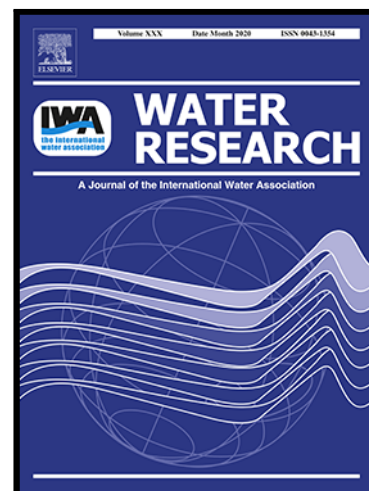# Journal Pre-proof

Combining multi-isotopic and molecular source tracking methods to identify nitrate pollution sources in surface and groundwater

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Highlights

- Multi-isotopic and molecular source tracking data show high agreement to identify nitrate sources.
- $\delta^{11}B$ in effluents of wastewater treatment plant can be affected by livestock wastes.
- Information obtained was employed by local authorities to establish new vulnerable zones.

Combining multi-isotopic and molecular source tracking methods to identify nitrate pollution sources in surface and groundwater

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Abstract

Nitrate (NO_3^-) pollution adversely impacts surface and groundwater quality. In recent decades, many countries have implemented measures to control and reduce anthropogenic nitrate pollution in water resources. However, to effectively implement mitigation measures at the origin of pollution, the source of nitrate must first be identified. The stable nitrogen and oxygen isotopes of NO_3^- ($\delta^{15}N$ and $\delta^{18}O$) have been widely used to identify NO_3^- sources in water, and

their combination with other stable isotopes such as boron ($\delta^{11}B$) has further improved nitrate source identification. However, the use of these datasets has been limited due to their overlapping isotopic ranges, mixing between sources, and/or isotopic fractionation related to physicochemical processes. To overcome these limitations, we combined a multi-isotopic analysis with fecal indicator bacteria (FIB) and microbial source tracking (MST) techniques to improve nitrate origin identification. We applied this novel approach on 149 groundwater and 39 surface water samples distributed across Catalonia (NE Spain). A further 18 wastewater treatment plant (WWTP) effluents were also isotopically and biologically characterized. The groundwater and surface water results confirm that isotopes and MST analyses were complementary and provided more reliable information on the source of nitrate contamination. The isotope and MST data agreed or partially agreed in most of the samples evaluated (79 %). This approach was especially useful for nitrate pollution tracing in surface water but was also effective in groundwater samples influenced by organic nitrate pollution. Furthermore, the findings from the WWTP effluents suggest that the use of literature values to define the isotopic ranges of anthropogenic sources can constrain interpretations. We therefore recommend that local sources be isotopically characterized for accurate interpretations. For instance, the detection of MST inferred animal influence in some WWTP effluents, but the $\delta^{11}B$ values were higher than those reported in the literature for wastewater. The results of this study have been used by local water authorities to review uncertain cases and identify new vulnerable zones in Catalonia according to the European Nitrate Directive (91/676/CEE).

1.   Introduction

Nitrate (NO_3^-) pollution in surface and groundwater lowers the quality and quantity of drinking water resources and can cause adverse impacts on ecological and human health (Vitousek et al.,

1997; Ward et al., 2005). Nitrate contamination linked to anthropogenic activities are mainly derived from both diffuse (non-point) sources, such as livestock and synthetic and organic fertilizer application, and point sources, such as septic systems and/or waste water treatment plant (WWTP) effluents (Peed et al., 2011). The EU Water Framework Directive has established stringent thresholds to achieve good chemical status in groundwater and good ecological status in surface waters (Directives 2000/60/CE and 2006/118/CE). Despite the measures implemented since 1991 to minimize agricultural nitrate pollution in water resources (Directive 91/676/CEE), nitrate concentrations still frequently exceed the surface and groundwater limits (European Environment Agency (EEA), 2019). For example, 13.2 % of groundwater control points in Europe exceeded the threshold of 50 mg/L between 2012 and 2015, whereas 5.7 % had nitrate concentrations of 40–50 mg/L (European Commission, 2018). In Catalonia (NE Spain), 59 % of groundwater bodies had poor chemical status, and 77 % of them had exceeded the nitrate threshold of 50 mg/L (Munné et al., 2016). The accurate identification of pollution sources and a detailed understanding of the processes affecting local contaminant concentrations are key for developing effective management practices for water quality preservation (Kendall et al., 2007).

Stable isotopes are commonly applied to identify the dominant sources of anthropogenic nitrate pollution due to the specific isotopic signatures of the different sources (Demlie et al., 2008; Palmer et al., 2007; Puig et al., 2013; Stuart et al., 2010). They are typically measured as the ratio between the heavier and most abundant isotope (lighter isotope) (e.g., ^{15}N against ^{14}N). Studies have successfully combined the stable isotopic ratios of NO_3^- (δ^{15}N, δ^{18}O) and boron (δ^{11}B) to characterize the predominant sources of pollution in surface and groundwater in a variety of hydrogeological settings (Guinoiseau et al., 2018; Sebilo et al., 2013; Vitoria et al., 2008; Widory et al., 2004). However, the isotopic characterization of nitrate pollution sources has some uncertainty due to overlapping isotopic ranges, mixing between sources, and/or isotopic

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fractionation related to physicochemical processes. To minimize these limitations, different approaches can be applied. For instance, some studies combined isotope data with halides and trace elements (Pastèn-Zapata et al., 2014). Other studies employed a Bayesian mixing model to define the contribution of nitrate sources (Xue et al, 2012, Matiattos (2016)). However, this mixing models can only be applied in well monitored areas where nitrate sources are well characterized, and processes are not affecting nitrate concentration. Seldom publications consider common processes affecting nitrate such as denitrification in the mixing models. Recently Paredes et al. (2020) included different denitrification scenarios in a Bayesian mixing modelling). A recent approach combined isotopic data with microbial source tracking (MST) markers (host specific bacteriophages and species from the Bacteroidales group) to improve nitrate source apportionment in polluted surface waters and karstic aquifers (Briand et al., 2017, 2013). This combined approach is a promising method for pollution source identification particularly when the pollution is linked to organic sources.

The assessment of fecal pollution in water is based on the enumeration of fecal indicator bacteria (FIB), such as *E. coli* and enterococci (European Commission, 2006, 1998; WHO, 2009). These parameters have been broadly used to indicate the presence of potential fecal pathogens in water. As these bacteria are widespread in animals, the use of MST markers over the last decades have accurately identified the source of fecal pollution in water (Blanch et al., 2015; Hagedorn et al., 2011). These markers are also widely used as part of various river and coastal water management strategies (Ahmed et al., 2008; Ballesté et al., 2020b; Boehm et al., 2003; Casanovas-Massana et al., 2015; Gourmelon et al., 2007; Reischer et al., 2008; Shanks et al., 2010). Although they have shown good performance in sewage samples, MST markers generally have low sensitivity in the environment due to dilution, mixing with other pollution sources, and masking by autochthonous bacteria. All of the assessed MST markers have shown 100 % specificity to date, particularly under boarder analyses that consider different geographical

areas (Mayer et al., 2018; Reischer et al., 2013; Yahya et al., 2017); this suggests that no single marker can be applied universally. It is, therefore, necessary to combine several markers using computational tools to improve source classification (Ballesté et al., 2020a; Casanovas-Massana et al., 2015; Henry et al., 2016). A number of studies have compared MST markers with chemical markers, such as fecal sterols and fluorescent whitening agents (Blanch et al., 2006; Devane et al., 2019; Gilpin et al., 2003); however, only a few studies to date have combined MST markers with stable isotopes (Briand et al., 2017, 2013).

In this study, we combined isotopic data with MST indicators to identify the main sources of nitrate pollution in surface and groundwater across Catalonia (NE, Spain). We developed a useful and operative approach to respond to the PILOT 7849/15/ENVI procedure initiated by the European Commission in 2015 with the goal to review criteria and delimit vulnerable zones of nitrate pollution. For this purpose, 187 water samples—including 39 surface water and 148 groundwater samples—from different hydrogeological locations were selected based on both water management and sanitary policy priorities. We combined hydrochemical, multi-isotopic, and MST marker analyses with hydrogeological and agronomical assessments at each water sampling point. Each data point was interpreted individually, as each water sample represented a different geographical (inland and coastal areas) and hydrogeological context (sedimentary, calcareous, granitic aquifers including free and confined). Moreover, we selected and characterized (isotopes and MST markers) 18 effluents from WWTPs as references.

This study had two main objectives: i) to accurately determine the main sources of nitrate in each water sample by combining multi-isotopic data, MST markers, traditional microbial indicators, and additional field information; and ii) to evaluate the effectiveness of this approach to identify nitrate sources by applying these multi-disciplinary methodologies (isotopes, MST markers, and field information) within a large-scale project. We aimed to identify the advantages

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and limitations of each method and provide analytical recommendations for their application in similar studies.

2. Methods

2.1 Site descriptions and sample collection

A total of 148 groundwater samples and 39 surface water samples were collected (Fig. 1). Hydrogeology information for each groundwater sample can be found summarized in Table SI-1. Samples were transported to the laboratory at 4 °C and analyzed within 24–48 h after sample collection. In addition, effluents of 18 WWTPs located upstream from some of the surface water samples and distributed along the studied area were also collected (Fig. 1). The WWTPs were selected based on their proximity to the sampling sites as well as the type of treatment employed: Seven WWTPs employed biological treatments (B); 3 WWTPs employed biological treatments and nitrogen elimination (BN); 1 WWTP employed phosphate removal and biological treatments (BP); 6 WWTPs employed biological treatments and nitrogen and phosphorus removal (BNP); and 1 WWTP employed biological treatment, nitrogen and phosphorus removal, and tertiary treatment (BNPT).

Figure 1

The Catalan region has a total area of 31,600 km^2 and is located in a Mediterranean climate area (precipitations ranging from 400 to 650 mm per year), with scarce water flows, particularly in summer, and sudden floods in spring and autumn. It includes a total of 367 river water bodies, as well as the associated coastal waters and groundwater. Defined river water bodies have a drainage basin of at least 10 km^2 and they contribute to a minimum volume of 3.15 hm^3 per year. Water resources are unevenly distributed and mainly concentrated in the northern basins, which receive more precipitation and have higher runoff coefficients. Total natural water

resources are estimated at approximately 5,836 hm^3 per year (period 1940-2008). Lowland areas close to the Mediterranean Sea are characterized by high population density (420 inhab. km^2), associated to strong urban and industrial pressures, whereas inland areas are dominated by agricultural activities and farms.

We used existing local and regional information from local councils and the Catalan Government (Departament d'Agricultura, Ramaderia, Pesca i Alimentació (DARP)) to characterize the agronomy and anthropogenic pressures in each site. The territorial analysis was based on both the SIGPAC network (Geographical Information System for Agricultural Parcels) and specific information provided by the DARP, which allowed us to work on a plot scale. The potential pressures in each working area according to the focused and/or dominant activity around each sampling point are defined as follows: dry and irrigated agriculture, livestock facilities, urban areas, and wastewater treatments plants. The detailed characterizations were completed within a radius of 1–2.5 km around each sampling point.

2.2 Chemical and Isotopic characterization

Surface and groundwater temperature (T), pH, electrical conductivity, redox potential (Eh), and dissolved oxygen (DO) parameters were measured *in situ* using portable electrodes. The measurements were directly taken from the water flow of springs and streams. Groundwater measurements were conducted using a flow-through chamber in the four piezometers to minimize the effect of air exchange. Agricultural wells. All the wells were purged prior to sampling by either removing a minimum of three well volumes or until the electrical conductivity stabilized. Surface water samples were obtained using an extendable pole (2 m long) with a HDPE bottle at one end. Before taking the samples, the bottle was rinsed 3 times with surface water at the sampling point. All the samples were then stored in the dark at 4 °C prior to further

analysis following the official standard methods (APHA-AWWA-WEF, 1998). Chemical parameters were determined via standard analytical techniques: major anions (Cl^-, NO_3^-, NO_2^-, SO_4^{2-}) were determined by ionic chromatography (IC) using an 861 Compact Metrohm Advanced ion chromatographer, and major cations and trace elements were measured by inductively coupled plasma-optical emission spectrometry (ICP-OES) using an ICAP 6500 DUO (Thermo Fisher Scientific). The chemical analyses were determined in the laboratories of the Centre D'Estudis de la Construcció I Anàllisi de Materials SLU (CECAM).

Stable isotopes are usually measured as the ratio between the rare isotope and the abundant isotope, e.g. ^{15}N against ^{14}N. The isotopic composition of a sample was established with respect to international standards using the delta notation (δ) equation (1),

$$\delta_{sample} = (R_{sample} / R_{std}) - 1,$$

where R = $^{15}N/^{14}N$, $^{18}O/^{16}O$, $^{11}B/^{10}B$ and $^2H/^1H$ in the sample and the standard (std) and is expressed as ‰ (Coplen, 2011). The following isotopic compositions were determined in this study: $\delta^{15}N$ and $\delta^{18}O$ of NO_3^-, $\delta^{15}N$ of NH_4^+, δ^2H and $\delta^{18}O$ of H_2O, and $\delta^{11}B$. The $\delta^{15}N$ and $\delta^{18}O$ of dissolved NO_3^- were determined using a modified cadmium reduction method (McIlvin and Altabet, 2005; Ryabenko et al., 2009). the $\delta^{15}N$ of NH_4^+ was analyzed by the hypobromite method (Zhang et al., 2007), and the $\delta^{15}N$ and $\delta^{18}O$ of produced N_2O were simultaneously analyzed using a Pre-Con (Thermo Scientific) coupled to a Finnigan MAT-253 isotope ratio mass spectrometer (IRMS, Thermo Scientific). All isotopic analyses were prepared at the MAIMA-UB laboratory and determined at the Centres Científics i Tecnològics of the Universitat de Barcelona (CCiT-UB). The δ^2H_{H2O} and $\delta^{18}O_{H2O}$ were analyzed in the University of Malaga by Wavelength Scanned Cavity Ringdown Spectroscopy (WS-CRDS) (L2120-i Picarro®) for isotopic water measurements. We measured six replicates for each sample, but only the last three replicates were selected for the statistical analysis. $\delta^{11}B$ were measured using a HR-ICP-MS (high-resolution inductively coupled

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plasma mass spectrometer) Element XR (Thermo Scientific) following the method by Gäbler & Bahr (1999) at the labGEOTOP Service, Laboratory of Elemental and Isotopic Geochemistry for Petrological Applications of ICTJA-CSIC. Isotope ratios were calculated using both international and internal laboratory standards. All standards and theoretical values are presented in the supporting information (Table SI-2). Notations were expressed as δ relative to the international standards (V-SMOW for δ^{18}O and δ^2H$_{H2O}$, atmospheric N$_2$ for δ^{15}N, and NBS-951 for δ^{11}B). The reproducibility of the samples was $\pm$ 0.5 ‰ for the δ^2H of H$_2$O, $\pm$ 0.2 ‰ for the δ^{18}O of H$_2$O, $\pm$ 1 ‰ for the δ^{15}N of NO$_3^-$, $\pm$ 0.5 ‰ for the δ^{15}N of NH$_4^+$, and $\pm$ 1.5 ‰ for the δ^{18}O of NO$_3^-$.

2.3 Enumeration of fecal indicator bacteria and bacteriophages

The enumeration of *Escherichia coli* (EC) and Enterococci (ENT) as FIB was performed using the membrane filtration technique. Different volumes of water depending on the bacterial concentration were analyzed by passing the samples through a 0.45 µm pore-sized filter (EZ-PAK, Millipore, Darmstadt, Germany). EC was enumerated in accordance with UNE-EN ISO 9308-1:2001 (ISO, 2000a) using the chromogenic agar media (CCA). ENT was enumerated in accordance with the ISO standard 7899-2:2000 (ISO, 2000b), and filters were placed on Slanetz and Bartley medium.

Somatic coliphages (SOMCPH) and bacteriophages that infect the human-associated *Bacteroides* GA17 strain (GA17PH) were enumerated using the double layer plaque assay according to ISO10705-2 (ISO, 2000) and ISO10705-4 (ISO, 2001), respectively. Briefly, MgCl$_2$ was added to 100 mL of surface water and groundwater to a final concentration of 0.05 M. The samples were then concentrated by filtration using 0.22 µm-pore-size mixed cellulose ester membrane filters (Merck Millipore, Cork, Ireland). The filters were transferred to flasks with 6 mL elution buffer (1 % Beef Extract, 0.5 M NaCl, and 3 % Tween 80), and the viruses were eluted

in an ultrasound bath for 4 min (Méndez et al., 2004). The elution solution was brought to a pH of 7 and then passed through a low protein binding 0.2-μm pore-sized PES syringe filter (Merck Millipore). One milliliter of the solution was quantified in triplicate with the corresponding host strain (*E. coli* WG5 for SOMCPH and *Bacteroides* spp. GA17 for GA17PH). Moreover, 1 mL of direct surface water samples with high bacterial concentrations and treated sewage was analyzed for SOMCPH. The results were expressed in plaque-forming units (PFU) 100 mL^{-1}.

2.4 Molecular source tracking analysis

2.4.1 Nucleic acid extraction

Different volumes of water were filtered for MST analysis depending on the origin of the sample (surface or groundwater) and the amount of suspended particles able to saturate the filter: i) a volume of 1.4–5 L (average of 4 L) for groundwater, ii) a volume of 0.35–2 L (average of 1.2 L) for surface water, and iii) 0.1 L for treated sewage. Each sample was concentrated by passing them through 0.22-μm pore-sized filters (SO-PAK, Millipore, Darmstadt, Germany) for DNA extraction. The filters were then placed in 0.5 mL GITC buffer (5 M guanidine thiocyanate, 100 mM EDTA [pH 8.0], 0.5 % sarkosyl) and frozen at -20 °C in a lysis buffer until DNA extraction. The DNA was extracted from the filters using the QIAamp DNA Blood Mini Kit (Qiagen GmbH, Hilden, Germany) with some modifications from previously reported methods (Gourmelon et al., 2007). Filtration and DNA extraction controls were run together with the samples.

2.4.2 MST marker quantification by molecular methods

The four previously-described source tracking markers were analyzed by real-time qPCR to target the 16S rRNA gene from the host specific *Bifidobacterium* species: HMBif, CWBif, PLBif (Gómez-Doñate et al., 2012), and the host-specific *Bacteroidales* Pig2Bac (Mieszkin et al., 2009).

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HMBif is typically associated with human sources, CWBif with ruminants, Pig2Bac with porcine, and PLBif with poultry. The primers, probes, and conditions applied were the same as those described previously. Amplification was performed in a 20 μl reaction mixture using TaqMan Environmental Master Mix 2.0 (Applied Biosystems, Foster City, CA, USA) in a StepOne Real-Time PCR System (Applied Biosystems). All samples, negative controls, and extraction and filtration blanks were run in duplicate, and analyses were repeated when discordance between duplicates was detected. The results were expressed as gene copies (GC) 100 mL^{-1}. Five points on the standard curves were included in duplicate for each run and were generated from different 10-fold serial dilutions of the linearized plasmid containing the target gene. The quantification limits for Pig2Bac was 70 GC per reaction and for *Bifidobacterium* markers were 45, 40, and 25 GC per reaction for the human, poultry, and ruminant assays, respectively. The mitochondrial source tracking marker Pomito was tested in samples positive for Pig2Bac using nested-PCR assays. The primers and the conditions used were the same as those described in Martellini et al. (2005).

2.5 Criteria for nitrate pollution identification using a multi-isotopic approach.

The theoretical ranges of potential nitrate sources must be established for accurate interpretations of isotopic results. These ranges are usually established using previously reported values from the literature as well as measurements of local sources (Vengosh et al., 1994; Vitòria et al., 2004; Widory et al., 2005; Xue et al., 2009). The criteria for defining these ranges and values in this study as well as the limitations in their interpretations are described in the supporting information and summarized in Figure 2. The δ^{15}N and δ^{18}O of nitrate were analyzed in 182 samples with sufficient concentration for the analyses. The δ^{11}B was analyzed in selected samples based mainly on two criteria: i) if the isotopic composition of N and O from NO$_3^-$ showed an organic or uncertain source of NO$_3^-$, and ii) if positive MSTs were detected.

Furthermore, $\delta^{11}B$ was also determined in 15% of the samples where these criteria were not meet.

Figure 2

3. Results

All of the physicochemical, isotopic and microbiological results from the WWTPs and field samples as well as the agronomic information are presented in the supporting information (Table SI-3, SI-4, SI-5, SI-6, SI-7, SI-8 and SI-9).

3.1 Chemical and isotopic results

3.1.1 WWTP effluents

The ranges and mean values of the most relevant parameters determined in sewage effluents are presented in Figure 3. Effluents were characterized to more accurately identify the urban effluents linked to nitrate pollution.

Although most of the studied WWTPs incorporate nitrogen removal treatments, nitrogen species were still detected in 17 of the 18 characterized sewage effluents: NO_3^- was detected in 15 effluents (up to 57 mg/L), NO_2^- was detected in 6 effluents (up to 5.6 mg/L), and NH_4^+ was detected in 9 effluents (up to 51.4 mg/L). B concentrations showed high variability with a mean value of 130.8 μg/L.

We analyzed the $\delta^{15}N$ and $\delta^{18}O$ of dissolved nitrate in 7 samples where nitrate concentrations exceeded 3 mg/L. $\delta^{15}N\text{-}NO_3^-$ and $\delta^{18}O\text{-}NO_3^-$ showed mean values of +17.6 ‰ and +6.5 ‰, respectively. The $\delta^{15}N\text{-}NH_4^+$ was analyzed in 8 WWTPs and showed a similar range to that of

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δ^{15}N-NO$_3^-$ at +9.7 ‰ to +23.6 ‰ (Fig. 3). Finally, δ^{11}B from WWTP effluents ranged from +2.9 ‰ to +20.4 ‰ (Fig. 3).

Figure 3

3.1.2 Surface water

The ranges and mean values of the most relevant surface water parameters are presented in Figure 4.

Figure 4

Seventy-six percent of surface water samples had NO$_3^-$ concentrations below 20 mg/L, indicating moderate to low nitrate pollution. Only 2 samples (5 %) had NO$_3^-$ concentrations over 50 mg/L. In contrast, significant NO$_2^-$ concentrations were observed in 20 % of the samples with values exceeding 1 mg/L. Similarly, NH$_4^+$ contents exceeded 1 mg/L in 15 % of surface water samples. The B content exceeded 100 µg/L in 25 % of the surface water samples and was especially high (> 200 µg/L) in 4 samples (SW-8, 10, 29, and 35). Isotopically, δ^{15}N-NO$_3^-$ values exceeded +8 ‰ in 88 % of samples, while δ^{18}O-NO$_3^-$ values exceeded +8.0 ‰ in only 28 % of samples. For δ^{11}B, 20 % of the surface water samples had values below +14 ‰, 41 % had values between +14 ‰ and +20 ‰, and 25 % showed values over +20 ‰.

3.1.3 Groundwater

The ranges and mean values of the most relevant groundwater parameters are presented in Figure 5.

Figure 5

NO$_3^-$ concentrations exceeded the established threshold of 50 mg/L in 56 out of 148 samples (38 %). A similar number of samples (36 %) had concentrations of 20–50 mg/L, while 26 % of samples

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had concentrations of < 20 mg/L. A total of 146 groundwater samples had sufficient nitrate concentrations for isotopic measurements. Thirty-six percent of the samples showed δ^{15}N-NO$_3^-$ values below +8.0 ‰, 49 % showed values between +8.0 ‰ and +15.0 ‰, and 18 % showed values higher than +15 ‰. Forty-eight percent of samples had δ^{18}O-NO$_3^-$ values below +6.0 ‰. For δ^{11}B, 25 % of groundwater samples had values below +14.0 ‰, 22 % of samples had values between +14 ‰ and +20 ‰, and most of the samples (55 %) showed values higher than +20 ‰.

3.2 Microbial source tracking markers

3.2.1 Wastewater

All studied WWTP effluents were positive for the fecal indicators EC, ENT, and SOMCPH (Table SI-9). EC abundances ranged from 2.92 to 6.56 log$_{10}$ CFU 100 mL^{-1} with a mean of 4.54 log$_{10}$ CFU 100 mL^{-1} (Fig. 6). ENT abundances ranged from 2.87 to 5.06 log$_{10}$ CFU 100 mL^{-1} with a mean of 3.97 log$_{10}$ CFU 100 mL^{-1}, and SOMCPH ranged from 3.10 to 6.28 log$_{10}$ PFU 100 mL^{-1} with a mean of 4.45 log$_{10}$ PFU 100 mL^{-1}. For the human MST markers, all effluent samples were positive for HMBif with a mean value of 5.38 log$_{10}$ GC 100 mL^{-1}. Fifteen samples were positive for GA17PH with a mean of 2.01 log$_{10}$ PFU 100 mL^{-1}, and 3 of them were below the limit of detection. Ten of the 18 samples were positive for the Pig2Bac marker, with a range of 1.51—4.35 log$_{10}$ GC 100 mL^{-1} and a mean of 2.32 log$_{10}$ GC 100 mL^{-1}. Three of the samples were also positive for the Pomito marker, and 2 of them corresponded to the highest concentration of Pig2Bac at > 4 log$_{10}$ GC 100 mL^{-1}. However, Pig2Bac was not detected in 2 samples that were positive for the Pomito marker. Five samples were positive for CWBif with values ranging from 2.11 to 3.29 log$_{10}$ GC 100 mL^{-1}, and all of them were positive for PLBif.

Figure 6

3.2.2 Surface water

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General microbial fecal indicators and Microbial Source Tracking markers

All 39 surface samples were positive for at least one of the general fecal indicators tested. EC and SOMCPH were detected in 87.2 % of the samples (34/39), and ENT was detected in 97.4 % (38/39). The MST markers were detected less often in surface samples (56.4 % of the samples). HMBif was detected in 10 of 29 samples (25.6 %) with an average concentration of 3.75 $\log_{10}$ GC 100 mL^{-1} (Fig. 7). Pig2Bac was detected in 43.6 % of the samples (17/39) but at a lower concentration of 1.20 $\log_{10}$ GC 100 mL^{-1} (1 of them was also positive for Pomito). None of the samples were positive for the bovine and poultry markers tested in this study. Moreover, the porcine marker abundance did not exceed 2.71 $\log_{10}$ GC 100 mL^{-1} in most of the samples, whereas the abundance of the human marker reached 5.29 $\log_{10}$ GC 100 mL^{-1}.

Figure 7

The sum of all MST markers (GC 100 mL^{-1} of HMBif + CWBif + PLBif + Pig2Bac) strongly correlated with the sum of the general indicators (CFU 100 mL^{-1} of EC + ENT + PFU 100 mL^{-1} of SOMCPH) (0.734, df = 20, P-value < 0.001). The sum of all MST markers also correlated with EC, ENT, and SOMCPH individually (EC: 0.653, df = 20, P-value = 0.001; ENT: 0.762, df = 20, P-value < 0.001; SOMCPH: 0.694, df = 20, P-value < 0.001). Moreover, we observed strong correlations between EC and ENT (0.792, df = 30, P-value < 0.001) and between HMBif and EC and ENT (EC: 0.748, df = 8, P-value = 0.01; ENT: 0.752, df = 8, P- value = 0.01).

3.2.3 Groundwater

General microbial fecal indicators and Microbial Source Tracking markers

Of all groundwater samples, 42.6 % were negative for all the general fecal indicators and MST markers, 39.9 % were positive for a general fecal indicator, 31.1 % were positive for an MST marker, and 13.5 % were positive for both markers. Moreover, 17.6 % of the samples were positive for EC, with a mean abundance of 1.44 CFU 100 mL^{-1} and a range of 0.60–3.48 log$_{10}$ CFU 100 mL^{-1}. Most samples were positive for ENT, and 32.4 % had similar abundances (a mean value of 1.45 log$_{10}$ CFU 100 mL^{-1} and a minimum and maximum value of 0.60 and 3.43 log$_{10}$ CFU 100 mL^{-1}, respectively). SOMCPH was positive in 10.1 % of samples (a mean value of 0.64 log$_{10}$ PFU 100 mL^{-1} and a minimum and maximum value of 0.30 and 2.05 log$_{10}$ PFU 100 mL^{-1}, respectively). When comparing the different culturable bacterial indicators, 64.9 % of the samples were negative for EC and ENT, 14.9 % were positive for both markers, only 4 samples (2.7 %) were positive for EC and not ENT, and 17.6 % were positive for ENT and not EC. Six samples were positive for all 3 markers (SOMCPH, EC, and ENT), 2 samples were positive for SOMCPH and ENT but not EC, and 7 samples were positive for SOMCPH (at the limit of the detection) but not EC and ENT.

MST markers were also tested in groundwater samples. The GA17PH marker targeting cultured human-associated bacteriophages was detected in just 2 of the 148 samples, whereas the molecular marker HMBif targeting a human-associated *Bifidobacterium* was detected in 18.9 % of the samples. The mean concentration of HMBif was 1.37 log$_{10}$ GC 100 mL^{-1} with values ranging from -0.41 (0.39 GC 100 mL^{-1}) to 3.00 log$_{10}$ GC 100 mL^{-1}. The Pig2Bac marker was detected in 16.9 % of the samples (25 of 148 samples) with values ranging from -0.77 to 1.94 log$_{10}$ GC 100 mL^{-1}. The CWBif and PLBif markers were detected in 10 of the 148 samples with values ranging from 0.11 to 2.29 log$_{10}$ GC 100 mL^{-1} and 0.03 to 1.76 log$_{10}$ GC 100 mL^{-1}, respectively.

For groundwater samples, we observed strong significant correlations between all FIB and HMBif (0.675, df = 10, p-value = 0.02) markers and between ENT and HMBif (0.692, df = 7, P-value = 0.04).

Figure 8

3.3 Agronomic characterization and analysis of pressures

The dominant pressures in surface and groundwater were the application of manure, chemical fertilizers and/or sewage sludge in the fields, the use of phytosanitary products, and industrial, urban, and untreated discharges (ACA, 2013). We identified the main land-use type, the number of proximal urban areas and WWTPs, and the number of livestock facilities surrounding each sampling site (Table SI-5 and 8).

The dominant land-use types close to the sampling sites were agricultural and livestock, which included 64 % of surface water sites and 67 % of groundwater sampling sites. Eighteen percent of surface water and 38 % of groundwater sampling sites were located in urban or industrial areas. In addition, although most sampling sites were not located within urban areas, 47 % of surface water samples and 81 % of groundwater samples were located less than 1 km from a city. Finally, 18 % of surface water and 20 % of groundwater sampling sites were located in forested areas.

We also quantified the abundance of livestock facilities within a 1-km radius of each sample point. For surface water, 85 % of samples had < 5 facilities nearby, whereas 15 % of samples had between 5 and 10 facilities nearby and were mainly linked to pig farms (30 % of the farms identified). For groundwater, 56 % of samples had < 5 farms, 25 % had between 5 and 10 farms,

13 % had between 15 and 30 farms, and 6 % had > 30 farms within a 1-km radius. Poultry farms were the most abundant (30 %), followed by pig farms (24 %).

3.4 Integration of isotope and MST marker data

Isotopic data were interpreted following the criteria described in section 2.5 (and detailed in the supporting information), and the results were compared to the MST marker information for each sample. Finally, we assessed the land use and nitrate pressures at each sampling point to validate and confirm the sources of nitrate pollution. Tables 1 and 2 show the number of surface and groundwater samples linked to different sources based on the isotope and MST data, respectively.

The main nitrate sources were organic in origin based on the isotopic results, including wastewater and/or manure (Table 1). The presence of MST was more common in samples linked to an organic pollution source. However, MST was also detected in some of the samples with isotopic values indicative of inorganic sources.

Table 1

Groundwater samples showed mixed sources of nitrate, with higher inorganic sources compared with that of the surface water samples. MSTs were mainly detected in samples with isotopic values indicative of organic pollution, but they were also detected in several samples with isotopic values indicative of inorganic fertilizers (Table 2).

Table 2

To determine the advantages and limitations of the coupled approach (multi-isotopic and MST), we compared the isotope-derived nitrogen sources (Table 1 and 2) with the MST data. The degree of correspondence between the two datasets was classified into 3 categories: i)

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agreement, ii) partial agreement, and iii) disagreement. The proposed scenarios and the number of samples classified into each category are summarized in Table 3. Briefly, the isotopes and biological markers were in agreement when the two datasets showed similar pollution sources as well as provided more specific information regarding the pollution source (e.g., the type of animal manure). The samples showed partial agreement when the isotopic and marker datasets were not contradictory but did not provide additional information: e.g., if the isotopically-derived source of nitrate was organic, but no microbial indicators were detected. Finally, a disagreement was attributed to samples in which the source interpretations from the MST and isotope data did not match.

Table 3

We found that 66 % of surface and 52 % of groundwater samples showed complete agreement with regards to pollution source. Partial agreement was observed in 5 % and 27 % of surface and groundwater samples, respectively, and 23 % of surface samples and 19 % of groundwater samples disagreed.

Discussion

4.1 Evaluation of isotopes as tracers for WWTP effluents

N was detected in the form of NO_3^- in 7 WWTP effluents, 5 of which incorporated N removal treatments (BNP and BNPT). Most of these samples showed high $\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$ values, which are indicative of denitrification. This reaction commonly occurs in WWTPs that incorporate biological treatments for nitrogen removal. An incomplete reaction will be reflected in the isotopic signature of the outflow, as the values will be out of the theoretical isotopic range.

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Moreover, the $\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$ values in WWTP effluents without specific N removal processes also showed slight denitrification, which may be due to the high organic carbon content in the wastewater. Incomplete denitrification in these effluents can lead to uncertainties in nitrate source identification once NO_3^- is incorporated into the environment. In contrast, nitrogen was detected in the form of NH_4^+ in 8 WWTP effluents, 4 of which incorporated nitrogen removal processes. We expected to observe higher $\delta^{15}N$-NH_4^+ values in WWTPs with N removal processes; however, the maximum $\delta^{15}N$-NH_4^+ value of +29.2 ‰ was observed in a WWTP with only biological treatment, while the minimum value of +9.7 ‰ was observed in a WWTP with N removal. Nevertheless, all $\delta^{15}N$-NH_4^+ values fell within the range of the $\delta^{15}N$ theoretical values reported for wastewater (Widory et al., 2005).

$\delta^{11}B$ values were within the bibliographic range for wastewater in ten of the WWTP effluents, while higher $\delta^{11}B$ values were observed in eight effluents. High $\delta^{11}B$ values have been previously observed in coastal areas due to the influence of B from marine intrusion and sea spray ($\delta^{11}B \sim$ +35.7 ‰) (Cary et al., 2015). This marine influence likely explains the slightly higher $\delta^{11}B$ observed in 3 WWTPs (WWTP-7, 17, and 18) in this study located close to the sea. However, it cannot explain the high $\delta^{11}B$ values observed in the remaining 5 WWTPs located away from marine environments. Adsorption-desorption processes caused by the interaction with clay minerals, organic matter, and iron and aluminum oxides can slightly increase $\delta^{11}B$ values (Xue et al., 2009 and references therein); however, these processes only cause limited fractionation of between 0 ‰ and +3 ‰ (Guinoiseau et al., 2018). As the measured $\delta^{11}B$ values in the five WWTPs were significantly higher than the theoretical range, the elevated values cannot be explained by adsorption-desorption processes alone. The presence of animal waste can also lead to higher $\delta^{11}B$ values, and MST markers for animal sources (Pig2Bac, CWBif) were detected in 13 of the WWTP effluents. A few samples also tested positive for pig mitochondrial DNA (Pomito), but this may been linked to human feces due to the human consumption of animal meat

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(Caldwell and Levine, 2009). At least three of the studied WWTPs receive waste effluents from abattoirs, food industries, and/or livestock activities (WWTP-3, 6, and 14). These 3 WWTPs had high values of both $\delta^{11}B$ (> +13.9 ‰) and Pig2Bac, which confirm the influence of animal wastewater. Similarly, another 2 WWTPs with $\delta^{11}B$ values exceeding the literature range for wastewater (WWTP-1 and 11) showed positive results for Pig2Bac or CWBif (Table SI-9). The effect of animal wastewater on $\delta^{11}B$ was clearly evident and should, therefore, be considered when $\delta^{11}B$ is used to discriminate between wastewater and manure pollution.

4.4 Evaluating the use of isotopes, MST, and land use for NO$_3^-$ source identification

The isotopes and MST results agree in most of the evaluated surface and groundwater samples (79 %). The complete agreement between isotopes and MST was particularly useful for nitrate pollution tracing in surface waters when the dominant nitrate source was linked to wastewater. MST analysis was also a suitable approach for groundwater—particularly for samples collected from relatively shallow wells (< 50 m) and/or when isotopes inferred organic influence from manure. The combination of both approaches also showed positive results in samples where the isotopic signal was masked. In those samples where the isotope and MST results agreed, the nitrate source identification was supported and finalized by land-use information. For instance, the surface water samples linked to human wastewater sources were located either within an urban area or downstream of a WWTP. Moreover, the origin of nitrate in samples classified as organic and positive for animal MST can be linked to either livestock activity effluents or manure (organic) fertilizer application when the samples are located close to livestock activities or agricultural areas, respectively.

This combined approach was mostly successful, but some samples did not entirely agree. Understanding the limitations of this approach is necessary for improving its future application.

Partial agreement was mainly observed in samples where nitrate was linked to an organic source but MSTs were not detected. This observation mainly occurred in groundwater samples. In these cases, the absence of MSTs may be attributed to their lower mobilization/infiltration in soil due to the soil structure, the nature of the target (bacteria, virus, or DNA), and/or the inactivation rate of MSTs (Foppen & Schijven, 2006; Mantha et al., 2017; Tran, Gin, & Ngo, 2015). Moreover, the low correlation between MST and FIB in groundwater samples suggests that the environmental conditions had a different effect on the indicators tested (Mantha et al., 2017). Microorganisms are more rapidly inactivated with time (Ballesté et al., 2018), and therefore, the absence of MST does not necessarily infer an absence of organic influence. Moreover, MST is likely to degrade during the lag time between fertilizer application to NO_3^- pollution in the aquifer (Sebilo et al., 2013). This is consistent with the predominant observation of MST in shallow groundwater samples as opposed to deep wells (data not shown) and in free aquifers compared to confined ones. Therefore, it is necessary to conduct further studies to analyze both soil infiltration and inactivation under groundwater conditions (low and stable temperatures, lack of irradiation, and low bacterial activity).

Partial agreement also occurred in samples where human and animal markers were detected and isotopic results were indicative of animal manure sources. This scenario was only observed in 3 samples (1 surface water and 2 groundwater). The surface water sample had significantly lower concentrations of HMBif compared with CWBif (1.1 and 2.9 $\log_{10}$ CFU mL^{-1}, respectively), which suggests that cattle manure was the main source of nitrate pollution. In contrast, the two groundwater samples had very low concentrations of both HMBif and GA17PH (0.5 $\log_{10}$ GC mL^{-1} and 0.8 $\log_{10}$ PFU mL^{-1}, respectively), inferring limited impacts from human pollution. Although the results do not perfectly agree, our findings suggest that the abundance of MST markers can confirm or discard potential organic contributions.

The most common disagreement scenario between the isotope and MST data occurred in samples where MST markers were detected and isotopes inferred a natural or inorganic NO_3^- pollution source. For some samples, the source of MST markers and/or $\delta^{11}B$ may not be directly related to the main source of NO_3^- pollution. For example, several WWTP effluents in this study showed high abundances of human markers but negligible N concentrations (either as NO_3^- or NH_4^+). In some cases, the low MST values can suggest a low influence from organic pollution. For instance, GW-7 had low concentrations of HMBif and CWBif but still tested positive for both markers (0.4 and 0.6 $\log_{10}$ GC 100 mL^{-1}, respectively). In some samples, anthropogenic pollution sources (both animal and human) were identified near the sample sites, which suggests that the positive MSTs were linked to a point pollution source. However, MST markers were relatively high in other samples where isotopes inferred an inorganic fertilizer source; this was particularly the case for cattle and poultry markers, with values of up to 2.29 and 1.76 $\log_{10}$ GC mL^{-1}, respectively. Compared with pig manure, cattle and poultry manure are typically less affected by NH_3 volatilization (Lockyer et al., 1989), which produces lower $\delta^{15}N$ values than the ranges of organic NO_3^- sources used in this study. For example, Widory et al. (2004) reported $\delta^{15}N$ values of +4.3 ‰ for cattle manure, which is within the defined range for inorganic fertilizer sources. Figure 9 shows the $\delta^{15}N$ and $\delta^{18}O$ of NO_3^- in samples with positive values of CWBif, PLBif, and/or Pig2Bac. The samples with CWBif and PLBif generally showed lower $\delta^{15}N$ values than those with Pig2Bac, which indicates that lower $\delta^{15}N$ values are expected in areas where the organic source is linked to cattle and/or poultry; this also overlaps with inorganic and soil N values. The identification of possible nearby cattle and poultry livestock facilities can therefore minimize uncertainties regarding the influence of these particular pollution sources.

Figure 9

The other disagreement scenario occurred in water samples (mainly in surface samples) where animal markers were detected but isotopic data inferred nitrate pollution from sewage. As

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previously mentioned, we identified the contribution of livestock industries in some of the WWTP effluents. We therefore expect some animal MSTs to occur in samples affected by sewage pollution.

In most of the cases, the isotopic and MST data agreed with land use and nitrate pressures in the surrounding area (Table SI-5 and SI-8). However, the behavior of NO_3^- in the environment and its retention time in soils must also be considered, as the long-term fate of nitrate in soils (Sebilo et al. 2013) is sometimes ignored in nitrate pollution studies. Consequently, current land use and anthropogenic pressures may not directly relate to nitrate in groundwater if agricultural activities have changed over the last decades. The land-use history and the lag time within the study area should therefore be considered for effective interpretations of the combined isotopic and biological tracer datasets. The presence of MST in groundwater can therefore be indicative of a dominant organic pollution source as well as rapid water infiltration into the aquifer, which is influenced by soil porosity and saturation.

Overall, MST indicators can complement the multi-isotopic approach for more accurate determinations of the dominant nitrate pollution sources as well as minimize generalized assumptions. The combination of both indicators particularly improved the identification of nitrate sources in all surface water samples and in groundwater samples influenced by an organic source. We therefore recommend the application of this novel approach for the effective identification of the dominant nitrate pollution sources. To successfully apply this approach, the $\delta^{15}N$ and $\delta^{18}O$ of dissolved NO_3^- should first be assessed. If the isotopic data infers a nitrate origin linked to an inorganic source, $\delta^{11}B$ should only be analyzed when relevant livestock activities are identified. MST markers—particularly for cattle and poultry manure—should be assessed if the $\delta^{11}B$ indicates animal contributions. However, $\delta^{11}B$ as well as MST indicators should be analyzed if the N and O isotopic composition of dissolved nitrate suggests an organic or uncertain source. Moreover, the depth of the well and type of aquifer must be

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considered, as the analysis of MST markers is only recommended for shallow wells and free aquifers rather than deep wells and confined aquifers. This proposed methodology can minimize analytical costs, as not all parameters need to be analyzed at each sampling site. The methodology can also successfully characterize the NO_3^- pollution of a single well. The accurate identification of nitrate pollution sources is crucial for water resources management authorities. As a result, the Catalan Water Agency (ACA) from the Catalan Government has reviewed a number of uncertain cases based on the conclusions of this study and subsequently defined new vulnerable zones of nitrate pollution in Catalonia according to the requirements of the EU Nitrate Directive (91/676/CEE).

Conclusions

The isotopic and MST marker datasets showed complete or partial agreement in 79 % of the surface and groundwater samples analyzed. This suggests that both analyses are complementary and can provide more reliable information on the source of nitrate contamination in both surface and groundwater. The main disagreement between the two methods was in areas where MST markers were detected but nitrate pollution was isotopically linked to an inorganic source (inorganic fertilizers). This discrepancy may occur when chemical fertilizers are the main source of nitrate; however, the contribution of manure cannot be entirely dismissed.

The isotopic and MST marker datasets showed higher agreement in areas with larger organic source contributions; therefore, their combined application is more robust in areas where nitrate contamination is predominantly organically-derived. The percentage of agreement between the two methods differed between the type of samples analyzed: for groundwater, stronger agreement occurred in samples that included animal MST markers and were

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isotopically linked to manure nitrate sources; for surface water, stronger agreement occurred in samples that included human MST markers and were isotopically linked to sewage sources.

Due to the lack of field studies for isotopic characterization, literature values are usually employed to define the isotopic range for sewage. However, the MST results demonstrated clear isotopic variability for sewage. It is, therefore, necessary to locally characterize the isotopic composition of sewage in order to effectively interpret the isotopic data for nitrate source identification. The MST analysis of the WWTP effluents provided important information on livestock and its effect on $\delta^{11}B$. As well as improving nitrate source identification, the combination of the two approaches can also provide additional pollution information, such as the presence of secondary pollution sources.

The methodology described in this study can be used by local water authorities to identify surface and groundwater nitrate sources, establish new vulnerable zones, and select specific sample points for periodic control. Due to the large variability of the hydrogeological and geographical contexts evaluated as well as the good general results obtained of the present approach, we conclude that this multidisciplinary methodology can be successfully applied for researchers and water managers in many different areas and contexts.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Table 1: Number of samples for each origin of nitrate identified in surface water samples considering isotopic, FIB and MST markers detected.

Classification δ^{15}N δ^{18}O		Classification δ^{11}B		FIB			MST	
				E. coli	Enterococci	Somatic coliphages	HM	Animal
							HM MST markers	Animal MST markers
Inorganic	4	Mix	**1**	0	1	1	0	0
		Animal	**2**	1	1	2	0	1
		Marine	***1***	*0*	*1*	*1*	*0*	*0*
Organic	16	Human	**5**	5	5	5	2	3
		Mix	**8**	7	7	6	5	3
		Animal	**2**	2	2	2	0	1
		Marine	***1***	*0*	*1*	*1*	*0*	*0*
Uncertain	16	Human	**4**	4	4	4	3	3
		Mix	**7**	7	7	6	2	4
		Animal	**3**	3	3	2	2	1
		Marine	***1***	*1*	*1*	*1*	*1*	*0*

Table 2: Number of samples for each origin of nitrate in groundwater samples identified considering isotopic data, FIB and MST markers detected.

Classification δ^{15}N δ^{18}O		Classification δ^{11}B		FIB			MST	
				E. coli	Enterococci	Somatic coliphages	HM MST markers	Animal MST markers
Soil - N	8	-	8	1	2	0	1	1
Inorganic	45	not analyzed	26	4	4	0	1	2
		Inorg. fertilizer	6	3	3	1	4	3
		Mix	4	0	0	0	2	1
		Animal	8	1	3	1	3	6
		Marine	1	0	0	1	1	0
Organic	29	Human	5	1	0	0	1	2
		Mix	6	1	2	0	0	3
		Animal	17	2	8	4	2	4
		Marine	1	0	0	0	0	0
Uncertain	64	not analyzed	16	2	7	0	1	1
		Human	16	4	5	1	4	6
		Mix	7	1	2	2	2	2
		Animal	22	5	8	4	3	7
		Marine	3	1	2	0	1	0

Table 3: Classification of the agreement between isotopes and MST considering different scenarios found in surface and groundwater samples.

Agreement	Conditions		Num Surface water samples	Num Groundwater samples
	Source of Nitrate using isotopes	Markers		
Complete	Inorganic/Chemical fertilizers	Without markers	1/39	47/149
	Organic nitrate related to manure	Animal marker	4/39	9/149
	Organic nitrate related to sewage	Human marker	5/39	1/149
	Mixed: organic and inorganic	Some marker (Human and/or animal)	17/39	20/149
Partial	Mixed: Inorganic and organic nitrate	Without markers	0/39	11/149
	Organic nitrate	Without markers	2/39	26/149
	Organic nitrate related to manure	With both, human and animal marker	0/39	2/149
Disagreement	Organic nitrate related to manure	Human marker	1/39	3/149
	Organic nitrate related to sewage	Animal marker	3/39	2/149
	Inorganic/Chemical fertilizers	Any marker	4/39	21/149
	Mixed: Inorganic and organic (manure)	Human marker	0/39	2/149
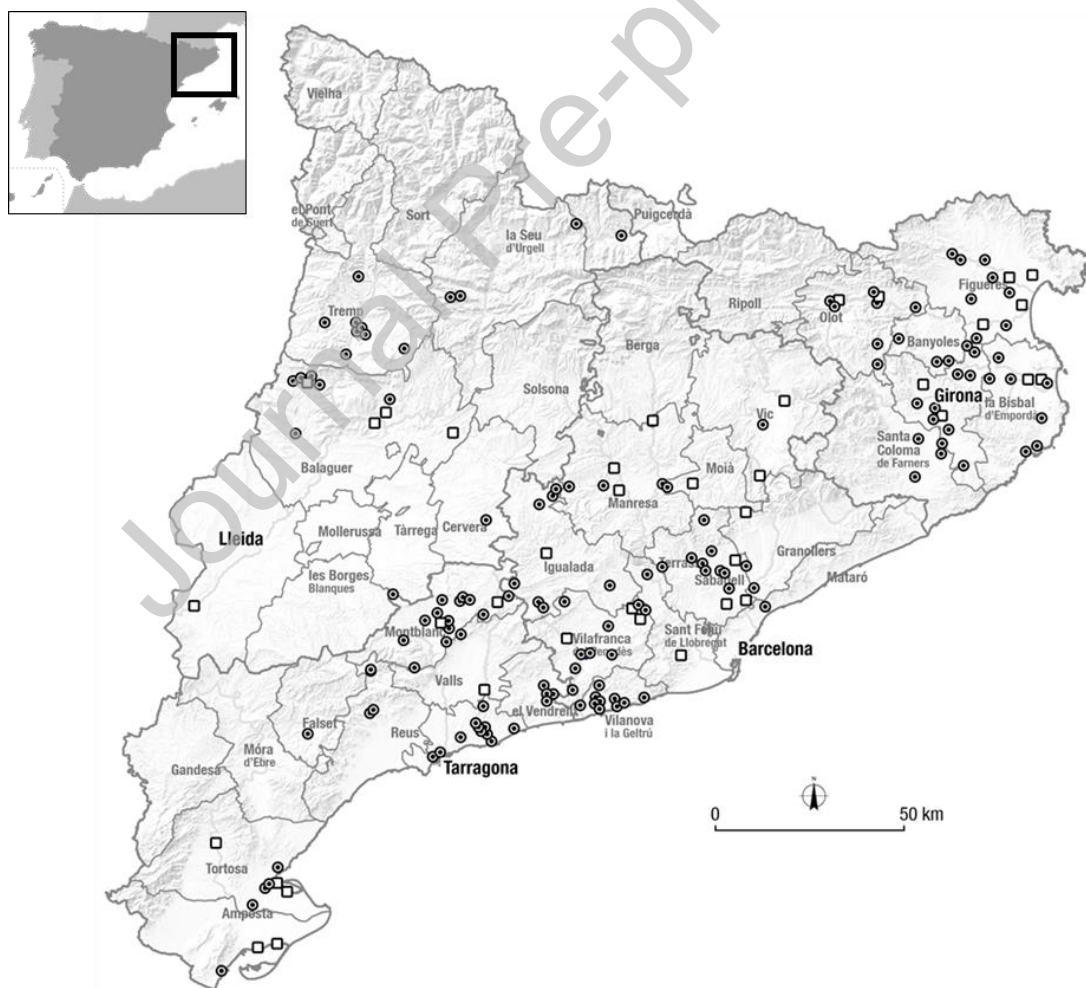
Fig. 1. Location map of the surface (squares) and groundwater samples (circles) analyzed in this study.
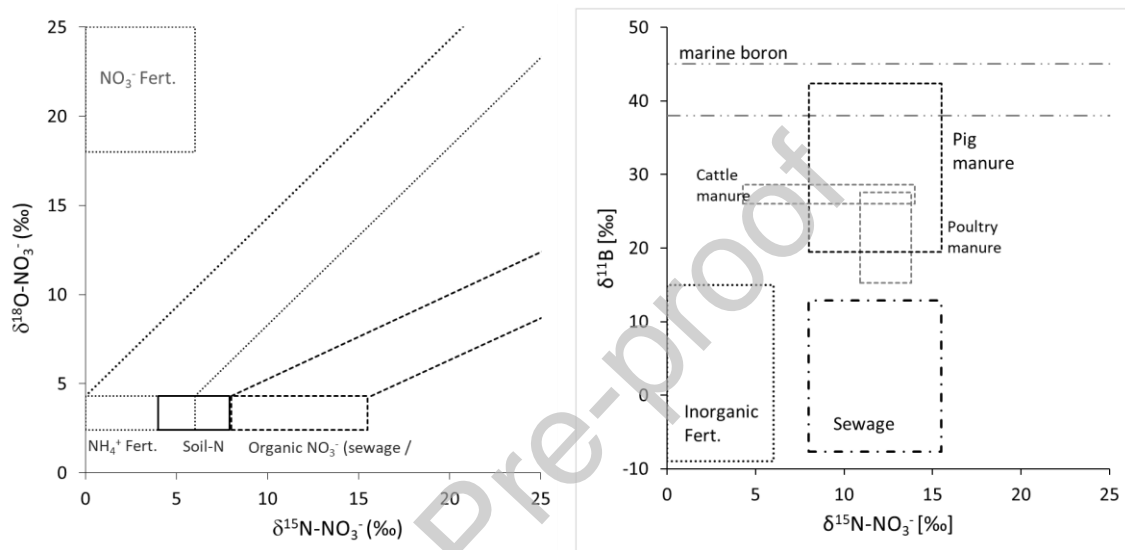
Fig. 2. A: the δ^{15}N-NO$_3^-$ vs. δ^{18}O-NO$_3^-$ ranges of the main nitrate sources (Xue et al., 2009, Mengis et al., 2001; Vitòria et al., 2004, 2008). The δ^{18}O values of nitrate derived from NH$_4^+$ nitrification were calculated following the experimental expressions of Andersson and Hooper (1983) and Hollocher (1984) using the δ^{18}O-H$_2$O range of the study area (-10.5–-5.1 ‰, n = 20) and a δ^{18}O-O$_2$ value of +23.5 ‰ (Kroopnick and Craig, 1972). The denitrification lines were calculated using the extreme εN/εO values of 2.1 (Böttcher et al., 1990) and 1.3 (Fukada et al., 2003) as reported in the literature. B: the δ^{15}N-NO$_3^-$ vs. δ^{11}B ranges of the main nitrate sources (Guinoiseau et al., 2018; Vengosh et al., 1994; Widory et al., 2005, 2004).
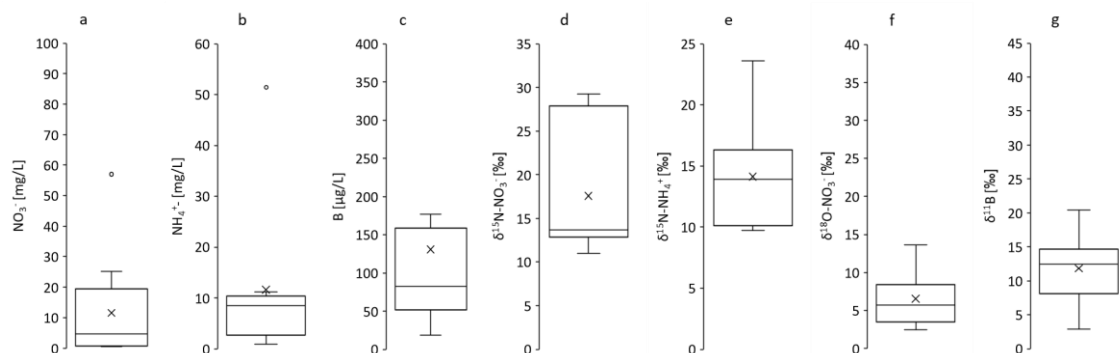
Fig. 3. Boxplots of nitrate (a), ammonium (b), boron (c), δ^{15}N-NO$_3^-$ (d), δ^{15}N-NH$_4^+$ (e), δ^{18}O-NO$_3^-$ (f), and δ^{11}B (g) in sewage effluents (x = mean, center line in box = median, lower and upper box sections = lower and upper quartiles, whiskers = 5 and 95 percentiles, o = outer values).
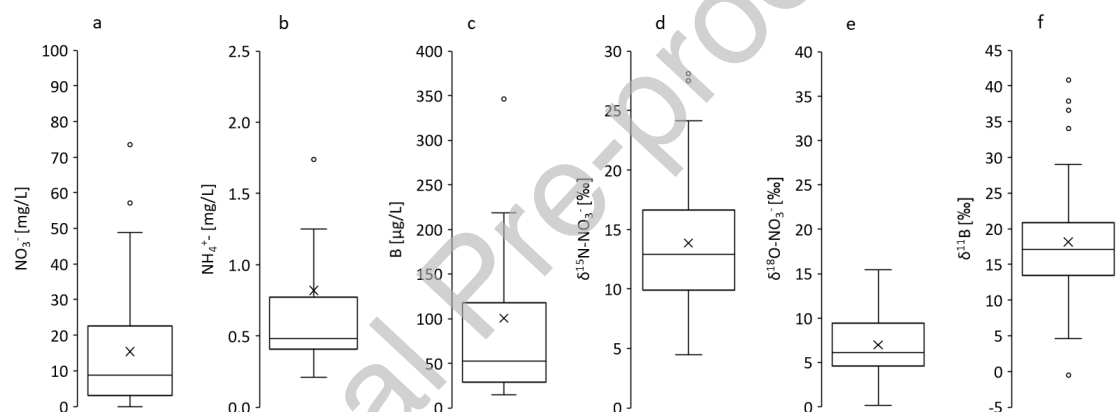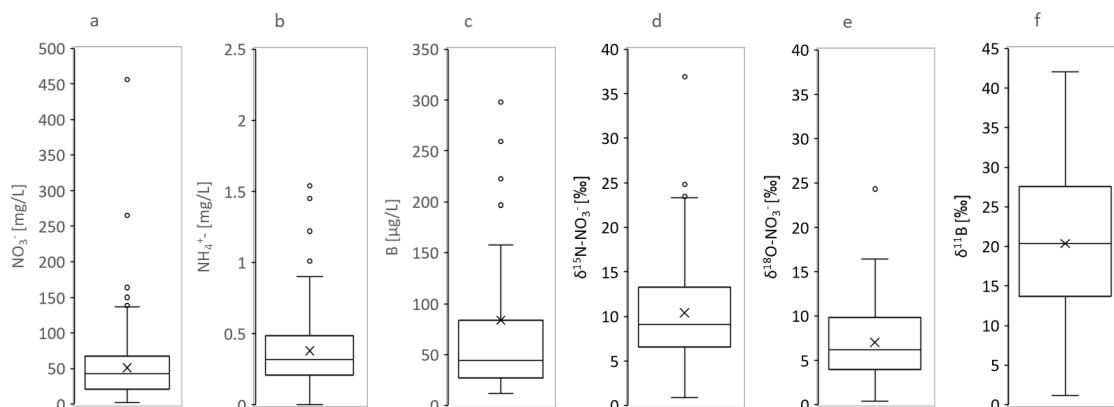

Fig. 4. Boxplots of nitrate (a), ammonium (b), boron (c), δ^{15}N-NO$_3^-$ (d), δ^{18}O-NO$_3^-$ (e), and δ^{11}B (f) in surface water samples (x = mean, center line in box = median, lower and upper box sections = lower and upper quartiles, whiskers = 5 and 95 percentiles, o = outer values.

Fig. 5. Boxplots of nitrate (a), ammonium (b), boron (c), δ^{15}N-NO$_3^-$ (d), δ^{18}O-NO$_3^-$ (e), and δ^{11}B (f) in groundwater samples (x = mean, center line in box = median, lower and upper box sections = lower and upper quartiles, whiskers = 5 and 95 percentiles, ○ = outer values.
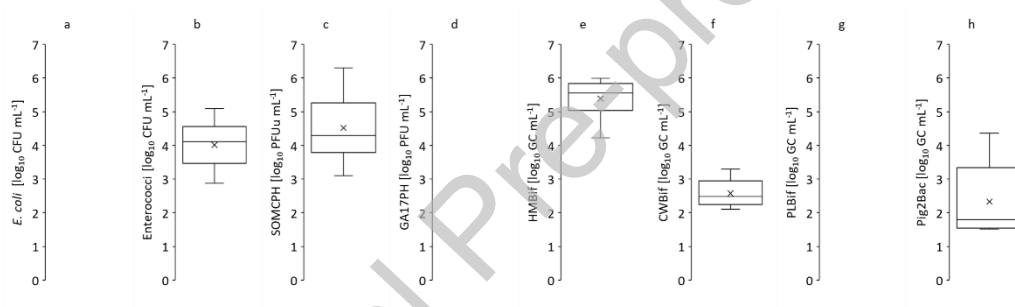
Fig. 6. Boxplots of *E. coli* (a), Enterococci (b), SOMCPH (c), GA17PH (d), HMBif (e), CWBif (f), PLBif, (g) and Pig2Bac (h) in sewage effluents (x = mean, center line in box = median, lower and upper box sections = lower and upper quartiles, whiskers = 5 and 95 percentiles, ○ = outer values.
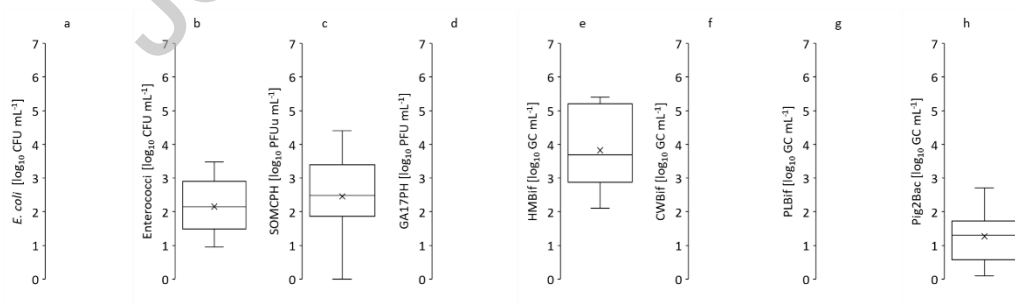
Fig. 7. Boxplot showing *E. coli* (a), Enterococci (b), SOMCPH (c), GA17PH (d), HMBif (e), CWBif (f), PLBif (g), and Pig2Bac (h) in surface water samples (x = mean, center line in box = median, lower

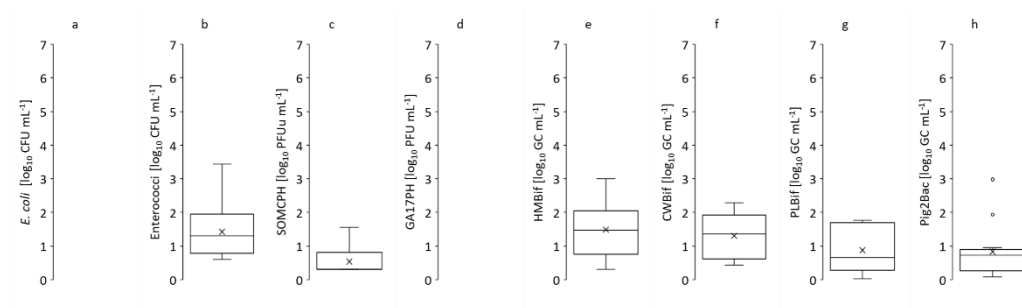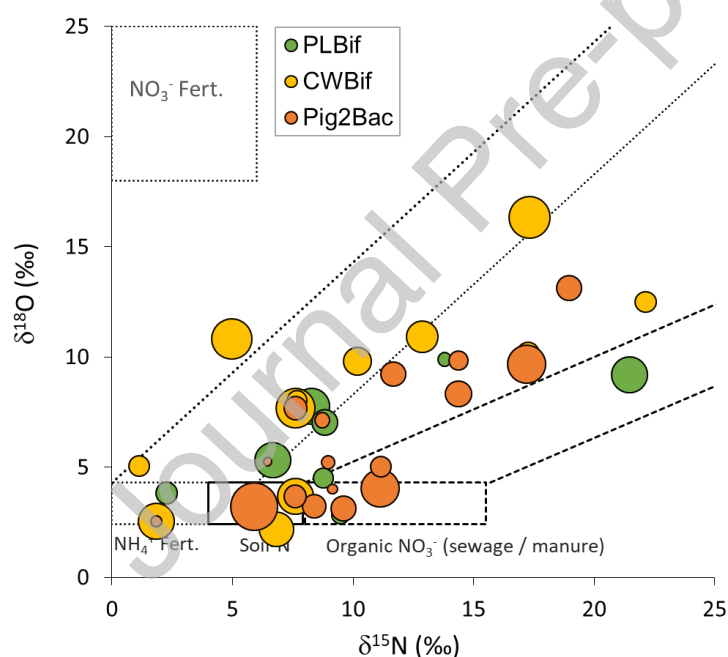and upper box sections = lower and upper quartiles, whiskers = 5 and 95 percentiles, ○ = outer values.

Fig. 8. Boxplots of *E. coli* (a), Enterococci (b) SOMCPH (c), GA17PH (d), HMBif (e), CWBif (f), PLBif (g), and Pig2Bac (h) in groundwater samples (x = mean, center line in box = median, lower and upper box sections = lower and upper quartiles, whiskers = 5 and 95 percentiles, ○ = outer values.
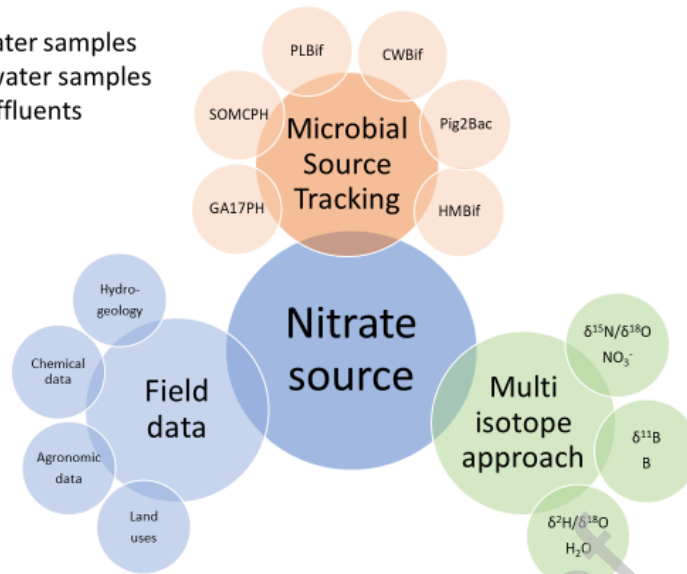

Fig. 9. δ^{15}N and δ^{18}O of NO_3^- in the groundwater samples that showed positive values for CWBif, PLBif, and Pig2Bac. The size of the points is proportional to the magnitude of the MST concentration.

149 grounwater samples
39 surface water samples
18 WWTP effluents

Graphical abstract