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

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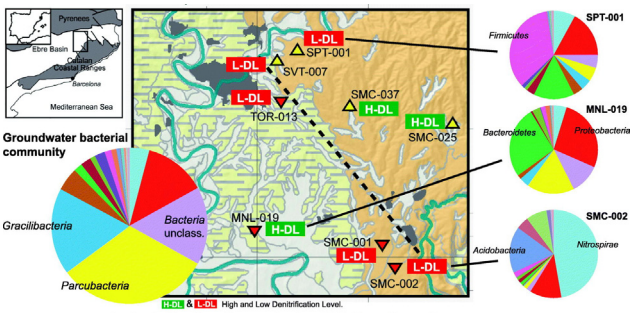
HIGHLIGHTS

- 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