Nitrate removal by combining chemical and biostimulation approaches using micro-zero valent iron and lactic acid

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HIGHLIGHTS

• A new nitrate removal method to solve issues raised by usual methods was developed.
• The use of ZVI as the sole reagent to remove nitrate produces the harmful ammonium.
• Ammonium is eliminated when ZVI combines with lactic acid causing the anammox process.
• Coupling ZVI and biostimulation of denitrifying flora effectively remove nitrate.

GRAPHICAL ABSTRACT

ABSTRACT

The occurrence of nitrate is the most significant type of pollution affecting groundwater globally, being a major contributor to the poor condition of water bodies. This pollution is related to livestock-agricultural and urban activities, and the nitrate presence in drinking water has a clear impact on human health. For example, it causes the blue child syndrome. Moreover, the high nitrate content in aquifers and surface waters significantly affects aquatic ecosystems since it is responsible for the eutrophication of surface water bodies. A treatability test was performed in the laboratory to study the decrease of nitrate in the capture zone of water supply wells. For this purpose, two boreholes were drilled from which groundwater and sediments were collected to conduct the test. The goal was to demonstrate that nitrate in groundwater can be decreased much more efficiently using combined abiotic and biotic methods with micro-zero valent iron and biostimulation with lactic acid, respectively, than when both strategies are used separately. The broader implications of this goal derive from the fact that the separate use of these reagents decreases the efficiency of nitrate removal. Thus, while nitrate is removed using micro-valent iron, high concentrations of harmful ammonium are also generated. Furthermore, biostimulation alone leads to overgrowth of other microorganisms that do not result in denitrification, therefore complete denitrification requires more time to occur. In contrast, the combined strategy couples abiotic denitrification of nitrate with biostimulation of microorganisms capable of biotically transforming the abiotically generated harmful ammonium. The treatability test shows that the remediation strategy combining in situ chemical reduction using micro-zero valent iron and biostimulation with lactic acid could be a viable strategy for the creation of a reactive zone around supply wells located in regions where groundwater and porewater in low permeability layers are affected by diffuse nitrate contamination.

1. Introduction

The presence of nitorgenous compounds (especially nitrate) is the most significant type of pollution affecting groundwater globally (Liu et al.,...
the poor condition of water bodies (Klement et al., 2017; Re et al., 2017). Moreover, the fact that this is pollution related to livestock-agricultural and urban activities (Dubrovsky and Hamilton, 2010; Ward et al., 2018) makes its presence in aquifers the cause of human and environmental impacts, at both spatial and temporal scales. The long exposure of subsurface materials to nitrate contamination accounts for the storage of a significant mass of nitrate in those materials (Ascott et al., 2016). This nitrate, which is difficult to remove, is dissolved in the porewater of layers of low hydraulic conductivity, into which it penetrated by molecular diffusion (Hinshaw et al., 2020). These layers will release nitrate to the environment by back-diffusion, even when the aquifers are cleared or the more or less continuous supply of the nitrogenous compounds to the medium is interrupted as a result of changes in land use.

As is well known, there are multiple sources of nitrate, mainly of anthropogenic character (Shukla and Saxena, 2018). Among these sources, those related to the following can be distinguished: agricultural activities (Xue et al., 2012; Levy et al., 2017; Merchán et al., 2020), wastewater (Zhang et al., 2014; Wang et al., 2017), and urban activities (Pael et al., 2016; Qin et al., 2019). Each of these sources can be identified by the isotopic composition (δ15N and δ18O) of nitrate (Guo et al., 2021).

The presence of nitrate in drinking water has a clear impact on human health (Ward et al., 2018; Kaur et al., 2020) since it causes methemoglobinemia (blue child syndrome) (Wongsanit et al., 2015; Karunanidhi et al., 2020) and is a major contributor to the health (Ward et al., 2018; Kaur et al., 2020) since it causes methemoglobinaemia (Bhateria and Jain, 2016; Lasagna et al., 2016), has a marked impact on the bodies (Bhateria and Jain, 2016; Lasagna et al., 2016), has a marked impact on the environment and/or the presence of a large mass of these species in different hydrogeological contexts requires the implementation of methods for minimizing these compounds. Traditionally, the most commonly used in situ techniques have been those related to the biostimulation of indigenous bacterial flora through the addition of an organic substrate, such as methanol, ethanol, acetate, and lactate, which acts as an electron donor and as a carbon source for these microorganisms (Park et al., 2014; Sheng et al., 2018). Bioaugmentation, as in situ bioremediation technique, has been used less frequently, given the large amount of denitrifying microorganisms in the medium (Ruan et al., 2020).

Although bioremediation techniques are among the most widely used, they present numerous problems. Among which is the fact that microbial overgrowth can occur, causing biologging (McLeod et al., 2018; Mohanadhas and Kumar, 2019; Zhang et al., 2020), which results in the following: i) reduced hydraulic conductivity of the aquifer (Zhong and Wu, 2013) and ii) decreased efficiency of the injection and extraction
wells of bioremediation systems. Another problem is that bacterial competition for available electrons is promoted, which often involves the displacement of the microbial communities of most interest for the biogeochemical process to be promoted (Bertrand et al., 2015).

Although, to a lesser extent, chemical nitrate reduction treatments by addition to the medium of Fe⁰ (metallic iron) in the form of micrometric-sized particles (micro-zero valent iron [mZVI]) have also been described (Liu and Wang, 2019). In this case, these are mainly abiotic reactions, including the Reaction 5 group (Chen et al., 2005; Rodríguez-Maroto et al., 2009), as follows:

\[
\text{Fe}^0 → \text{Fe}^{3+} + 2e^- \quad \text{Reaction 5 group (1)}
\]

\[
5\text{Fe}^0 + 2\text{NO}_3^- + 12\text{H}^+ → 5\text{Fe}^{3+} + \text{N}_2 + 6\text{H}_2\text{O} \quad \text{Reaction 5 group (2)}
\]

\[
4\text{Fe}^0 + 2\text{NO}_3^- + 10\text{H}^+ → 4\text{Fe}^{3+} + \text{NH}_4^+ + 3\text{H}_2\text{O} \quad \text{Reaction 5 group (3)}
\]

\[
\text{Fe}^0 + 2\text{NO}_3^- + 4\text{H}^+ → \text{Fe}^{2+} + 2\text{NO}_2^- + 2\text{H}_2\text{O} \quad \text{Reaction 5 group (4)}
\]

\[
6\text{Fe}^{2+} + 2\text{NO}_3^- + 8\text{H}^+ → 6\text{Fe}^{3+} + \text{NH}_4^+ + 2\text{H}_2\text{O} \quad \text{Reaction 5 group (5)}
\]

Under acidic conditions:

\[
\text{Fe}^0 + 2\text{H}^+ → \text{Fe}^{2+} + \text{H}_2 \quad \text{Reaction 5 group (6)}
\]

Under neutral conditions:

\[
\text{Fe}^{2+} + \text{Fe}^{3+} + \text{H}_2\text{O} → \text{iron oxides} + \text{H}^+ \quad \text{Reaction 5 group (7)}
\]

\[
\text{Fe}^0 + 2\text{H}_2\text{O} → \text{iron oxides} + \text{H}^+ \quad \text{Reaction 5 group (8)}
\]

Among the above reactions, it is noteworthy, on the one hand, that when Fe⁰ oxidizes it produces H₂ gas, which is one of the most important electron donors in the medium. These electrons can be used by the microorganisms, which favor autotrophic denitrification (Liu et al., 2018). However, on the other hand, some of the nitrate degradation reactions in the presence of Fe⁰, e.g., Reaction 5 (3), result in the formation of ammonium, which is toxic at high concentrations (Di Lorenzo et al., 2015).

The use of combined in situ chemical reduction (ISCR) and biostimulation methods in aquifers contaminated by nitrogen species is not a frequent practice. However, this combination of methods has shown satisfactory results in the treatment of other types of contamination, such as those shown by Herrero et al. (2019) in chloroethenes.

The working hypothesis of the current study is that, in regional diffuse contamination, nitrate concentration in groundwater can be decreased using combined methods with mZVI and biostimulation with lactic acid. To demonstrate this hypothesis, a treatability test was performed in the laboratory with microcosm experiments to study the decrease in the nitrate concentration in groundwater in the capture zone of a water supply well. It is intended that the laboratory test results could be used to analyze combined in situ remediation strategies in future field-scale implementation. These strategies would consist of the creation of reactive zones around groundwater supply wells. This would minimize the treatment costs of nitrate-contaminated groundwater before it is extracted by these wells.

The following specific objectives were set for studying the decrease in nitrates in the above-mentioned capture zone: (1) analysis of denitrification rates for the different remediation methods tested, based on chemical, isotopic, and molecular techniques; (2) establishment of the sequence of hydrogeochemical and biogeochemical processes occurring according to the different scenarios created; and (3) assessment of the efficiency of nitrate mobilization depending on the remediation method tested to establish the most suitable strategy.

A field zone was chosen the municipality of Torrefeta and Florejacs (La Segarra shire), to obtain sediment and groundwater from the capture zone of a well for the supply of drinking water to carry out the treatability test. This municipality is located in the nitrate pollution vulnerable zone #6 of Catalonia (Spain, Fig. 1A). This shire was declared a vulnerable zone for nitrate contamination from agricultural sources by Decree 283/1998 of October 21, 1998.

2. Site description

The monitoring network of the Catalan Water Agency (CWA) in this vulnerable zone is formed by two municipal supply wells for the village (W1 and W2, Fig. 1B). In fact, well W1 is formed by two wells, 3.7 and 7 m deep (both with a diameter of 1.5 m) and is connected by a 3 m gallery. Both wells are located in Tertiary (Oligocene) and Quaternary (Pleistocene and Holocene) materials of the Central Catalan Depression (Fig. 1B). The lower Oligocene materials (Fig. 1B) consist mainly of red clays and gray marls with interbedded sandstones and limestones. The Quaternary
The mentioned nitrate pollution vulnerable zone #6, where sediments and groundwater for the treatability test were taken, has been characterized by a significant increase in nitrate concentration over the years. In particular, in supply well W1 (Fig. 1C), nitrate concentrations remain above 100 mg/L (Fig. 1D), and in recent years a trend of sustained growth in the minimum values recorded has been observed, along with occasional increases in ammonium.

3. Materials and methods

3.1. Design, setup, and dimensioning of the microcosm-type treatability test with combined remediation methods

The treatability test consisted of four microcosm experiments in which the following scenarios were simulated (Table 1): i) natural attenuation (NAT), ii) biostimulation of the microbial flora with the addition of lactate (BLA, in the form of lactic acid) as the major carbon source and electron donor, iii) ISCR abiotic degradation with the addition of mZVI (ZVI), and iv) simulation of a biostimulation strategy with lactic acid combined (COMB) with ISCR using mZVI.

Each microcosm experiment was performed in duplicate; therefore, there were two actives and two controls per experiment (i.e., four bottles for each experiment). In the actives, microbial activity was present, while in the controls microorganisms were killed.

Table 1

<table>
<thead>
<tr>
<th>Microcosm experiment</th>
<th>Treatment performed</th>
<th>Bottles (duplicates are included)</th>
</tr>
</thead>
<tbody>
<tr>
<td>#1</td>
<td>Natural attenuation (NAT)</td>
<td>A-NAT 1, A-NAT 2, C-NAT 1, C-NAT 2</td>
</tr>
<tr>
<td>#2</td>
<td>Biostimulation with lactate as electron donor using lactic acid (BLA)</td>
<td>A-BLA 1, A-BLA 2, C-BLA 1, C-BLA 2</td>
</tr>
<tr>
<td>#3</td>
<td>ISCR with Fe(^{0}) microparticulate (ZVI)</td>
<td>A-ZVI 1, A-ZVI 2, C-ZVI 1, C-ZVI 2</td>
</tr>
<tr>
<td>#4</td>
<td>Combination of biostimulation and mZVI (COMB)</td>
<td>A-COMB 1, A-COMB 2, C-COMB 1, C-COMB 2</td>
</tr>
</tbody>
</table>

3.2. Water sampling protocols in the bottles of the experiments. Conservation procedures. Sample pretreatments. Parameters determined

To conduct the treatability test, once the bottles of the microcosm experiments were prepared, they were placed in the anaerobic chamber, where the water of the bottles was periodically monitored. An integration of chemical, isotopic, and molecular analyses was performed. Table 2 shows the samples taken during the treatability test.

The sampling protocols followed during the monitoring of water in the bottles of the microcosm experiments consisted of extracting a specific volume of water (Table 2) using syringes and opening the Minivert® valve of the bottle to be monitored.

Further information on chemical and isotopic analyses and molecular determinations conducted in microcosm water samples can be found in the SM document. This document also includes detailed information on analytical techniques to perform chemical and isotopic analyses and methodologies for molecular determinations.

3.3. Data processing

3.3.1. Processing of chemical and isotopic analytical data

The interpretation of the treatability test was performed by integrating the evolution in the microcosm experiments of the concentrations of nitrogen compounds. This integration was carried out with respect to the redox conditions characterized from the analysis of the redox sensitive species, similarly to what was done by Munz et al. (2019). In addition, the isotopic fractionation of the $^{15}$N of nitrate was studied, similarly to that performed by Vavilin and Rytov (2015), which allowed for characterization of the enrichment factor ($\varepsilon$) produced by the biotic denitrification process (Wells et al., 2019) and abiotic reduction of nitrate produced by the addition of mZVI (Grau-Martínez et al., 2019) as the main mechanisms of nitrate mobilization. Subsequently, all of these results obtained were interpreted in the framework of the molecular determinations (see Section 3.3.2).
3.3.2. Processing of data from molecular determinations

Once the analyzed genes were identified, the software of the equipment was used to determine the threshold cycle (Ct) value (which is the cycle in which the DNA concentration exceeds the established threshold). Specifically, the threshold values were 0.0224 for the nirS, nirF, and nosZ primers; 0.0143 for the narG and 16S (1055f-1392r) primers; and 0.0172 for the 16S (341f-785r) and Hzo primers. No presence was detected in the HszA, HszB and nor primer sets. In all cases, the average and standard deviation of the data were calculated (in semiquantitative units of cycles where the threshold value was reached).

For interpretation, values from the same set of primers are related to each other since all samples were analyzed together and under the same conditions (however, it is not possible to compare different sets of primers).

In addition, an increase in the Ct value indicates that there is a lower concentration of microorganisms, so that the concentration of DNA (or microorganisms) is inversely proportional to the Ct.

4. Results and discussion

4.1. Evolution of the concentrations and isotopic composition of nitrogen compounds in the microcosm experiments

4.1.1. Natural attenuation

The evolution of nitrate concentrations in the active NAT experiment (Fig. 2A, NAT) shows how the indigenous microbial communities have a high nitrate degradation capacity under natural conditions (Park et al., 2016) since after 3 days (4320 min) the nitrate concentration decreased by 81.76%, with a value below the parametric limit of 50 mg/L established by the European Union legislation for drinking water (European Union Directive, 2020), above which human health is at risk. The degradation rate throughout the NAT experiment was 0.23 day\(^{-1}\) or 0.02 h\(^{-1}\). This degradation rate, although below the value of 0.2 h\(^{-1}\) reported by Ghane et al. (2015), shows that denitrification did indeed take place under conditions of natural attenuation. The decrease in nitrate concentrations was accompanied by a maximum concentration of nitrite of 4 mg/L after 1 day (1440 min), which is consistent with the fact that denitrification is often favored by the complete mineralization of the initial nitrate to N\(_2\) gas (Tiedje, 1994; Istok et al., 2007; Keiner et al., 2015).

The mentioned degradation rate of nitrate in the active NAT experiment was also demonstrated by comparing the nitrate percentage mobilization between active and control experiments. In the former, the value was 95.33% at the end of the experiment, while in the latter it was only 13.95%, similar to what Ashok and Hai (2015) described.

In the NAT experiment, denitrification produced isotopic fractionation in nitrate (with an increase in \(\delta^{15}N\)) mainly in the active experiments (Fig. 3). Considering that experiments were conducted in duplicate, on day 0 (0 min, start of the treatability test) the \(\delta^{15}N\) nitrate value was 4.06‰ (Fig. 3), with practically no value dispersion. In contrast, on day 10 (14,400 min, when experiments finished) the range of \(\delta^{15}N\) values varied between 16.2‰ and 25.0‰. This range of values resulted in denitrification between the first and final day, leading to a range of \(\Delta\delta^{15}N\) values in nitrate that varied between 12.1‰ and 20.09‰, respectively, with a mean value of 16.1‰. With the same \(\delta^{15}N\) values between day 0 (0 min) and day 10 (14,400 min), the resulting E factor was \(-5.2 \pm 0.3\)‰, which is in the range of isotopic enrichment factors between \(-5\) and \(-30\)‰ of groundwater described by Aravena and Robertson (1998) and Wenk et al. (2014), among others.

4.1.2. Lactic acid biostimulation

The biostimulation experiment with lactic acid (Fig. 2C, BLA), as an electron donor in the form of lactate ion, generated overgrowth of the set of microbial consortia present in groundwater and sediment (see, for example, Fig. 4A in Section 4.3), similar to what was observed by Puigserver et al. (2016a,b), which generated a decrease in the efficiency of nitrate degradation. This implies that the nitrate concentrations did not fall below the parametric value of 50 mg/L until 7 days (10,080 min) after the experiment was initiated (Fig. 2C), which was accompanied by a high accumulation of nitrite (with maximum values of 5 to 6 mg/L), similar to what was observed by Chen et al. (2012).

The different degree of development of bacterial communities (see Fig. 4 in Section 4.3) was also revealed by a large dispersion of degradation rates ranging from 0.25 to 0.39 days\(^{-1}\), which showed that there was heterogeneity in the distribution of the microbial flora, especially that living in the sediment (Pedersen et al., 2015). This was also observed in the different percentages of decreases in nitrate, with values in the active experiment ranging from 94.43 to 98.26%, whereas in the controls the mobilization percentage was much lower, with an average value of 18% and with little dispersion.

The \(\delta^{15}N\) values in the BLA experiment were higher than those recorded in the case of the NAT experiment (Fig. 3). Thus, in the BLA experiment, \(\Delta\delta^{15}N\) values ranging from 22.07 to 25.26‰, with a mean value of 23.67‰, were observed. With the same \(\delta^{15}N\) values between day 0 (0 min) and day 10 (14,400), the resulting E factor was \(-6.8 \pm 0.5\)‰. Although biostimulation allows the microbial activity to favor nitrate degradation, the existence of bacterial competition for electrons supplied by lactate leads to a loss of efficiency in the total mineralization of nitrate.

4.1.3. Chemical reduction with micro-zero valent iron

The evolution of nitrate concentrations in the ZVI experiment, with mZVI as a reducing agent (Fig. 2E, ZVI), showed a higher degradation rate (varying between 0.42 and 0.32 days\(^{-1}\), with a mean value of 0.38 days\(^{-1}\)) than in the case of the BLA experiment. This is attributable to the fact that the use of mZVI partially inhibited the activity of denitrifying microorganisms (Xie et al., 2017), especially immediately after mZVI was placed with the sediment in the bottles of this experiment on day 0 (0 min). However, the progressive consumption of mZVI promoted the recovery of microbial activity (Cullen et al., 2011). In contrast to the NAT and BLA experiments, the control experiment showed a higher nitrate degradation rate because of the abiotic degradation caused by the reduction promoted by mZVI (Y. Zhang et al., 2019).

According to Reaction 5 (3), the degradation of nitrate in the presence of mZVI as a reducing agent was accompanied by an increase in ammonium concentration, as observed by Jia et al. (2017), Yang et al. (2018), Liu and Wang (2019), and Su et al. (2020). This was observed in both active
It is noteworthy that in the two actives (duplicates) ammonium production was lower than in the two controls, which suggests that there are microorganisms capable of degrading ammonium. In contrast, the nitrite concentrations recorded in the active experiments (with maximum values up to 2.6 mg/L) were lower than those recorded in the NAT and BLA experiments.

The isotopic evolution of $\delta^{15}N$ of nitrate (Fig. 3) showed a higher isotopic fractionation in the ZVI experiment than in the NAT experiment. Thus, in the active ZVI experiments, the nitrate $\Delta\delta^{15}N$ value varied between 40.7 and 44.6 ‰, which represents a mean value of 42.7 ‰ and is about 20 units more than in the NAT experiment. This higher isotopic fractionation was also evidenced by a greater $^{15}$N nitrate $\epsilon$ factor, which was $-11.6 \pm 0.2$ ‰, below that reported by Grau-Martínez et al. (2019), who recorded a value of $-29.5 \pm 2.7$ ‰.

### 4.1.4. Combination of mZVI and biostimulation

The evolution of nitrate concentrations in the COMB experiment, with the combined strategy of mZVI and biostimulation with lactic acid (Fig. 2G, COMB), showed an evolution quite similar to that in the ZVI experiment (Fig. 2E, ZVI). Thus, nitrate concentrations were below 50 mg/L 2 days (2880 min) after the start of the experiment. However, the biostimulation of bacterial communities in the COMB experiment (Fig. 2C) slightly decreased the nitrate degradation rate (ranging from 0.27 to 0.34 days $^{-1}$), similar to what was observed in the experiment exclusively with biostimulation (Fig. 2C, BLA). Nevertheless, in counterpart, no remarkable ammonium production was observed in the COMB experiment (Fig. 2G, COMB), as was the case in the ZVI experiment (Fig. 2E, ZVI). This confirms the greater efficiency of combining the use of mZVI with biostimulation in reducing toxicity since the decrease in nitrate is accompanied by little formation of toxic ammonium. Due to the absence of microbial activity in the control experiments, the controls of the COMB experiment showed a similar evolution of nitrate concentrations to those of the ZVI controls.

Isotopic fractionation was also recorded in the COMB experiment (Fig. 3), with $\Delta\delta^{15}N$ values higher than those in the other experiments. Thus, $\Delta\delta^{15}N$ values varied in the COMB experiment between 54.3 and 64.3 ‰, with a mean value of 59.4 ‰, which, specifically compared to the ZVI experiment, were higher than in this experiment. This agrees with the mentioned higher degradation efficiency because of the combination of biotic and abiotic degradation. Furthermore, although in the COMB experiment, the degradation of nitrate to nitrite (or ammonium) followed the same pathway as in the case of the ZVI experiment. In the COMB experiment, the $\epsilon$ factor was higher ($-17.4 \pm 0.2$ ‰) than in the ZVI experiment. Fig. 3 also shows that a large part of the isotopic fractionation in the COMB experiment was caused by the abiotic nitrate degradation reaction caused by the presence of mZVI in that experiment.
4.2. Evolution of redox-sensitive species

In general, an increase in Mn$^{2+}$ concentrations was observed in the BLA and COMB experiments (Figs. 2D, H), up to 2.5 and 40.5 mg/L, respectively, which confirms that Mn reduction occurred (Trouwborst et al., 2006; McMahon et al., 2018).

In the case of Fe$^{2+}$ concentrations, small increases were detected in the NAT and BLA experiments (Fig. 2B, D), up to 2 and 2.5 mg/L, respectively, and high increases in the COMB experiment (Fig. 2H) up to 60 mg/L were observed. This reveals that in the NAT and BLA experiments Fe-reducing conditions were reached, which is in agreement with Abiriga et al. (2021), and more reducing conditions were found in the case of COMB.

The evolution of sulfate concentrations showed that in the NAT experiment there was a decrease of 30% of the initial concentration after 3 days (4320 min), which remained constant thereafter (Fig. 2B, NAT). In the BLA experiment, sulfate concentrations did not significantly decrease until 7 days (10,080 min, Fig. 2D, BLA).

In the ZVI experiment, sulfate decreases were slightly higher than those recorded in the NAT experiment—about 43% after 3 days (4320 min)—but, unlike in the NAT experiment, concentrations continued to decrease up to 50% at the end of the experiment on day 10 (14,400 min, Fig. 2F, ZVI). The decrease in sulfate was accompanied by low Fe$^{2+}$ concentrations (that did not reach 1 mg/L throughout the experiment; Fig. 2F, ZVI). This reveals the precipitation of iron sulfide as a result of sulfate reduction (Christensen et al., 2000; Karimian et al., 2018).

In the case of the COMB experiment (Fig. 2H, COMB), sulfate concentrations were below the detection limit after 7 days (10,080 min), implying that 100% was eliminated because of highly reducing conditions, possibly even methanogenic.

4.3. Evolution of genes characteristic of denitrification and anammox processes

The analysis of the evolution of the microbial communities detected with the 16S primers (1055f-1392r, in Table 1 in the SM document) showed that the highest degree of microbial development occurred in the BLA experiment (Fig. 4A) between 7 and 10 days (10,080 and 14,400 min, respectively), similar to what was observed by Puigserver et al. (2016a,b).

In some cases (Fig. 4A), the abundance of bacterial communities in the NAT and COMB experiments decreased. This is what is shown in Fig. 4A, where the height of the colored bar corresponding in the plot to the different experiments decreases because of the number of PCR cycles required to assess the amount of communities would need to be increased (see the Fig. 4 caption). This decline in bacterial communities was caused by the increase in reducing conditions, in which many of the microorganisms cannot survive.

In contrast, in the case of the ZVI experiment (Fig. 4A), a progressive increase in the abundance of microbial communities was observed. This increase is attributable to the fact that the initially high amount of Fe$^{0}$ (in the form of mZVI) was toxic to many of the microbial communities (Xue et al., 2018; Daraei et al., 2019). The consumption of the initially introduced mZVI and the adaptation of the microbial communities to the presence of this iron allowed these communities to increase their presence throughout time (Summer et al., 2020; Xu et al., 2021).

The narG gene (nitrate reductase, Table 1 in the SM document) was detected from the start of the experiments. This is consistent with the presence of nitrite in the water sampled at the beginning of the experiments, proving that microorganisms capable of performing the denitrification process naturally exist in the medium (Zielińska et al., 2016; K. Zhang et al., 2019), which further demonstrates that this process takes place at the site.

According to what was observed with the evolution of the concentrations of the nitrogenous compounds (see Section 4.1), a significant increase in the communities encoding the narG gene was observed in the NAT experiment (Fig. 4B) and, to a lesser extent, in the COMB experiment (with the exception of the time of 7 days, 10,080 min, Fig. 4B). By contrast and in accordance with what was observed in the evolution of the concentrations of the nitrogen compounds (see Section 4.1), although a slight increase in the communities encoding this gene was observed in the BLA experiment, after the start of the experiments, the presence of these communities did not show significant differences in general. This, together with the increase in abundance recorded with the 16S (1055f-1392r) primers, demonstrate
that biostimulation alone is not a strategy that particularly favors the passage of nitrate to nitrite by denitrification (Garousin et al., 2021).

The reduction of nitrite to NO identified with the nirS gene (nitrite reductase, Table 1 in the SM document) was detected from time 0, which is new evidence that the denitrification process occurs naturally (Zhou et al., 2016; Li et al., 2019; H. Zhang et al., 2019). This contrasts with the absence at time 0 of the nirS gene (nitrite reductase, Table 1 in the SM document, Fig. 4D).

Among all the experiments conducted, the COMB experiment was the one with the highest abundance of the nirS gene (Fig. 4C); although, it decreased slightly throughout the experiment, which confirms that throughout the experiment the denitrification process was at a more advanced stage, favoring the total mineralization of nitrate (Espenberg et al., 2018). This pattern was similar to that observed in the NAT experiment; although, in the latter there was a certain recovery of the presence of this gene at the end (Fig. 4D).

The nirS gene was not detected at the beginning of the experiments. Its presence was observed in the NAT, BLA, and COMB experiments, especially after 3 days (4320 min), decreasing slightly throughout the test (Fig. 4D). This suggests that the denitrifying communities change throughout the experiment, similar to what was observed by Feng et al. (2019). The analysis of this gene shows that the passage of nitrite to NO is frequent in the NAT and COMB scenarios. In contrast, this passage was only observed after 10,080 min (7 days) in the ZVI experiment, which agrees with the toxicity effect of mZVI on bacterial communities. The reduction of NO to NO2 was not identified as the cnorB gene was practically not observed. However, the presence of the nosZ gene (nitrous oxide reductase) was observed (Fig. 4E), which is in accordance with the observations of Zhang et al. (2021). The progressive increase in this gene in the COMB experiment and its decrease at the end of the experiments is noteworthy (reduction process of NO2 to N2 gas). The detection of the nosZ gene from the beginning of the experiments agrees with complete denitrification, even under natural attenuation conditions (Hallin et al., 2018); although, Fig. 4E suggests that the reducing conditions at the end of the experiments favored the increase in this gene in the COMB experiment and its decrease in the NAT, BLA, and ZVI experiments (Song et al., 2015; Wang et al., 2020; Yuan et al., 2021).

The presence of the nirS gene from the beginning of the experiment, the presence of the nirS gene after 3 days (4320 min), and the increase in the abundance of this gene in the COMB experiment (Fig. 4C), accompanied by the decrease in ammonium (Fig. 2G, COMB), originated from the abiotic degradation of nitrate by mZVI, suggesting the existence of anaerobic...
ammonium oxidation processes such as anammox (Shu et al., 2016; Wang et al., 2019; Podder et al., 2020) in the COMB strategy.

Although the HzsA (hydratase synthase) and HzsB (hydratase synthase) genes were analyzed (Table 1 in the SM document), they were not sufficiently detected throughout the experiments, which does not allow for determination of whether NH₄⁺ synthesis from NO and ammonium occurred. By contrast, the presence of the Hzo gene (hydratase oxidoreductase) was detected in some of the samples from a time of 3 days (4320 min) (Fig. 4F). Among the different experiments, the presence of this gene stands out in the ZVI experiment (where an increase in its presence was observed especially at the 7 days) (10,080 min, Fig. 4F). It was also observed that in the COMB experiment this gene (which is related to the occurrence of the anammox reaction, see Section 4 in the SM document) remained constant, which confirms that this reaction occurred very early in the experiment, probably from the beginning, while in the ZVI experiment it occurred significantly later (i.e., at 7 days, 10,080 min), coinciding with the substantial decrease in ammonium concentrations (Fig. 2E, ZVI), similar to what was observed by Wang et al. (2019) and Connan et al. (2016).

### 4.4. Evolution of chemical and biochemical processes

The processes identified throughout the treatability test were mainly denitrification, Mn and Fe reduction, sulfate reduction, and anammox.

In the case of denitrification, it was observed that this process occurred completely in all the microcosm experiments, but it was in the COMB experiment that it was most effective and with a greater presence of the nosZ gene, which is indicative of the passage from NO to NO₂.

In the case of Mn- and Fe-reduction processes, they were detected in all the experiments, while sulfate reduction was only significantly detected in the COMB experiment and, to a lesser extent, in the ZVI experiment.

Although the anammox process could potentially have occurred in the NAT experiment (as indicated by the presence of enzymes such as those expressed by the Hzo gene), the low ammonium concentrations in the waters sampled at the beginning of the experiment did not make it possible for the process to occur in the NAT and BLA experiments. By contrast, ammonium occurred in the ZVI and COMB experiments, where ammonium was produced as a consequence of abiotic nitrate degradation, which generated toxicity problems in the water. Mobilization of ammonium occurred in the COMB experiment because of the more extreme reducing conditions created and the biostimulation of the microbial communities, which gave rise to the anammox process.

#### 4.5. Comparative analysis of the efficiency of the different remediation strategies tested for decreasing nitrate concentrations

Table 3 shows how the most efficient strategy in removing 80 % of the original nitrate (representing a nitrate concentration below the parametric value of 50 mg/L, European Union Directive, 2020) was ZVI, followed by COMB. However, the high production of ammonium generated in the ZVI experiments should be recalled. This cation is toxic (see Sections 1 and 41.4). Hence, it is considered that the most efficient strategy is that which, in addition to removing nitrate, is also capable of eliminating the toxic ammonium, which is achieved with the COMB strategy (Table 3).

However, the high efficiency of the combined remediation strategy requires appropriate dimensioning of the reagents to be added according to the characteristics of the site. An excess of reagents, and especially of lactic acid for biostimulation, can lead to extremely reducing conditions in the medium. Under these conditions, overgrowth of other microorganisms that do not result in denitrification can occur, and even bioclogging of aquifer effective porosity, or the mobilization of metal ions such as Fe²⁺ and Mn⁴⁺ from iron or manganese oxide minerals. These ions, under some specific circumstances, can re-precipitate. For example, this can occur with Fe²⁺ (including that from the oxidation of mZVI) if redox conditions favor it. Neof ormation pyrite can precipitate if there is dissolved sulfate in the medium and the redox conditions are sulfate-reducing. This can result in a loss of effective porosity and hydraulic conductivity, as in the case of bioclogging. As a consequence, the preferential flow paths may change, so that there may be areas that are no longer accessible to the reagents and therefore untreated. It would therefore be recommendable that, in a second phase, a pilot test be conducted in the field to address these and other key possible issues arising from implementing the COMB strategy in the real world (more information can be found in the SM document).

### 5. Conclusions

The use of mZVI for the abiotic degradation of nitrate promotes the formation of ammonium (an undesirable compound due to its high toxicity). By contrast, the strategy resulting from the combination of the use of mZVI coupled with biostimulation of the microbial flora present in the medium causes the anammox process to degrade the harmful ammonium.

The integrated analysis of chemical, isotopic, and biological data shows that the combined strategy favors the denitrification process and results in total mineralization of the original nitrate, which is accompanied by good microbial development of the microorganisms performing this reaction.

The treatability test with microcosm experiments on a laboratory scale shows that the in situ remediation strategy combining ISCR using mZVI and biostimulation with the addition of lactic acid (lactate acting as an electron donor) is a viable strategy for the creation of a reactive zone around supply wells located in regions where groundwater is affected by diffuse nitrate contamination.

The high efficiency of the combined strategy requires an adequate dimensioning of reagents according to the characteristics of the site, since an excess, especially lactic acid, can lead to extremely reducing conditions in the environment. This could favor overgrowth of non-denitrifying bacterial communities, bioclogging of the porosity, mobilization of metal ions such as Fe²⁺ and Mn⁴⁺, and even precipitation of neof ormation minerals, such as pyrite that (as in bioclogging) can result in a loss of effective porosity and hydraulic conductivity. As a result, preferential flow paths may change, leading to zones that may become inaccessible to the reagents and remain untreated. For this reason, it would be advisable to conduct a pilot study in the field to evaluate how to deal with the problems that may arise during the implementation of the COMB strategy.

### CRediT authorship contribution statement

**Diana Puigserver:** She designed the treatability test, executed and testified the research boreholes. She collected the groundwater and sediments needed for the test at those boreholes. She also sampled the waters in the anaerobic chamber in which the test was carried out and performed the analyses of nitrogen species and other sensitive redox species. She also processed the data, participated in the discussion meetings of the results obtained with the other two authors, and wrote the article.

**Jofre Herrero:** He performed the enzyme detection analyses and the different molecular techniques necessary for this purpose in the waters sampled during the test. He also carried out the determination of the nitrogen isotopic fractionation of nitrites in these waters to verify that denitrification was occurring. He also carried out the corresponding processing of the data obtained in the enzyme determination and isotopic fractionation. He participated in the discussion meetings of the results obtained together with the other two authors.
José M. Carmona: He participated in the execution of the research drilling boreholes, in the testification, and in their equipment as piezometers. He also supervised the progress of the research and participated in the meetings to discuss the results obtained with the other two authors.

**Declaration of competing interest**

The authors of this research paper (Diana Puigserver, Jofre Herrero and José M. Carmona) 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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**Appendix A. Supplementary data**

Supplementary data to this article can be found online at [https://doi.org/10.1016/j.scitotenv.2022.156841](https://doi.org/10.1016/j.scitotenv.2022.156841).

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