionales de cuantificación de solutos (Kendall et al. 2008, Kohzu et al. 2008). Además, los análisis isotópicos son una herramienta que permite un procesado de muestras y análisis rápido y ofrece una precisión analítica alta. Los resultados de la presente Tesis confirman la utilidad y viabilidad de los análisis isotópicos en aguas superficiales en Doñana y su entorno y por ello se recomienda su integración en los protocolos de seguimiento de la calidad de las aguas. En Estados Unidos, por ejemplo, cuentan con programas de seguimiento rutinario de la calidad del agua a escala regional y nacional en los que se han integrado con éxito los análisis de isótopos estables (Kendall et al. 2010). Aunque en esta tesis nos hemos centrado en la aplicación de los isótopos estables para estudiar la contaminación por N en Doñana, esta herramienta también podría usarse para estudiar la contaminación por P, a través del análisis isotópico del O del fosfato disuelto ($PO_4^{3-}$) (Smith et al., 2011; Li et al. 2011). El estudio de la composición isotópica del H y O del agua también sería muy útil para estudiar la dinámica hidrológica de la marisma


**Figura 12**. Medidas específicas, tanto correctoras como preventivas, que pueden contribuir a la reducción del problema de la eutrofización en la marisma de Doñana y sus cuencas vertientes.

y arroyos vertientes, ayudando a distinguir, por ejemplo, entre aguas de lluvia enriquecidas por evaporación y aguas procedentes directamente de los excedentes de riego (Clay et al. 2004). Los datos obtenidos podrían incorporarse a una base de datos específica sobre información isotópica en estudios ambientales en Doñana.

### *Incrementar el conocimiento sobre la calidad y la cantidad de las aguas*

En un contexto de cambio global es necesario que la investigación científica siga aportando conocimiento para entender y detectar los efectos de este cambio en los ecosistemas acuáticos y en el proceso de eutrofización en Doñana. Estudios como la presente Tesis aportan información muy valiosa sobre la variación espaciotemporal de las concentraciones de nutrientes y las fuentes contaminantes de nitrógeno que es fundamental para el desarrollo de una gestión del agua más efectiva, que se adapte al dinamismo del sistema y que más que corregir, pueda anticiparse a los problemas del futuro proponiendo soluciones preventivas. Para ello se propone, en primer lugar, asegurar la continuidad a largo plazo de los programas de seguimiento rutinario existentes (ej. Confederación Hidrográfica del Guadalquivir, Espacio Natural de Doñana y Estación Biológica de Doñana) y plantear la posible unificación de los mismos para una mejor coordinación. Estos programas deben contar con una adecuada resolución espaciotemporal y estar adaptados a la alta variabilidad hidrológica de la marisma y de los arroyos vertientes. En estos programas no deben faltar medidas simultáneas tanto de calidad como de cantidad: concentración de nutrientes (aguas superficiales y subterráneas), clorofila (no solo fitoplancton sino también perifiton, especialmente en arroyos), red fiable de medición de caudales (WWF 2016), volumen de descarga de agua del acuífero a la superficie, origen de los nutrientes y procesos biogeoquímicos principales (ej. mediante isótopos estables) y todos los demás indicadores físico-químicos, biológicos e hidromorfológicos requeridos según lo establecido por la Directiva Marco del Agua.

### *Facilitar el acceso a la información sobre calidad de las aguas*

Crear un repositorio de acceso público, que integre tanto la información sobre el área del Parque Nacional de Doñana como sus cuencas vertientes, donde se recopilen de forma estandarizada y organizada todos los datos numéricos y espaciales, brutos o elaborados, relacionados con la calidad de las aguas superficiales y subterráneas (ej. datos isotópicos, concentración de nutrientes) y otros parámetros indicadores del cambio climático (ej. información meteorológica) recogidos por los distintos organismos implicados (ej. Confederación Hidrográfica del Guadalquivir, Espacio Natural de Doñana y Estación Biológica de Doñana). Esta medida ayudaría a agilizar enormemente el acceso a los datos a todos los agentes implicados en la investigación, uso y gestión del agua tanto dentro de los límites de la Reserva de la Biosfera, donde se incluyen tanto las zonas con una protección más estricta (Parque Nacional) como las cuencas, donde se concentran las mayores presiones humanas y donde menor protección ambiental hay.

### *Restauración de las zonas de ribera*

En las cuencas de Doñana, particularmente en el arroyo del Partido, y la cabecera de las cuencas de La Rocina y Los Sotos, los bosques de ribera se encuentran altamente degradados debido principalmente a la deforestación y ocupación de las márgenes. Además, la expansión de la agricultura de regadío ha generado un fuerte proceso de erosión por el que se producen mayores aportes de nutrientes y arrastre de sedimentos a los arroyos, particularmente durante los eventos torrenciales típicos de esta región, acelerando a su vez la colmatación de la marisma (PORN, 2016). Teniendo en cuenta que las zonas de ribera poseen una alta capacidad para retener y eliminar el exceso de nutrientes derivados de la actividad agrícola (Weigelhofer et al., 2012; Teufl et al. 2013; Pinay et al. 2018), y además ayudan a frenar la erosión de las márgenes, sería prioritario restaurar todos aquellos tramos de los arroyos cuyas riberas estén degradadas y tomar medidas para frenar la erosión a nivel de cuenca, como por ejemplo se logró con el Corredor Verde del Guadiamar (Junta de Andalucía, 2005b). Actualmente, la Confederación Hidrográfica del Guadalquivir está llevando a cabo actuaciones de restauración del tramo final del arroyo del Partido (proyecto "Restauración hidrogeomorfológica y naturalización del tramo final del

Arroyo del Partido") para reducir avenidas, favorecer la recarga del acuífero en esta zona y restaurar la cubierta vegetal autóctona. Con este proyecto se espera mejorar la calidad de las aguas superficiales y la reducción de los procesos erosivos.

### *Construcción de humedales artificiales*

Para reducir la cantidad de nutrientes que provienen de la contaminación difusa de la agricultura, una medida complementaria a la restauración de los bosques de ribera podría ser crear una red de humedales artificiales a lo largo de los principales arroyos para que el contenido de nutrientes en las aguas superficiales se pueda mantener dentro de un rango aceptable, especialmente antes de entrar al Parque Nacional (Tonderski et al. 2017). Existen numerosos ejemplos de humedales construidos con el objetivo de reducir la eutrofización en ecosistemas acuáticos de alto valor (Viaroli et al. 2016), como por ejemplo en humedales de importancia internacional Ramsar como *L'Albufera de Valencia* (Martín et al. 2013, Hernández-Crespo et al. 2017) o los *Everglades* de Florida (Chimney & Goforth, 2006). El valor añadido de crear humedales artificiales es su bajo coste de mantenimiento, el alto valor paisajístico y ayudar a conservar la biodiversidad de los ecosistemas húmedos (Mitsch & Gosselink, 2015).

### *Mejorar la depuración de las aguas residuales*

A pesar de la mejora sustancial de los sistemas de depuración de aguas residuales en el Espacio Natural de Doñana durante las últimas dos décadas (PORN 2016), sigue siendo necesario asegurar la correcta depuración de las aguas que se vierten al entorno de Doñana, por ejemplo, aplicando tratamientos terciarios cuando sea necesario (ej. tecnologías de biorreactores de membrana –MBR), aumentando la dilución de los efluentes o haciendo que la descarga de las aguas residuales tratadas se produzca de una manera más difusa para evitar que el impacto se concentre en un único punto del cauce receptor (WWF 2016; Bernal et al. 2020). También es necesario tener un control continuo de los efluentes donde los valores máximos de nutrientes establecidos por la legislación no sean fijos sino que se adapten al caudal de los arroyos receptores de forma estacional, como proponen Bernal et al. 2020, ya que la capacidad de dilución de los nutrientes en periodos secos (i.e. bajo caudal) se ve considerablemente reducida. No menos relevante sería facilitar el acceso a los datos diarios sobre concentración de nutrientes y otros parámetros de relevancia medidos en las aguas de entrada y salida de las depuradoras. Por último, también sería necesario un control más estricto de las aguas residuales generadas por las numerosas viviendas y establecimientos que no cuentan con sistemas de depuración o que los que tienen no ofrecen la capacidad suficiente para tratar la cantidad de aguas residuales generada, especialmente en momentos de gran afluencia de personas como en la romería del Rocío, en la que se llegan a congregar durante un periodo corto de tiempo cientos de miles de personas a orillas de la marisma de Doñana.

### *Cambio en el modelo agrícola*

En las últimas décadas, la rápida expansión de los cultivos de invernadero (**Capítulo 1**), así como la intensa extracción de aguas subterráneas (Custodio et al. 2009) han supuesto dos de las grandes amenazas para cantidad y calidad de las aguas superficiales y subterráneas de Doñana y sus cuencas. Este problema se ha visto además agravado por el aumento progresivo de prácticas agrícolas ilegales que ha resultado en un importante conflicto entre agricultores y administraciones públicas. Como una de las posibles herramientas para intentar solucionar el problema, la Junta de Andalucía aprobó en diciembre de 2014 el denominado *Plan Especial de ordenación de las zonas de regadíos ubicadas al norte de la Corona Forestal de Doñana.* Esta y otras medidas que se han propuesto para disminuir las presiones antrópicas sobre el acuífero de Doñana (la declaración del acuífero como "sobreexplotado", Plan Anual de Extracción de Agua o la adquisición de derechos del agua; WWF 2016) pueden contribuir también a reducir el problema de la eutrofización gracias a que si el acuífero descarga más cantidad de agua en los arroyos, estos pueden aumentar su capacidad de dilución. Sería necesario complementar estas medidas con otras de mejora de las prácticas agrícolas, como por ejemplo, usar menos fertilizantes químicos y priorizar los abonos orgánicos, crear un sistema para reciclar o retener los nutrientes y el agua que se pierden con los excedentes de riego o instalar sondas u otros equipamientos que ayuden a controlar el riego para evitar la pérdida de nutrientes por infiltración al acuífero (WWF 2015). Más allá de estas medidas, y teniendo en cuenta los potenciales escenarios futuros de cambio climático y crecimiento de la población mundial, es necesario un cambio urgente y profundo en el modelo agrícola que realmente reduzca desde la raíz el impacto de la agricultura sobre los ecosistemas, especialmente sobre aquellos de tan alto valor ecológico como Doñana (Habel et al. 2019).

### *Mejorar la gobernanza del agua*

Es necesario asegurar una **buena gobernanza multinivel** en la que exista una coordinación fluida entre los diferentes niveles (internacional-nacional-regional-local) de administraciones públicas, organismos de investigación y organizaciones no gubernamentales implicados en la conservación, planificación y gestión del agua en Doñana. Además es imprescindible implicar a la población local y a los usuarios del agua en la toma de decisiones, pues la conservación de este patrimonio natural es principalmente fuente de calidad de vida y actividad económica sostenible para ellos (WWF 2016).

Como conclusión final de este apartado, el conjunto de estas medidas se podría considerar como una primera propuesta para crear un plan de actuación específico que proporcione soluciones al problema de la eutrofización en el ámbito de Doñana y otros humedales mediterráneos en similares circunstancias, y con el que se consiga una visión más integradora de la problemática, fomentando el intercambio de información y datos entre científicos y gestores.

# Conclusiones generales

1 La cuantificación de la evolución de cultivos bajo plástico revela que el área cubierta por invernaderos en las cuencas de la Rocina, los Sotos y el Partido aumentó un 487% desde 1995 hasta 2016. La superficie cubierta por invernaderos en las cuencas de la Rocina y los Sotos fue casi tres veces superior a la del Partido, aunque en relación al área total de la cuenca, el Partido presenta el mayor porcentaje. Estos resultados sugieren que el fuerte desarrollo de la agricultura en las cuencas es uno de los motores principales de la contaminación por nutrientes en los arroyos y la marisma.

2 Durante el periodo de estudio, las concentraciones de nutrientes en las aguas superficiales de la marisma fueron consistentemente más bajas que en los arroyos. El arroyo del Partido presentó un 63% de muestras cuyas concentraciones se clasificaron dentro de la categoría de mala calidad del agua. Los arroyos de la Rocina y los Sotos presentaron concentraciones más bajas, aunque aproximadamente un tercio de las muestras también indicaron mala calidad del agua. Las aguas del Guadiamar mostraron los valores más bajos entre los arroyos. Estos resultados muestran que la eutrofización afecta de forma desigual a la marisma y a sus arroyos vertientes, sin embargo, en todos los casos representa uno de los principales problemas de origen antrópico que amenaza gravemente la integridad y el alto valor ecológico de los ecosistemas acuáticos de Doñana.

3 Los análisis isotópicos de nitrógeno en dos especies de plantas helófitas (*Bolboschoenus maritimus* y *Typha domingensis*) sugieren que la fuente principal de nitrógeno en la marisma es de origen natural, es decir, que proviene del propio ecosistema. Por el contrario, los análisis isotópicos en helófitas y nitratos disueltos en los arroyos, sugieren que las fuentes de nitrógeno predominantes en estos sistemas tienen un origen antrópico, principalmente derivado de fertilizantes agrícolas y en el caso del arroyo del Partido, también de aguas residuales depuradas. Estudios con una mayor resolución espacio-temporal serían necesarios para determinar la variabilidad anual de la contribución de las fuentes principales de nitrógeno.

4 Los resultados isotópicos de nitratos sugieren que la desnitrificación es un proceso predominante en los puntos de arroyos y lagunas muestreados. Se estima que este proceso podría estar eliminando hasta un 80% de nitratos en algunos puntos, actuando como un importante mecanismo natural de atenuación de la contaminación por nitrógeno.

5 Desde el punto de vista de la gestión, es necesario contar con información actualizada y detallada sobre la calidad del agua en los ecosistemas acuáticos de Doñana y sus cuencas para poder proponer medidas adecuadas y adaptadas a los continuos cambios derivados de las sinergias entre las presiones antrópicas y el cambio climático. En este contexto, la investigación científica en las líneas de limnología y ecología de isotopos es fundamental para arrojar luz y encontrar soluciones adecuadas al problema de la eutrofización en Doñana.

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