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Isotope and microbiome data provide complementary information to identify natural nitrate attenuation processes in groundwater

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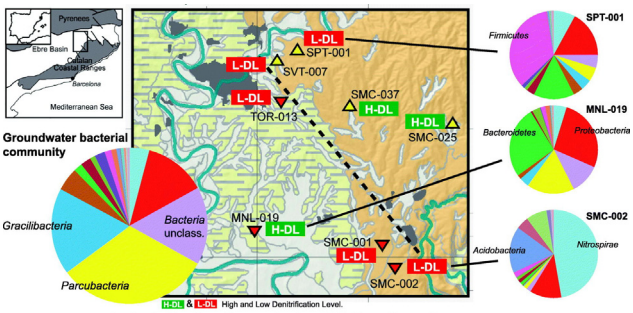
HIGHLIGHTS

- Isotopic data assess the occurrence and rate of denitrification at the aquifer scale.
- Denitrification gene abundance but not genera correlate with isotopic values.
- Gene information characterizes the occurrence of denitrification near the well.
- Isotopic and gene information contribute to the design of induced attenuation.

GRAPHICAL ABSTRACT



Isotopic fractionation and microbial diversity analyses are complementary in explaining denitrification level in groundwater.

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ABSTRACT

Natural attenuation processes alleviate the impact of fertilization practices on groundwater resources. Therefore, identifying the occurrence of denitrification has become a requirement for water quality management. Several approaches are useful for this purpose, such as isotopic and microbiological methods, each of them providing distinct but complementary information about denitrification reactions, attenuation rates and their occurrence in the aquifer. In this paper, we investigate the contribution of both approaches to describe denitrification in a consolidated rock aquifer (limestone and marls), with a porosity related to fracture networks located in the northeastern sector of the Osona basin (NE Spain).

Isotopic methods indicated the origin of nitrate (fertilization using manure) and that denitrification occurred, reaching a reduction of near 25% of the nitrate mass in groundwater. The studied area could be divided in two zones with distinct agricultural pressures and, consequently, nitrate concentrations in groundwater. Denitrification occurred in both zones and at different levels, indicating that attenuation processes took place all along the whole hydrogeological unit, and that the observed levels could be attributed to a larger flow path or, in a minor extent, to mixing processes that mask the actual denitrification rates.

Microbiological data showed a correlation between denitrifier genes and the isotopic composition. However, the groundwater microbiome and the distribution of denitrifying bacteria did not reveal a major influence on the denitrification level observed by isotopic methods. This focuses the interest of microbiological analysis to identify functional genes within the bacteria present in the aquifer.

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Results indicated that isotopic methods provide information of the overall denitrification ability of the hydrogeological unit, and that genomic data represent the processes actually acting nearby the well. A combination of both approaches is advised to support induced in situ attenuation actions in polluted sites.

1. Introduction

Groundwater nitrate pollution, as a general worldwide issue, is a current topic in scientific research and water planning forums (e.g. Galloway et al., 2008; Sutton, 2011). Mostly originated by the intensive use of fertilizers produced by livestock raising, nitrate consequences on human health (WHO, 2016) as well as those to the environment (Vitousek et al., 1997; Wilson et al., 1999; Mason, 2002) have been broadly exposed. In front of the resilience and persistence of nitrate in groundwater due to ancient and current fertilization practices in agricultural areas (Böhlke et al., 2002), the occurrence of natural attenuation processes, mainly due to denitrification (Rivett et al., 2008), must be identified and the conditions under they exist should be maintained or enhanced. Therefore, denitrification studies gain importance as a means to understanding the management of nitrate pollution.

Nitrate mass removal in groundwater can occur by autotrophic or heterotrophic denitrification, depending on the availability of organic matter in the environment and redox conditions. Denitrification occurs as a series of sequential enzymatic reactions, mainly catalysed by microorganisms, that causes reduction of nitrate to nitrogen gas in a step-wise process (Zumft, 1997; Rivett et al., 2008). Several methods exist to infer denitrification occurrence in the subsurface. Among these methods, isotope pairing and molecular tools are the most used.

Denitrification can be identified in its occurrence and extent by a coupled isotopic analysis of nitrogen and oxygen of the nitrate molecule. This process causes an increase of the $\delta^{15}N$ and $\delta^{18}O$ values of the residual nitrate by means of isotopic fractionation (e.g., Kendall, 1998), with a $\varepsilon_N/\varepsilon_O$ ratio that ranges from 1.3 to 2.1 (Böttcher et al., 1990; Fukada et al., 2003); where ε is the isotopic fractionation. Autotrophic denitrification in which pyrite (FeS_2) acts as the main electron donor in carbon-limited systems can also be traced with the use of sulfur and oxygen isotopes (Moncaster et al., 2000; Vitòria et al., 2008; Otero et al., 2009). Otherwise, in dissolved organic carbon rich environments, heterotrophic denitrification can be identified by nitrate isotopes and carbon isotopes in bicarbonate derived from the oxidation of organic matter (Aravena and Robertson, 1998); yet other sources of alkalinity may mask the imprint of denitrification in the carbon isotopic signature (Puig et al., 2017).

Denitrification activity, being predominantly a microbial process, can be inferred by measuring the abundance of key genes in the process (García-Lledó et al., 2011). In this sense, a suite of molecular probes targeting functional genes involved in denitrification have been designed to study the abundance and diversity of denitrifying microbial communities (Hallin et al., 2009; Jones et al., 2013). Among these molecular markers, genes coding for nitrate reductases (*narG* and *napA*), dissimilatory nitrite reductases (*nirS* and *nirK*), nitric oxide reductases (c*norB* and q*norB*), and nitrous oxide reductases (*nosZ* types I and II), have been widely used in many environments and sample types (Graf et al., 2014; Jones et al., 2013; Philippot, 2002; Zumft, 1997). The complete set of genes may exist in a bacterial species, but most likely denitrifying organisms possess only truncated pathways which reinforces the need of synergistic relationships among different bacterial species to complete nitrate reduction to nitrogen gas (Jones et al., 2008, 2013). Truncated denitrification is a relevant source of the greenhouse gas N_2O (Müller et al., 2014). In fact, different authors showed that NO_2^- and N_2O could accumulate in aquifers due to incomplete denitrification thus contributing to the emission of N_2O to the atmosphere (Barrett et al., 2013; Böhlke et al., 2002; Otero et al., 2009). Some researchers have demonstrated the role of sediment composition and texture together with the groundwater oxidation state as key factors influencing the abundance of denitrifiers (Santoro et al., 2006; Sei et al., 1999). In addition, Barrett et al. (2013) showed that the abundance of denitrifying genes in groundwater was not strictly dependent on land management strategies, although the increase in DOC and the depth of the water table were positively correlated with increasing *nir* and *nosZ* denitrifier abundances.

Groundwater environments are usually characterized by low organic matter content, especially in consolidated rock aquifers. Therefore, nitrate attenuation is usually achieved by autotrophic denitrification (Jahangir et al., 2013). There are evidences that reduction of nitrate by Fe^{2+} can occur either biotically or abiotically in groundwater. Biotic nitrate reduction in the presence of Fe^{2+} is mainly catalysed by denitrification coupled to microbial oxidation of pyrite. This has been widely studied in groundwater and lab-scale fermenters and, in most cases, pyrite dependent denitrification can be catalysed by different *Proteobacteria*, *Bacteroidetes*, *Firmicutes* and *Verrucomicrobia* (Pu et al., 2015; Rivett et al., 2008; Torrentó et al., 2011).

Previous work in the Osona region (Central Catalonia, NE Spain) have proved the occurrence of denitrification processes in a severely nitrate polluted aquifer originated from manure fertilizers (Otero et al., 2009; Menció et al., 2011a). This region lays on sedimentary formations of transitional and marine origin that constitute multi-layer fractured aquifers, together with localized alluvial formations. Groundwater supplies the main agricultural and farming water demand. Nitrate content in groundwater is easily above the 50 mg NO_3/L limit for drinking, reaching up to 500 mg NO_3/L in some wells (Menció et al., 2011a; Boy-Roura et al., 2013b). Because of such intense pollution, natural springs also show concentrations above this limit (Menció et al., 2011b), and their nitrate content remains uniform, even during strong rainfall events, due to the effect of long time intense fertilization practices (Boy-Roura et al., 2013a). Urban and domestic supply is thus restricted to surface water because of the high pollution levels in groundwater resources. In this sense, the Osona region presents the appropriate hydrogeological conditions, as well as social and management concerns where to confront isotopic and microbiological data with the aim to better characterize the processes that control denitrification in its aquifers.

The goal of this study was to elucidate the complementarity between isotopic and microbiological information; that is, to show how these data mutually supply each other's lack, and contribute to characterize the denitrification potential along the groundwater flow path. In this sense, data from the isotope-based approach are compared to the microbiological information for which key molecular markers (genes) for bacterial denitrification are quantified. Furthermore, the structure of the microbial community is also analysed as a means to identify the bacteria potentially participating in the regional nitrate transformation pathways. According to the authors' knowledge, there are very few scientific reports combining these two approaches in groundwater research (Kim et al., 2015). Our study is based in samples from eight wells of the Osona area, already monitored in previous studies (Otero et al., 2009; Menció et al., 2011b; Boy-Roura et al., 2013b), suspected to contain specific bacteria for pyrite based autotrophic denitrification; hence, an accurate approximation to the nitrate reduction potential in this environmentally sensitive area, based on both approaches, is expected.

2. Geographical and geological setting

The Osona region is located approximately 60 km to the north of Barcelona (NE Spain), in the inland basins of Catalonia (Fig. 1). It constitutes a geomorphological basin surrounded by ranges that attain

1300 m asl in its north-eastern and eastern limit. The basin is drained by the Ter River that frames the study area in its west and south boundaries (see inset in Fig. 1). With an area of 1260 km^2 and a total population of 154,000 inhabitants, this is an intensive agricultural and livestock production area with >740,000 head of pigs, and 65,000 head of cattle (Menció et al., 2016). Slurry and manure produced by husbandry activities are used as the main fertilizer for crops, although synthetic fertilizers are also applied. Because of orographic reasons, most of the arable land in Osona is located at the basin rather than in the surrounding ranges. Consequently, a major application of manure is expected at low altitude areas. Osona has been classified as Nitrate Vulnerable Zone, according to the Nitrate Directive (Directive 91/676/EC).

The geological setting of the Osona region consists of a sequence of Paleogene sedimentary layers with a total thickness of approximately 1500 m, which overlies the igneous and metamorphic rocks of the Hercynian basement (Fig. 1). The study area is situated at the north-eastern part of Osona, where the sedimentary formations comprise a thick (≈500 m) basal level of conglomerates, overlain by an alternation of carbonate formations, with calcareous, marl and carbonate sandstone layers (≈1000 m; see stratigraphic column and geologic cross-section in Abad, 2001, and Menció et al., 2011a). In particular, the wells chosen for this study exploit productive levels constituted by silt, sandstones and marls layers. No major faults or tectonic structures affect neither the geology of the studied area, nor its hydrogeological dynamics. Joints and fractures affect most of the sedimentary rocks and they constitute the main porosity of these layers. Mean annual rainfall is about 585 m, and actual evapotranspiration reaches 480 mm.

3. Methodology

Eight selected wells from a wider database from previous studies were sampled in summer 2014 to analyse the denitrification processes,

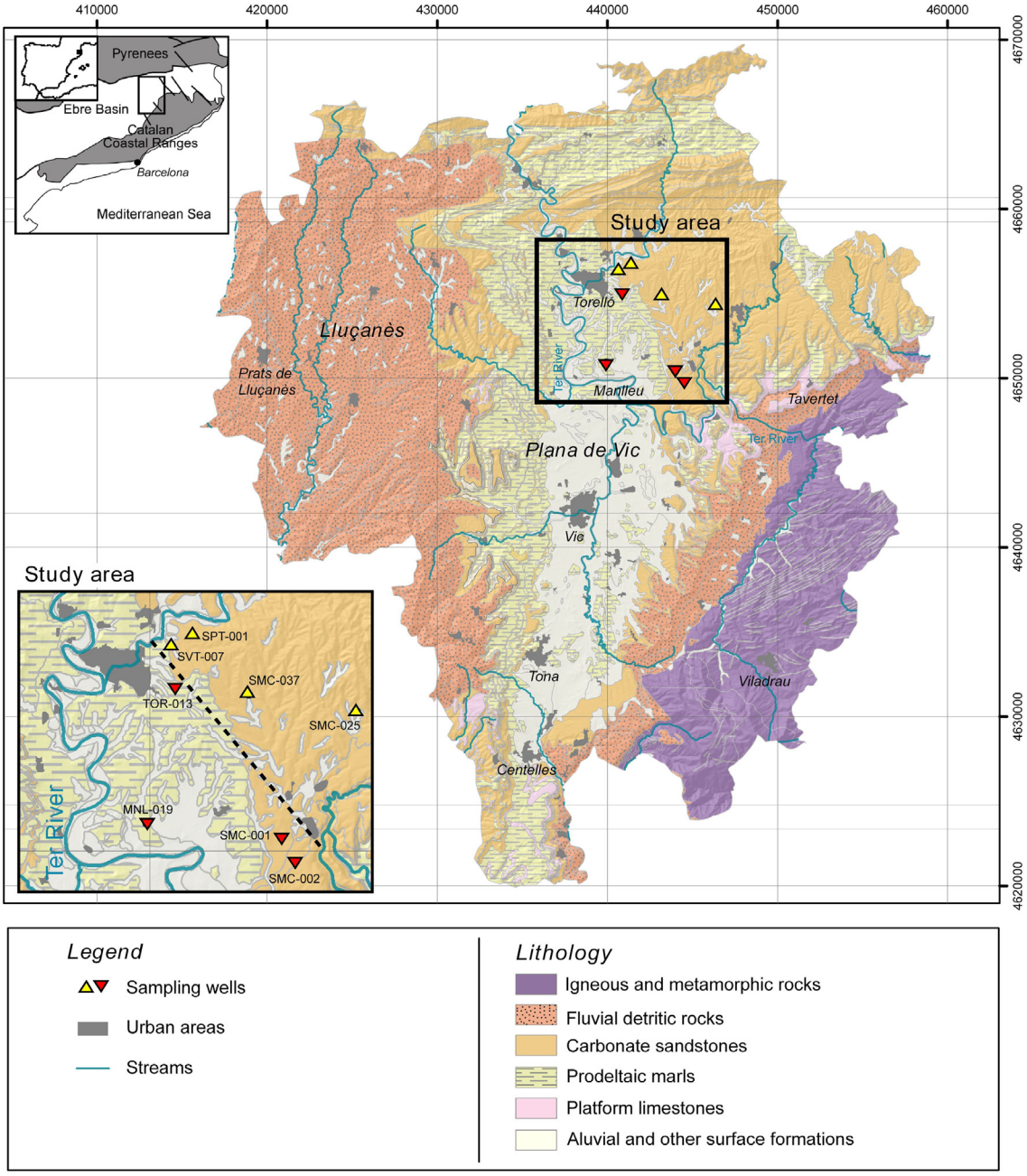

Fig. 1. Geographic and geologic setting of the study area. Geological map after ICGC (2017). Dashed line in the zoomed image indicates separation between NE and SW wells.

as an example of autotrophic denitrification in a fractured aquifer, and using isotope and microbiology approaches. Samples have been grouped in two distinct sets according to their geographical location: a first set includes wells located in the NE zone (SMC-025, SMC-037, SPT-001, SVT-007), and the second those in the SW zone (MNL-019, SMC-001, SMC-002, TOR-013; Fig. 1). Well depths ranged from 60 to 115 m (Table 1). Boreholes are usually uncased below the surface formations, so withdrawn water becomes a mixture of all the productive levels intercepted by the well. Potentiometric levels could not be recorded because boreholes are usually sealed. Regional hydraulic head distribution is taken from Menció et al. (2011a).

Supplementary Material 1 (SM1) provides the methodological details on hydrochemical, isotopic and microbiological analyses. As a brief summary, physico-chemical parameters (pH, Eh, electrical conductivity (EC), dissolved oxygen (DO) and temperature) were measured in situ with a flow cell to avoid contact with the atmosphere. Samples were stored at 4 °C in a dark environment for subsequent analyses of the hydrochemical parameters in the laboratory.

Isotopic data ($\delta^{18}O$ and δD) of water samples are expressed in terms of the ‰ deviation of the isotope ratio of the sample relative to that of the V-SMOW standard. Reproducibility of the samples are ±0.06‰ for $\delta^{18}O$ and ±0.7‰ for δD. $\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$ analysis of dissolved NO_3^- are expressed in terms of δ (‰) relative to that of AIR (atmospheric N_2) as the international standard for $\delta^{15}N$, and with respect to V-SMOW for $\delta^{18}O$. Precision (≡1σ) are ±0.3‰ and ±0.4‰, for $\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$, respectively.

For microbiological analyses, total DNA was extracted from filters, and DNA extracts were stored at −20 °C. DNA concentration was determined using Qubit® 2.0 fluorometer (Invitrogen, Molecular Probes Inc., Oslo, Norway). The abundance of denitrifying bacteria was estimated by quantification of key functional genes (*nirK*, *nirS*, *nosZI* and *nosZII*) using quantitative PCR (Supplementary Material 2). Abundance of dissimilatory nitrite reductase gene (*nrfA*) was also inferred to estimate the potential dissimilatory nitrite reduction to ammonia (DNRA) in the water samples. In addition, 16S rRNA gene was quantified and used as a proxy to the total abundance of bacteria. qPCR efficiency ranged between 80 and 106% in all reactions. Controls without templates gave null or negligible values.

The analysis of microbial community structure was conducted after the sequencing of 16S rRNA gene. The prokaryotic 16S rRNA V3-V4 region was amplified using dual indexed Illumina compatible primers Pro341F/Pro806R as described previously (Takahashi et al., 2014).

Alpha-diversity indicators of richness (Observed richness) and diversity (Shannon and phylodiversity index) were calculated after normalization of the number of sequences in each sample by randomly selecting a subset corresponding to the lowest amount of sequences found in a sample. Weighted and unweighted Unifrac distances were calculated and samples clustered in a PCoA in order to analyse beta-diversity of microbial communities.

4. Results and discussion

4.1. Hydrogeological dynamics and hydrochemistry

The NE zone of the study area is located on carbonate sandstone formations, while the wells of the second group were drilled in prodeltaic marls. Both lithologies own their main porosity to the fracture network. Alluvial formations, and therefore agricultural land uses, are more extended in the SW zone, within the Osona basin. Wells in the NE zone are located in a hilly area that forms the surrounding ranges of the Osona basin. In a broad sense, it constitutes the up gradient area of the regional flow field, where a general flow path NE-SW can be drawn for the regional groundwater system. Local orography and the influence of the Ter River in the south-western boundary control local scale flow systems that might affect the capture zone of the shallowest wells (60 m depth). In summary, it is not possible to delineate a flow line that links all (or part of) the sampled wells; as they were selected based on data from previous research and they are sparsely distributed in the study area.

Given the similar mineralogy of both carbonate sandstones and prodeltaic marls, a comparable hydrochemical composition of all samples should be expected. Accordingly, the major hydrochemical facies is calcium-bicarbonate, yet samples from the SW zone show a significant increase of sulphate (TOR-013 and MNL-19; Fig. 2, Table 1), and two samples (SMC-025 and MNL-019), one from each zone, present a larger percentage of sodium. Despite hydrochemical similarity, small differences among samples are

Table 1
Hydrochemical properties of sampled wells obtained in July 2014.

Area	NE area					SW area				
Sampling point	SMC-025	SMC-037	SPT-001	SVT-007	Mean values	SMC-001	SMC-002	MNL-019	TOR-013	Mean values
Well depth	80	67	100	90		110	115	60	70	
EC (μS/cm)	968	802	832	668	818 ± 61.5[a]	949	889	1215	1061	1029 ± 71.7[a]
pH	7.25	7.26	7.20	7.52	7.31 ± 0.07[a]	7.09	7.03	7.23	7.16	7.13 ± 0.04[a]
pE	6.09	5.76	6.41	5.72	6.00 ± 0.16	6.76	7.29	5.08	7.61	6.69 ± 0.56
O_2 (mg/L)	6.60	1.65	0.26	8.42	4.23 ± 1.95	1.04	0.81	0.07	3.13	1.26 ± 0.66
T (°C)	16.0	16.4	18.0	18.2	17.2 ± 0.6	17.1	18.0	16.2	15.3	16.7 ± 0.6
HCO_3^- (mg/L)	468.5	414.8	417.2	400.2	425.2 ± 14.9	414.8	461.2	419.7	392.9	422.2 ± 14.3
Cl^- (mg/L)	32.8	30.4	22.8	11.3	24.3 ± 4.8[a]	48.2	35.9	71.1	48.8	51.0 ± 7.3[a]
SO_4^{2-} (mg/L)	98.4	91.2	68.4	33.9	73.0 ± 14.5	144.6	107.7	213.3	146.4	153.0 ± 22.0
Na^+ (mg/L)	65.1	26.8	19.2	11.3	30.6 ± 11.9	35.1	25.1	90.9	21.4	43.1 ± 16.2
K^+ (mg/L)	17.1	2.7	3.4	2.8	6.5 ± 3.5	2.2	4.1	8.0	4.1	4.6 ± 1.2
Mg^{2+} (mg/L)	41.5	32.6	35.4	25.6	33.8 ± 3.3[a]	45.9	29.1	56.2	44.0	43.8 ± 5.6[a]
Ca^{2+} (mg/L)	118.5	128.2	136.5	123.2	126.6 ± 3.8[a]	139.8	157.3	133.6	176.9	151.9 ± 9.7[a]
IC (mg/L)	104.1	92.2	110.1	88.0	98.6 ± 5.1	92.2	102.5	93.3	86.2	93.6 ± 3.4
TOC (mg/L)	6.8	2.8	1.3	2.1	3.3 ± 1.2	1.9	1.8	1.9	2.4	2.0 ± 0.1
TC (mg/L)	110.9	95.0	111.4	90.1	101.9 ± 5.5	94.1	104.3	95.2	88.6	95.6 ± 3.3
NH_4^+ (mg/L)	0.007	0.006	0.038	0.034	0.021 ± 0.009	0.007	0.007	0.007	0.090	0.028 ± 0.021
NO_2^- (mg/L)	<0.004	<0.004	<0.004	0.010	0.010	<0.004	0.025	0.226	0.004	0.085 ± 0.061
NO_3^- (mg/L)	16.6	25.8	23.5	26.3	23.1 ± 2.2[a]	54.6	71	69.6	89.17	71.09 ± 7.08[a]
TN (mg/L)	3.8	6	5.5	6.1	5.4 ± 0.5[a]	13.1	17.1	16.8	21.6	17.2 ± 1.7[a]
P-PO_4^{2-} (mg/L)	0.032	<0.003	0.005	0.003	0.013 ± 0.008	<0.003	<0.003	<0.003	0.003	0.003
TOC:TN	29.38	15.88	20.37	14.74	20.09 ± 3.33	7.19	6.10	5.66	4.10	5.76 ± 0.64
H_2S (mg/L)	0.040	0.007	0.006	0.023	0.019 ± 0.008	0.009	0.028	0.020	0.023	0.020 ± 0.004

Legend: mean values, mean ± standard error.
[a] Parameter with significant difference between areas (p-value < 0.05, Mann-Whitney *U* test).

consistent with the general flow field; and more importantly, both groups of samples largely differ on their nitrate content, which can be initially attributed at a larger proportion of arable land in the SW zone. Mean nitrate concentration in the NE zone wells is of 23 ± 2 mg/L, and in the SW zone wells of 71 ± 7 mg/L. Besides sulphate and nitrate, DO, EC also show a notable difference between those zones. Cations, as sodium and calcium, also present differentiable means (Table 1).

DO and pe values are relevant since they are related to the occurrence of denitrification processes. While DO shows varying values at the NE zone (mean: 4.2 ± 2.0 mg/L), samples of the SW zone are considerably depleted in oxygen (mean: 1.3 ± 0.7 mg/L). pe values, as indicators of the redox potential of groundwater, do not show a neat difference between zones, ranging from 5.08 to 7.61. These values are representative of groundwaters where dissolved oxygen has been consumed by degradation of organic matter, but SO_4^{2-} is not yet reduced (e.g., Stumm and Morgan, 1996). EC mean values increase from 818 ± 61 to 1029 ± 71 μS/cm from the NE to SW zones, (Table 1). A similar pattern was observed for Ca^{2+}, Mg^{2+}, SO_4^{2-}, Cl^- and NO_3^- concentrations with lowest values present in the NE zone wells (ranges of 118.5–136.5 mg Ca^{2+}/L, 25.6–41.5 mg Mg^{2+}/L, 33.9–98.4 mg SO_4^{2-}/L, 11.3–32.8 mg Cl^-/L, and 16.6–26.3 mg NO_3^-/L), and the highest in SW zone samples (ranges of 133.6–176.9 mg Ca^{2+}/L, 29.1–56.2 mg Mg^{2+}/L, 107.7–213.3 mg SO_4^{2-}/L, 35.9–71.1 mg Cl^-/L, and 54.6–89.17 mg NO_3^-/L; Table 1).

The relationship between $(Ca^{2+} + Mg^{2+})$-$(HCO_3^- + SO_4^{2-})$ vs. Na^+-Cl^- is plotted to analyse the possible origin of Na^+ (Fig. 3a). When groundwater hydrochemistry is driven by calcite/dolomite and gypsum/anhydrite dissolution, the amount of Ca^{2+} and Mg^{2+}, in meq/L, compensates the HCO_3^- and SO_4^{2-} concentrations, and samples tend to be found close to the Y-axis origin. In the same way, when halite dissolution dominates, Na^+ is compensated by Cl^- concentrations, in meq/L, and samples should be found close to the X-axis origin. However, when cation exchange takes place, Ca^{2+} is hold in clays and Na^+ is released. Under these conditions, samples would be displaced following the 1:−1 ratio. If reverse cation exchange occurs, samples follow the −1:1 ratio. In the studied area (Fig. 3a), samples with the highest Na^+ concentrations (MNL-019 and SMC-025) reveal the occurrence of cation exchange. In addition, there is a displacement from the origin of most of groundwater samples with ratios ≈0:1, meaning that the larger proportion of $Ca^{2+} + Mg^{2+}$ with respect to $HCO_3^- + SO_4^{2-}$ is not related to sulphate or carbonate mineral dissolution. Similarly, Fig. 3b indicates that such unexplained Ca^{2+} increase is attributed to nitrate pollution, which affects the overall hydrochemical composition of the water (Böhlke et al., 2002; Menció et al., 2016).

4.2. Denitrification level according to isotopic fractionation

As regards isotopic data (Table 2), samples showed light values of $\delta^{18}O_{H2O}$ and δD, ranging from −6.8 to −6.37‰ and from −44.77 to

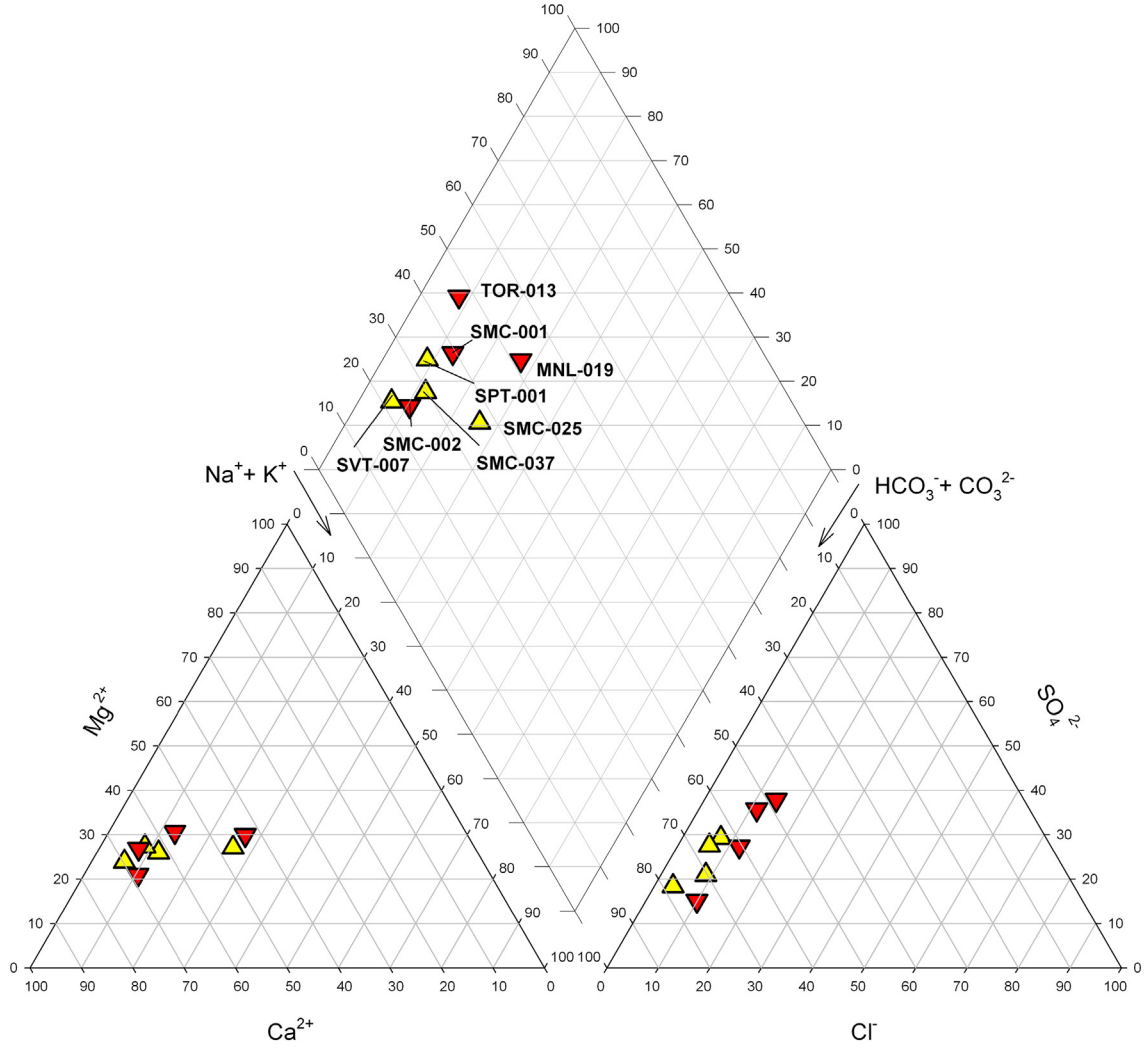

Fig. 2. Piper diagram of July 2014 campaign. Legend: yellow triangles, wells located in the NE area; and red upside down triangles, wells located in the SW area. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

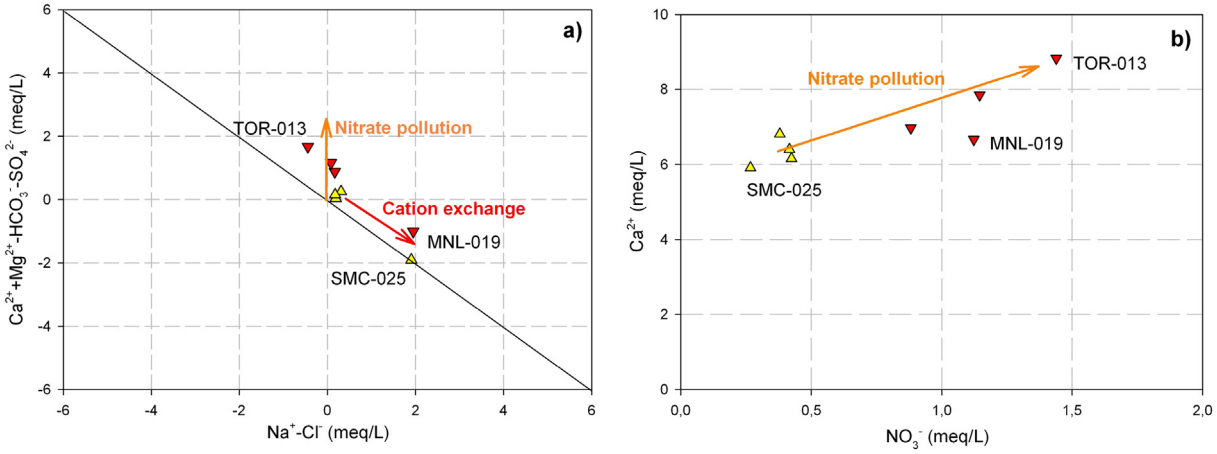

Fig. 3. Bivariate relationships of: a) $Ca^{2+} + Mg^{2+} - HCO_3^- - SO_4^{2-}$ vs. $Na^+ - Cl^-$; b) Ca^{2+} vs. NO_3^-. Legend: yellow triangles, wells located in the NE area; and red upside down triangles, wells located in the SW area. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

−41.99‰, respectively, being representative of high altitude recharge areas in the Osona region (Menció et al., 2011a). Sample SMC-037 exhibited heavier values probably caused by evaporation (almost 5%) (Gonfiantini's, 1986) originated by a recent modification of the well withdrawal mechanism. This result contrasts with those from the preceding sampling campaigns conducted in 2005, 2006 and 2010, when this well showed similar values to the rest of the samples in this area (Menció et al., 2011a; Boy-Roura et al., 2013b). From a regional perspective, water stable isotopes indicate a rainfall recharge from the surrounding ranges and, more importantly, wells located at lower altitudes, as those in the SW zone, also present a large contribution from this recharge origin. Nevertheless, their higher nitrate content indicates that local infiltration from nearby arable land must also contribute to the sampled groundwater. In consequence, groundwater samples, especially those from the SW zone are constituted by a mixture of regional flow paths intercepted at the deeper parts of the uncased wells, and local infiltration from the upper levels. Given that stable isotope data mostly correspond to high altitude recharge, it can be inferred that regional flows are the dominant resource that dilutes a local high-nitrate recharge. Mean nitrate values around 70 mg/L in the SW zones (Table 1) are considerably lower than the high nitrate concentrations registered in shallow wells and natural springs (Menció et al., 2011a, 2011b; Boy-Roura et al., 2013a, 2013b), supporting the mentioned dilution process.

The $\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$ isotopic signatures of NO_3^- in groundwater provide evidence of denitrification. In the studied wells, $\delta^{15}N_{NO3}$ ranged between +10.4 and +26.9‰, and $\delta^{18}O_{NO3}$ between +3.8 and +12.3‰, with nitrate concentrations between 16.5 and 89.2 mg/L. Isotopic values of dissolved NO_3^- in groundwater, plotted in Fig. 4 together with the isotopic ranges of the main NO_3^- sources, confirm the manure and/or sewage origin of the nitrogen. Given the intense agricultural and husbandry activity in the region, manure application as fertilizer stands as the main nitrogen source in groundwater. The lowest $\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$ values are found in SVT-007 (with +10.4‰ and +3.8‰, respectively). The rest of samples showed heavier $\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$ values displayed along a positive trend, consistent with denitrification isotopic enrichment paths that have their origins in the range of manure nitrogen source.

In Fig. 4 the percentage of natural denitrification has also been represented, according to the N and O isotopic enrichment factors (ε_N of −26.3 ± 1.8‰, and ε_O of −20.4 ± 1.3‰) determined by Torrentó et al. (2011) in lab experiments using Osona rock cores and groundwater. For instance, according to the $\delta^{18}O_{NO3}$ enrichment factor, samples SPT-001, TOR-013 and SMC-001 present a degree of denitrification lower than 10%; SMC-002, approximately of 15%; and SMC-037, SMC-025 and MNL-019, a degree that can be even higher than 25%. Such denitrification percentages are more conservative than those using the enrichment factors estimated by Otero et al. (2009), which would give a rank between 12 and 50%.

Furthermore, the plot between $\delta^{15}N_{NO3}$ and NO_3^- in logarithm scale differentiates between each of the two geographical zones, being their samples properly aligned following a denitrification trend of slope close to that of the nitrogen enrichment factor ($\varepsilon_N = -26.3$‰; Fig. 4b). This result is particularly interesting from the hydrodynamic perspective as it distinguishes two distinct nitrate source areas: the NE area, located in the hills with a lower nitrate input, and the SW area, located in the basin with a larger proportion of arable land and, therefore, a larger manure input that results in higher nitrate concentrations. As derived from the geological setting and the water stable isotopes, nitrate concentration is a mixing of the distinct aquifer levels intercepted

Table 2
Isotopic data of sampling points obtained in July 2014. Any of the recorded variables was found to be significantly different between areas according to Mann-Whitney U test.

Area	NE area					SW area				
Sampling point	SMC-025	SMC-037	SPT-001	SVT-007	Mean values	SMC-001	SMC-002	MNL-019	TOR-013	Mean values
$\delta^{18}O_{H2O}$ (‰ VSMOW)	−6.70	−4.76	−6.46	−6.80	−6.18 ± 0.48	−6.45	−6.37	−6.50	−6.45	−6.44 ± 0.03
δD (‰ VSMOW)	−43.88	−35.74	−42.95	−44.77	−41.84 ± 2.07	−43.18	−42.61	−42.26	−41.99	−42.51 ± 0.26
δ-Excess (‰ VSMOW)	9.72	2.38	8.74	9.65	7.62 ± 1.76	8.40	8.39	9.76	9.57	9.03 ± 0.37
$\delta^{18}O_{NO3}$ (‰ VSMOW)	12.00	10.90	6.20	3.80	8.23 ± 1.94	7.00	8.50	12.30	6.70	8.63 ± 1.29
$\delta^{15}N_{NO3}$ (‰ AIR)	26.90	16.90	12.90	10.40	16.78 ± 3.63	17.30	14.10	19.80	14.80	16.50 ± 1.30

Legend: mean values, mean ± standard error.

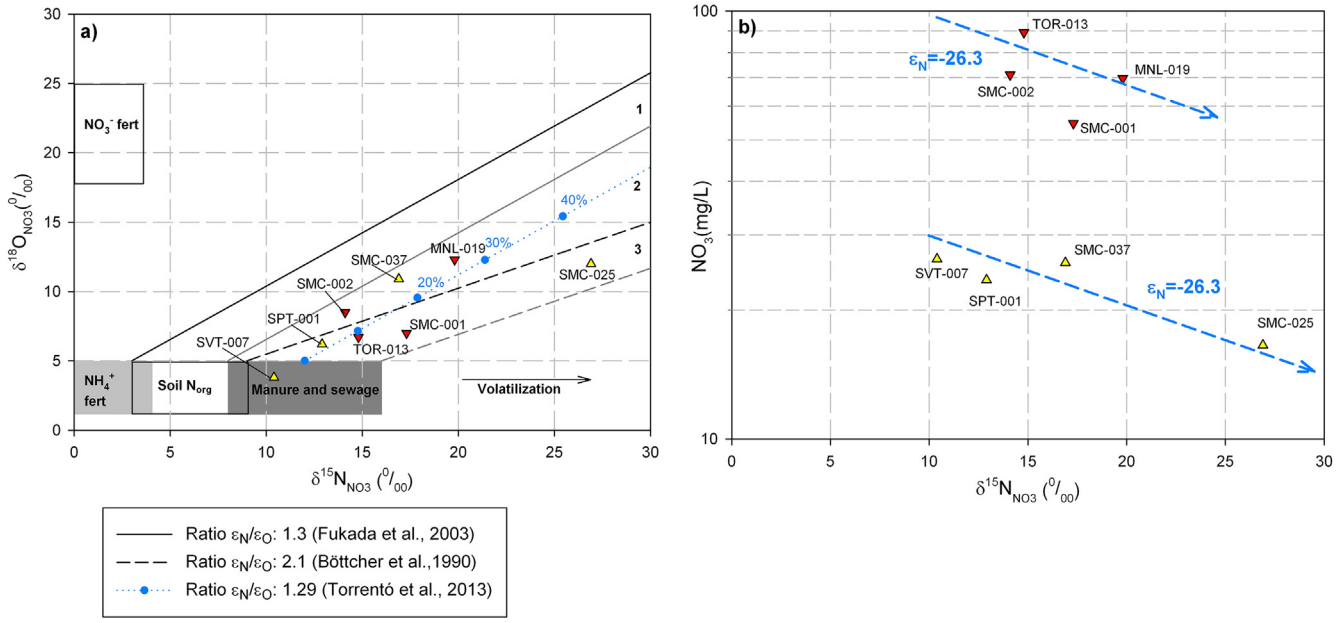

Fig. 4. Relationship between: a) $\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$ for the nitrate in groundwater samples with estimated isotopic enrichment factors of Böttcher et al. (1990) and Fukada et al. (2003); ranges of local potential NO_3^- sources from Puig et al. (2013), Vitòria et al. (2004) and Vitòria et al. (2008); and percentage of natural denitrification quantified according to Torrentó et al. (2011). Legend: 1, denitrified samples from soil N_{org}; 2, denitrified samples from both sources; and 3, denitrified samples from manure and sewage N, considering a 1:3 slope for the minimum $\delta^{15}N_{NO3}$ value and a 2:1 slope for the maximum $\delta^{15}N_{NO3}$ described for each source; b) $\delta^{15}N_{NO3}$ and NO_3^-, showing the denitrification trends based on the enrichment factor estimated by Torrentó et al. (2011). Note that a logarithm scale is used for the Y axis. Yellow triangles, wells located in the NE area. Red upside down triangles, wells located in the SW area. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

by the boreholes, especially in the SW zone. Fig. 4b demonstrates that wells from each zone have a common hydrogeological setting related to recharge areas and the mixing of flow lines, which finally determine their hydrochemical composition and denitrification potential expressed by isotopic values. The dispersion from an expected lineal enrichment trend shown by samples from both zones can be attributed to the effect of mixing flow lines in the sampling borehole. It would then be erroneous, from a hydrogeological perspective, drawing a single flow path according to an increasing denitrification level. The unsolved question so far is whether such denitrification level is the consequence of a large flow distance, equivalent to a longer residence time within the aquifer, or to the specific conditions within the capture zone of each well (including mixing of flow lines) that will enhance denitrification up to distinct degrees.

In synthesis, wells are classified in two generic groups according to isotopic analyses, which are consistent with geographical location, hydrogeological features of the aquifer system, and land use spatial distribution. However, since we focus on the denitrification process, its occurrence and its extent, we also group samples as those with low (SPT-001, TOR-013, SMC-001, SVT-007 and SMC-002) and high denitrification levels (SMC-037, SMC-025 and MNL-019; Fig. 4b), naming them as Low-$DL_{isotope}$ and High-$DL_{isotope}$, depending whether the denitrification percentage is less or >20%, according to the isotopic thresholds $\delta^{18}O_{NO3}$ > 10‰. Such sorting based on the percentage of nitrogen mass removal summarizes the contribution of the isotopic data to the estimate of natural attenuation levels of nitrate in groundwater, regardless mixing effects. Denitrification levels, as defined here, will later on be used to contrast isotopic with microbiological information.

4.3. Abundance of 16S rRNA and denitrification genes

The concentration of bacterial 16S rRNA gene ranged from $5.03 \cdot 10^3$ to $3.12 \cdot 10^5$ copies/ng DNA (Table 3). As predicted, 16S rRNA gene abundance was always higher than any other functional gene. There were no significant differences in the abundance of total bacteria between the studied wells (Kruskal-Wallis test, $p > 0.05$). All studied functional genes, albeit at different relative abundances, were found in all groundwater samples. Abundance of *nirS* and *nirK*, genes implied in nitrite reduction, was highly variable and ranged between $1.41 \cdot 10^2$ and $1.54 \cdot 10^5$ copies/ng DNA, and from $5.12 \cdot 10^1$ to $7.57 \cdot 10^4$ copies/ng DNA, respectively. Previous surveys in groundwater also detected high and significant abundances of *nirS* compared to *nirK* (Barrett et al., 2013). *nrfA* gene, responsible of the DNRA process, occurred at low abundance, with values ranging from $2.90 \cdot 10^1$ to $7.26 \cdot 10^3$ gene copies/ng DNA. Nitrous oxide reductases *nosZI* and *nosZII* ranged from $3.96 \cdot 10^2$ to $1.80 \cdot 10^4$ copies/ng DNA, and from $2.46 \cdot 10^2$ to $1.30 \cdot 10^5$ copies/ng DNA, respectively. In wells SMC-001, SMC-002, SPT-001 and MNL-019, *nosZII* was not detected (<20 copies/ng DNA). However, in samples where *nosZII* was present, this gene appeared at higher concentrations than *nosZI* (Mann Whitney test, $p < 0.05$). In all wells, gene copy abundance values were similar to those found in other groundwater studies (Barrett et al., 2013; Herrmann et al., 2017), and lower than those found in other environments characterized by higher concentrations of organic carbon such as constructed wetlands, estuaries and agricultural soils (García-Lledó et al., 2011; Hallin et al., 2009; Lindemann et al., 2016).

In general, 16S rRNA gene correlated positively (Spearman's correlation, $p < 0.01$) with all functional genes except *nirK*, indicating that the relative abundance of potential denitrifiers remained constant in the studied wells (Table 4). This hypothesis was confirmed by the positive pair-wise correlations between functional genes. *nrfA* abundance appears positively correlated to *nirS* (Spearman's correlation, $p < 0.01$), but not to *nirK*, pointing to a selection of bacteria harbouring either nitrite reductase for specific environmental conditions in groundwater. *nosZ* gene abundance (*nosZI* + *nosZII*) correlated positively to *nirS*, and negatively to *nirK* (Spearman's correlation $p < 0.01$).

In order to analyse differences in gene abundances according to physicochemical characteristics of the water, pair-wise correlation tests were performed (Supplementary Material 4). H_2S correlated positively with *nrfA* and *nosZ* genes (Spearman's test, $p < 0.01$), and negatively with *nirK* (Spearman's test, $p < 0.05$). Brunet and Garcia-Gil (1996) showed that denitrification can be inhibited by the presence of H_2S, which provokes an accumulation of ammonia due to DNRA. In this sense, the higher correlation between *nrfA* and H_2S ($R = 0.811$,

Table 3
Mean abundance values and standard deviation of studied genes (number of copies ($\times 10^3$)/ng of DNA extracted) at the studied wells. DL means denitrification level according to isotopic analyses. Values were obtained from two replicates for each sample, except for SMC-001, MNL-019 and TOR-013.

Area	NE area				SW area			
Sampling point	SMC-025	SMC-037	SPT-001	SVT-007	SMC-001	SMC-002	MNL-019	TOR-013
DL	High	High	Low	Low	Low	Low	High	Low
q16S rRNA	213.31 ± 99.04	14.76 ± 9.73	37.41 ± 24.21	69.47 ± 0.31	111.93	88.83 ± 18.00	165.43	74.46
q*nirS*	17.64 ± 9.99	1.45 ± 1.31	0.65 ± 0.01	4.94 ± 1.93	153.74	28.76 ± 5.45	42.63	1.4
q*nirK*	0.21 ± 0.16	7.41 ± 3.27	3.55 ± 2.81	0.36 ± 0.18	4.06	1.50 ± 0.47	1.13	5.29
q*nrfA*	2.11 ± 0.79	0.10 ± 0.03	0.05 ± 0.02	1.04 ± 0.11	0.59	6.55 ± 0.71	3.52	0.32
q*nosZI*	13.04 ± 4.99	0.50 ± 0.11	4.03 ± 0.82	4.85 ± 2.36	9.88	13.84 ± 2.83	8.62	1.44
q*nosZII*	100.13 ± 30.11	0.72 ± 0.47	<0.02	24.15 ± 20.19	<0.02	<0.02	<0.02	3.52

$p < 0.01$) suggested that DNRA might have contributed to the nitrate reduction in some wells, as it was previously observed in limestone aquifer (Herrmann et al., 2017). NO_3^- was not correlated to any studied gene, while NO_2^- was positively correlated to *nrfA*. Abundances of *nirS* and *nirK* genes were positively correlated to $\delta^{15}N$ and $\delta^{18}O$ (Spearman's test, $p < 0.05$), which might indicate a relationship between denitrification level ($DL_{isotope}$) and gene abundances.

Despite the changes in abundance of denitrification genes and the existing correlation between them, no significant differences were detected when samples were grouped according to the denitrification level based on isotopic analyses (Mann-Whitney test, $p > 0.05$; Table 3). There is then a lack of consistency between molecular and isotopic analyses, used as proxies for potential denitrification. Although it was reasonable to think that the presence of denitrifying bacteria and denitrification reactions could be concurrently detected (Kim et al., 2015), actual results in Osona brings in the need of a thoughtful discussion on the hydrodynamic aspects of these observations. Nitrate, as a dissolved compound, flows at the pore water velocity. Conversely, bacteria, being a particulate material, possibly remain attached to surfaces and form biofilms, or occur as aggregates having a lower mobility (Griebler and Lueders, 2009; Williamson et al., 2012). Unfortunately, biofilm sampling was not possible due to methodological constraints. Previous experiments comparing attached and free-living bacteria in groundwater revealed significant differences in the microbial community, probably affecting denitrifying bacteria (Herrmann et al., 2017). Lastly, significant differences in denitrifiers may occur between wells, since bacteria with this metabolism are well scattered in the phylogenetic tree (Philippot and Hallin, 2005). Most of denitrifying bacteria do not contain all genes necessary for complete denitrification (Jones et al., 2008, 2013) and, in addition, the same set of genes in two different bacteria may lead to considerable changes in activity. According to this, it is reasonable to think that information derived from isotopic signatures and abundance of genes may not coincide in reporting denitrification reactions (Mann-Whitney test, $p > 0.05$). Both results derived from isotopic and microbial data corroborate the occurrence and extent of denitrification, yet they fail to indicate at which location/moment along the flow path these reactions had taken place.

Table 4
Spearman correlation coefficients between 16S rRNA and functional genes abundances.

	q16S rRNA	q*nirS*	q*nirK*	q*nrfA*	q*nosZI*	q*nosZII*	q*nirS* + q*nirK*	q*nosZI* + q*nosZII*
q16S rRNA	1.000							
q*nirS*	0.819 **	1.000						
q*nirK*	−0.516 n.s.	−0.385 n.s.	1.000					
q*nrfA*	0.698 **	0.747 **	−0.522 n.s.	1.000				
q*nosZI*	0.703 **	0.813 **	−0.467 n.s.	0.764 **	1.000			
q*nosZII*	0.893 **	0.929 **	−0.857 *	0.893 **	0.964 **	1.000		
q*nirS* + q*nirK*	0.571 *	0.775 **	0.071 n.s.	0.582 *	0.670 *	0.250 n.s.	1.000	
q*nosZI* + q*nosZII*	0.791 **	0.692 **	−0.742 **	0.747 **	0.797 **	1.000 **	0.434 n.s.	1.000

n.s., not significant.
* $p < 0.05$.
** $p < 0.01$.

4.4. The groundwater microbial community structure

In order to infer the main taxonomical groups responsible for denitrification in Osona wells, microbial communities were studied on the basis of the 16S rRNA gene sequence. A total of 69,092 sequences passed quality filtering. Roughly on average 4600 sequences were obtained per sample (ranging from 1249 to 8526). For one of the replicates from TOR-013 no sufficient number of sequences was obtained and was removed from the study. A subset of 1150 sequences per sample was randomly obtained and used for diversity analysis. Despite the lower number of sequences used, rarefaction curves revealed a reasonable coverage of bacterial richness (Supplementary Material 5). Observed richness (Sobs, number of OTUs) varied between 109 ± 5 and 377 ± 3 (Table 5). Diversity of microbial community was estimated by Shannon and phylodiversity indices (H′ and PD), which varied from 3.12 to 5.45 and from 3.97 to 11.22, respectively. No significant differences (Mann-Whitney test $p < 0.05$) were found between samples grouped according to $DL_{isotope}$ for alpha diversity indicators.

Bacteria were clearly dominant over *Archaea*, which ranged from 0.1% (SMC-002) to 6% (MNL-019) of the total sequences (Fig. 5). Two bacterial phyla (*Proteobacteria* and *Parcubacteria*) accounted for almost 40% of all sequences in all samples, except those retrieved from SMC-002. In the majority of wells, 5 to 20% of the sequences remained as unclassified *Bacteria*. A closer inspection of these sequences, using updated NCBI nucleotide collection database for cultured bacteria, yielded high similarity values to *Candidatus* Parcubacteria, *Candidatus* Gracilibacteria and *Candidatus* Saccharibacteria. These phyla, unknown until recently, have been described in many environments, including groundwater (Kindaichi et al., 2016; Kutvonen et al., 2015). Some members within these phyla have been also reported as potential denitrifiers according to whole genome sequences (Albertsen et al., 2013).

Groundwater microbiome varied notably in three of the wells, revealing a higher relative abundance of specific phyla. In particular, *Firmicutes* (mainly *Clostridium* sp.) accounted for approximately 25% of total sequences in SPT-001. Secondly, SMC-002 was characterized by high abundance of *Nitrospirae* (about 40%) and *Acidobacteria* (about 15%) which were less represented in other wells. Finally, MNL-019 had a high relative abundance of *Bacteroidetes*. In relation to denitrification capacity, all these four phyla contain denitrifying members (Heylen et al., 2006; Lücker et al., 2010; Lee et al., 2013; Rösch et al., 2002).

Statistical correlation between microbial communities was analysed using a Principal Coordinates Analysis (PCoA), based on the Unifrac

Table 5
Alphadiversity values and potential denitrification level of studied wells. Values were obtained from two replicates for each sample, except in TOR-013 where only for one of the replicates had optimal results.

Area	NE area				SW area			
Sampling point	SMC-025	SMC-037	SPT-001	SVT-007	SMC-001	SMC-002	MNL-019	TOR-013
DL	High	High	Low	Low	Low	Low	High	Low
#sequences	5076	12706	6996	12706	10591	15337	11069	1249
H′	4.82 ± 0.10	5.07 ± 0.16	4.75 ± 0.06	5.41 ± 0.06	4.71 ± 0.01	3.12 ± 0.02	3.67 ± 0.32	5.45
phyloDiversity	9.37 ± 0.64	9.20 ± 2.86	7.40 ± 0.10	7.28 ± 4.69	6.47 ± 1.17	5.71 ± 0.05	6.51 ± 01.17	3.97
Sobs	280.59 ± 0.24	358.68 ± 24.34	274.28 ± 10.57	327.04 ± 24.10	240.03 ± 11.11	123.18 ± 20.42	181.45 ± 3.98	344.09

#sequences: number of sequences obtained; H′: Shannon diversity index; phyloDiversity: phylogenetic diversity index, Sobs: number of OTUs observed.

weighted distance matrix. Distances were calculated from OTU distributions accounting for their relative abundance and their phylogenetic relationships (Fig. 6). PCoA clustering confirmed the different microbial community composition of well SMC-002. Significantly, the position of this sample in the PCoA correlated positively (Spearman's correlation, $R^2 > 0.6$) with redox potential (Eh). All other samples clustered in two groups: a first one correlated with pH, O_2 (SMC-037, SMC-025; TOR-013, and SVT-007; Spearman's correlation, $R^2 > 0.6$) and a second group correlated with S-SO_4^{2-} (SMC-001, MNL-019, and SPT-001; Spearman's correlation, $R^2 > 0.6$). Oxygen and pH have previously been shown to be key drivers of denitrification in different environments (García-Lledó et al., 2011; Graham et al., 2010; Jurado, 2017). Correlation with SO_4^{2-} may be interesting in Osona wells, since this compound is produced as the result of the oxidation of sulphides, appearing in the form of pyrite in the region, by autotrophic denitrifiers (Pauwels et al., 2000; Otero et al., 2009).

Analyses of similarities (ANOSIM) test is a distribution-free analogue of one-way ANOVA, used to test spatial differences in community's structure (Clarke, 1993). At the studied wells, results show that the distribution of microbial communities resulting from PCoA is not related to the denitrification level ($DL_{isotope}$), as defined according to isotopic analyses (ANOSIM R-value 0.011, p-value 0.293). On the contrary, the ANOSIM value was higher (R-value 0.159) and slightly significant (p-value 0.049; Fig. 6) when microbial communities were grouped according to hydrogeological area (NE or SW). Statistical results suggest that microbial communities are shaped by the hydrogeological features of the aquifer; whereas the $DL_{isotope}$ activity, as a denitrification proxy, integrates all geochemical reactions/processes occurred along the flow path.

Supporting this hypothesis, the abundance of denitrifying genes (*nirS*, *nirK*, *nosZI* and *nosZII*) in well showed some coincidence to the distribution according to sampling zones. q*nirS* + q*nirK* showed a significantly higher presence in wells located in the SW area (Mann-Whitney test, $p < 0.05$), in the discharge zone, specifically due to the high abundance of *nirS*. Generally *NirS*-type denitrifiers are predominant compared to *NirK*-type (Barrett et al., 2013; Lindemann et al., 2016). Even though the two nitrite reductases are functionally equivalent, denitrifiers harbouring either nitrite reductase seem to show a preference for certain environments (García-Lledó et al., 2011; Jones and Hallin, 2010). Distinct groundwater physico-chemical properties could determine the presence of the two types of nitrite reductases in each zone (NE and SW), probably due to differential niche preferences. More precisely, NO_3^- and TOC:TN ratio were greater in SW wells than in NE, and this suggests a positive selection of *nirS*-type denitrifiers (Gao et al., 2016; Herrmann et al., 2017). Moreover, wells with higher *nir* abundance were negatively correlated to oxygen concentration and pH which agrees with several studies at different environments including groundwater (García-Lledó et al., 2011; Graham et al., 2010; Jurado, 2017).

Similarly to what has been observed for *qnirS* + *qnirK*, the ratio (*qnirS* + *qnirK*)/(*qnosZI* + *II*), a proxy of N_2O accumulation (García-Lledó et al., 2011; Saarenheimo et al., 2015), was higher in the SW zone wells (Fig. 6). In wells located in the NE zone, *nosZII* gene

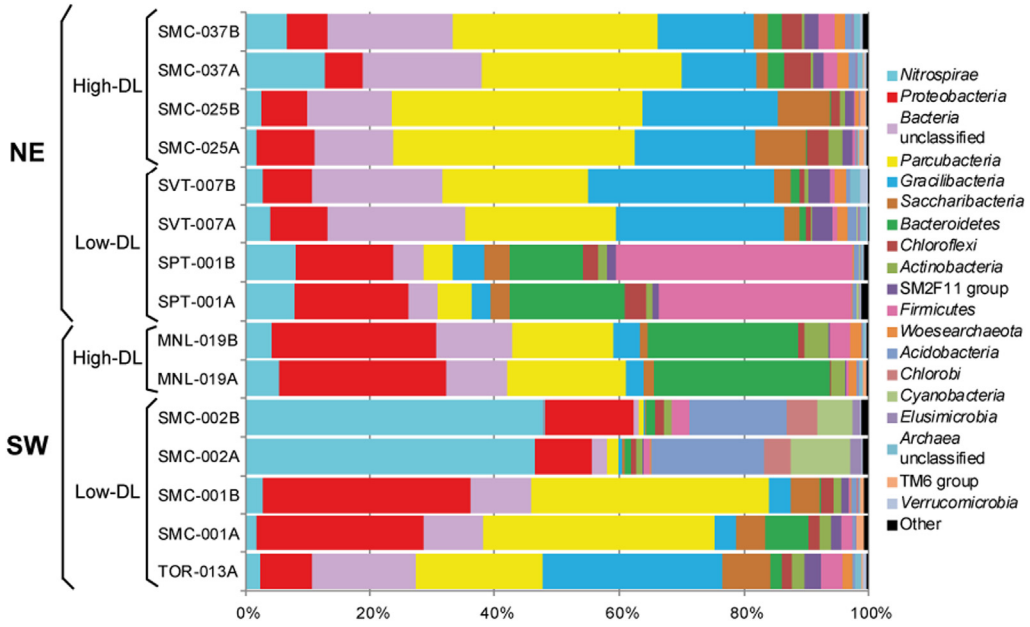

Fig. 5. Relative abundances of main phyla (found >1% of sequences) in each replicate in the studied wells. Bars are grouped according to the location of the sampled well (NE and SW) and the Potential denitrification level according to isotopic fractionation (High-DL and Low-DL). "Other" refers to phyla that represented <1% of total sequences in all samples.

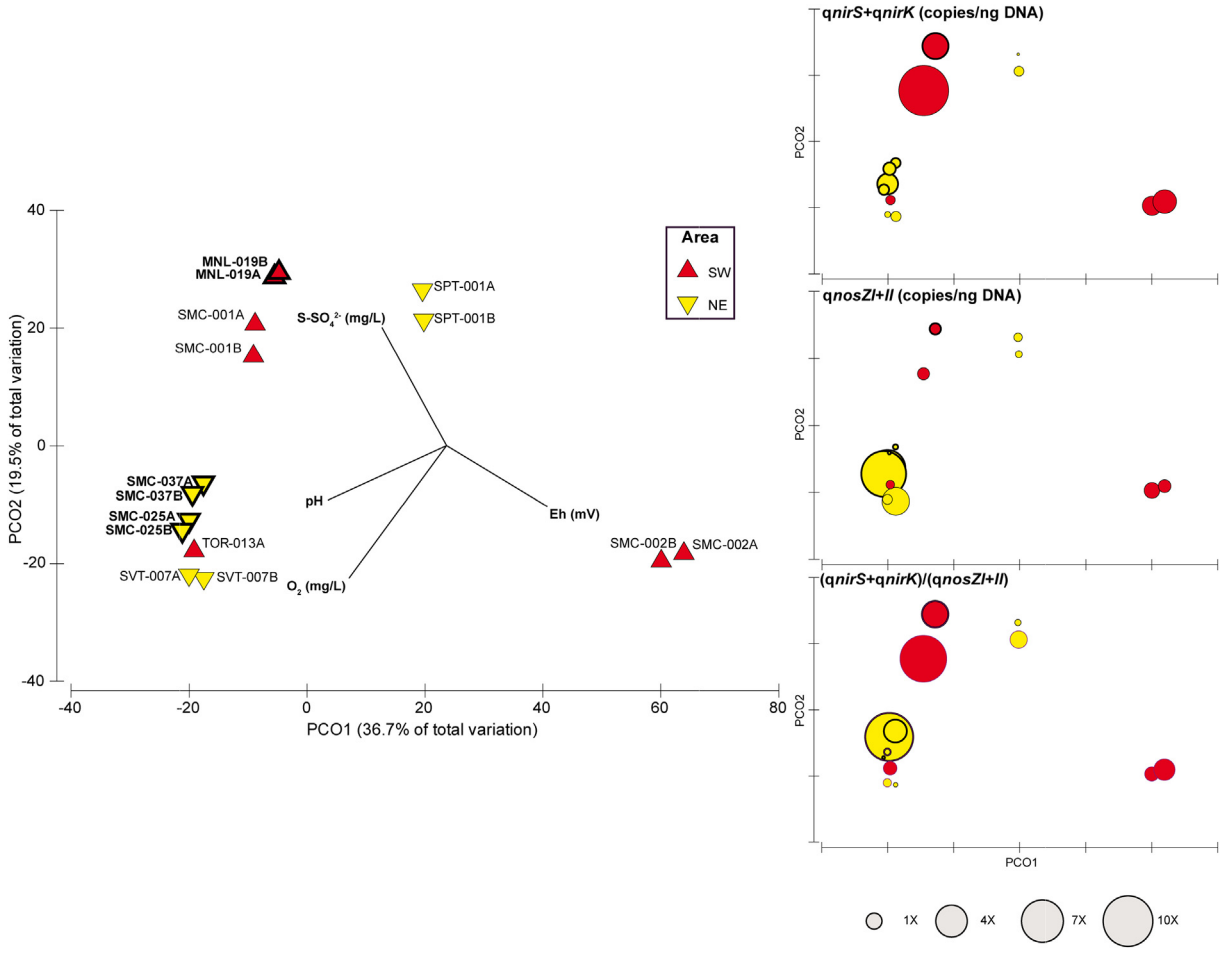

Fig. 6. Left: PCoA distribution of OTUs microbial community and environmental parameters (vectors) correlated with this distribution (Spearman's correlation, R > 0.6). Bold lines indicate high $DL_{isotope}$. Right: distribution of abundances of *nirK* + *nirS* and *nosZI* + *II*, and (*qnirS* + *qnirK*)/(*qnosZI* + *II*) ratio according to PCoA of microbial community. In the legend, 1X refers to $2 \cdot 10^4$ (copies/ng) or 2 (ratio).

was significantly more abundant compared to the SW (Mann-Whitney test, $p < 0.05$), whereas *nosZI* remained at similar abundance in both areas. This was in agreement with the fact that the majority of the typical *nosZI*-type containing bacteria have the complete set of denitrification genes, while the latter occurs in less than half of the known *nosZII*-carrying microorganisms. In fact, *nosZII*-type reductase was referred to a "non-denitrifier nitrous oxide reductase" (Sanford et al., 2012). The lack of nitrous oxide reductase in some denitrifying bacteria, which results in high (*qnirS* + *qnirK*)/(*qnosZI* + *II*) ratios, was reported more than a decade ago when the *Agrobacterium tumefaciens* genome was sequenced (Wood, 2001). A later survey of bacterial genomes confirmed that approximately 1/3 of denitrifying bacterial isolates have a truncated pathway (Jones et al., 2008). Similarly to *nir* genes, the relative importance of *nosZ* genes seems to systematically differ between habitats and with environmental conditions (Jones et al., 2013), yet the exact controls that modulate their relative abundance in nature are uncertain. At wells with high (*qnirS* + *qnirK*)/(*qnosZI* + *II*) ratios, nitrate concentration was generally high, leading to a possible N_2O accumulation. This has been previously shown in groundwater (Jahangir et al., 2013; Jurado, 2017). The dominance of *nirS* over *nirK* and *nosZ* in SW wells, can also be due to changes in trace metals rather than other variables frequently considered to alter denitrifiers abundance and activity, i.e. nitrate, organic carbon, pH and oxygen. For instance, the bioavailability of copper (Cu) and iron (Fe) is hypothesized to control the expression and activity of nitrite and nitrous oxide reductases and was defined as a selection variable explaining the dominance of *nirS* denitrifiers in Cu limited boreal lakes (Saarenheimo et al., 2015).

4.5. Distribution of potential denitrifying genera

Between 68.7 and 93.7% of representative sequences could be classified at the genus level (Fig. 7), and between 13.4% and 67.4% of the total analysed sequences belonged to genera containing putative denitrifying bacteria. In low organic matter environments as Osona, autotrophic dissimilatory nitrate reduction is achieved by oxidation of pyrite, using it as an electron donor which in turn is oxidized to SO_4^{2-}. In the studied wells, SO_4^{2-} was predominantly found in the SW area. Coupling of autotrophic denitrification to sulfide or iron oxidation has been known since at least twenty years ago, and it has been proven in many isolates and pure cultures (Straub et al., 1996; Weber et al., 2006). According to the microbial community composition, *Acidiferrobacter* and *Sideroxydans* were proportionally more abundant in the SW area (Fig. 7). *Acidiferrobacter* has been described as an anaerobic iron- and sulfur-oxidizer able to reduce nitrate autotrophically to nitrogen gas (Niu et al., 2016). However, this bacterium is acidophilic and remains uncertain if the described reactions can be performed at pH values ranging from 7.03 to 7.52 (Table 1). *Sideroxydans* is a well-known iron dependent nitrate reducer (Blöthe and Roden, 2009). Collectively these results suggest that at least for these specified genera a relationship between pyrite and denitrification must exist in the studied zone, as already proved by Vitòria et al. (2008) and Otero et al. (2009) based on a multi-isotopic approach. Moreover, also related to pyrite oxidation, *Clostridium* was found in some studied wells, but it was mainly abundant in a single well of NE zone (SPT-001). This genus is able to reduce nitrate using sulfur as electron donor and it was previously found in groundwater (Pu et al., 2015).

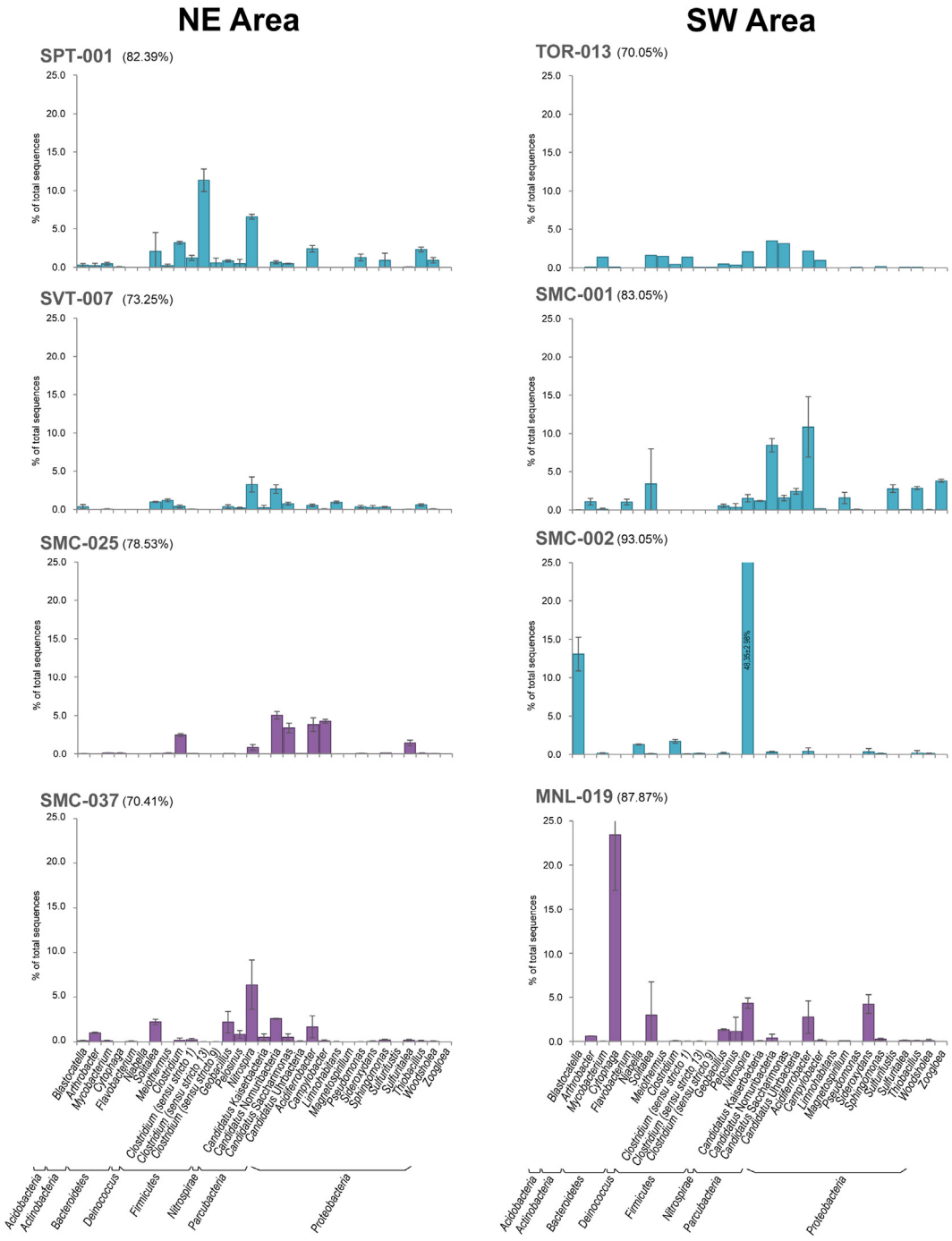

Fig. 7. Relative abundances of sequences belonging to suspected denitrifying bacteria at the genus level. Mean abundances (bars) and SD (whiskers) of two replicates are shown for each well. Wells were organized according to their location (NE and SW). Blue bars, wells with Low-DL. Purple bars, wells with High-DL. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Nitrospira species were found almost exclusively in a single well (SMC-002; 48% of total sequences) revealing a higher niche selection for certain bacteria due to changes in the hydrochemical properties. Taking into account the NH_4^+ and O_2 concentrations found in SW zone (Table 1), nitrification reactions are not expected and *Nitrospira* could be related to denitrification. Other denitrifying genera as *Cytophaga* were found in MNL-019, located in the SW zone, and *Clostridium* related species appeared to be more abundant in SPT-001, in the NE zone. Interestingly, the presence of these potentially denitrifying genera is a determinant factor explaining the sample distribution in the PCoA. Despite the changes in relative abundance observed for denitrifying genera in some wells, no significant differences in the relative abundances of any of the identified potentially denitrifiers could be detected between NE and SW zones (Mann-Whitney test, $p > 0.05$; Fig. 7). The later indicates that, at least for the selected genera, enrichment is promoted by local conditions.

Moreover, it is remarkable that less abundant but well-known denitrifying genera, such as *Geobacillus*, *Solitalea*, *Campylobacter* and *Candidatus* Saccharimonas were detected mainly in wells with High-$DL_{isotope}$. This is in agreement with previous works in which members of these genera were found in groundwater (Albertsen et al., 2013; Kuppardt et al., 2014; Stanley et al., 1998). Unfortunately, we do not have enough experimental evidence to relate the presence of these species to the higher denitrification levels measured by isotopic

fractionation. The relationship between taxonomic and isotopic data in groundwater has been recently shown in an agricultural area in Korea (Kim et al., 2015). This relationship was not so clear in the selected area of the Osona basin. However, in the studied region, nitrate attenuation by denitrification seems to be highly correlated with specific conditions of water. In this sense, disturbances in hydrogeological parameters could affect groundwater microbial stability and denitrification processes (Baho et al., 2012). Additional experimental measurements, such as sequencing of *nirS* and *nirK* genes and implementing methods directed to analyse active bacteria specifically (mRNA determinations), would successfully help in linking the identification of microbial processes that produce denitrification, and providing a better link between isotopic and microbiological information.

5. Conclusions

Natural nitrate attenuation in groundwater has been traced using isotopic and microbiological data to identify denitrification processes, and to build up a conceptual model based on the complementarity of both approaches. Hydrogeological data (geological setting, hydrochemistry and water stable isotopes) determine a common hydrogeological framework for the study area. Nitrate stable isotopes identify the source of nitrate as organic manure, and allow the estimation of the degree of denitrification. More relevantly, nitrate concentration and isotopic data point to the presence of two zones (named NE and SW) having different input source conditions, yet both attain high degrees of denitrification, which reach up to a 25% according to experimental enrichment factors.

As regards denitrification, sampling locations at each zone present both low and high denitrification levels ($DL_{isotope}$). This finding shows that: 1) ideal denitrification conditions (mainly due to autotrophic denitrification as shown by previous research; Otero et al., 2009) can be found all over the study area; and 2) the degree of denitrification is determined by either the residence time (or length of the flow path) and the mixing proportions of the distinct flow lines intercepted by the well capture zone. This dataset indicates that, in a region as Osona, the complexity of the flow system within the same hydrogeological unit permits, at a local scale, finding wells with distinct degrees of denitrification, even though denitrification processes occur ubiquitously at similar rates (Fig. 4b).

Since denitrification is a microbiological-driven process, genetic information will provide complementary evidence of its occurrence in groundwater. First of all, sampled wells present a rather uniform abundance of total bacteria, indicating some kind of uniformity between the hydrogeological unit. However, the microbial community structure in groundwater reveals that the occurrence of specific phyla or genera does not compare with the denitrification level (Figs. 5 to 7). Conversely, the abundance of denitrifying nitrite reductases, *nirK* and *nirS*, can be statistically correlated with isotopic data, though these gene abundances were not different between denitrification levels ($DL_{isotope}$). This is attributed to the fact that many different genera share similar genes and, consequently, they are able to strengthen denitrification processes.

From a hydrogeological perspective, the diversity of the groundwater bacteria is associated with local subsurface ecological conditions, even though the occurrence of denitrification is known to be related not to phyla, but to specific genera and genes. For instance, local high TOC:TN ratio enhances the occurrence of *nirS*-type denitrifiers. Assuming that gene and specific genera dominance is representative of the microbial community in the vicinity of the borehole, it provides evidence whether a specific location is prone to actively attenuate nitrate concentration in that spot, whereas isotopic data integrate all the processes that had taken place along the flow path. It can thus be concluded from this introductory research that isotopic approaches are valuable to set up the contextual setting for the occurrence of denitrification, and microbiological methods (particularly, gene analysis) reports on the local conditions around the well. Future studies, based on RNA and in-situ activity analyses, will contribute to a better understanding of denitrification. From a practical point of view, combined isotopic and microbiome data are essential to ascertain the ability and success of induced in-situ attenuation methods designed to reduce groundwater nitrate content at polluted sites.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2017.09.018.

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