Nitrogen, carbon, and sulfur isotopic change during heterotrophic (Pseudomonas aerofaciens) and autotrophic (Thiobacillus denitrificans) denitrification reactions

Takahiro Hosono¹*, Kelly Alvarez², In-Tian Lina, Jun Shimadab

¹ Priority Organization for Innovation and Excellence, Kumamoto University, 2-39-1 Kurokami, Kumamoto 860-8555, Japan.
² Graduate School of Science and Technology, Kumamoto University, 2-39-1, Kurokami, Kumamoto 860-8555, Japan.

*Corresponding author.
Tel & Fax: +81-96-342-3935 Email address: hosono@kumamoto-u.ac.jp (T. Hosono)

ABSTRACT

In batch culture experiments, we examined the isotopic change of nitrogen in nitrate ($\delta^{15}$N$_{NO_3}$), carbon in dissolved inorganic carbon ($\delta^{13}$C$_{DIC}$), and sulfur in sulfate ($\delta^{34}$S$_{SO_4}$) during heterotrophic and autotrophic denitrification of two bacterial strains (Pseudomonas aerofaciens and Thiobacillus denitrificans). Heterotrophic denitrification (HD) experiments were conducted with trisodium citrate as electron donor, and autotrophic denitrification (AD) experiments were carried out with iron disulphide (FeS$_2$) as electron donor. For heterotrophic denitrification experiments, a complete nitrate reduction was accomplished, however bacterial denitrification with T. denitrificans is a slow process in which, after seventy days nitrate was reduced to 40% of the initial concentration by denitrification. In the HD experiment, systematic change of $\delta^{13}$C$_{DIC}$ (from -7.7‰ to -12.2‰) with increase of DIC was observed during denitrification (enrichment factor $\epsilon$N was -4.7‰), suggesting the contribution of C of trisodium citrate ($\delta^{13}$C = -12.4‰). No SO$_4^{2-}$ and $\delta^{34}$S$_{SO_4}$ changes were observed. In the AD experiment, clear fractionation of $\delta^{13}$C$_{DIC}$ during DIC consumption ($\epsilon$C = -7.8‰) and $\delta^{34}$S$_{SO_4}$ during sulfur use of FeS$_2$-S (around 2‰), were confirmed through denitrification ($\epsilon$N = -12.5‰). Different pattern in isotopic change between HD and AD obtained on laboratory-scale are useful to recognize the type of denitrification occurring in the field.

Keywords: heterotrophic denitrification; autotrophic denitrification; isotope fractionation; C, N, S isotopes; batch culture experiments

© 2015. This manuscript version is made available under the Elsevier user license http://www.elsevier.com/open-access/userlicense/1.0/
1. Introduction

Controlling and reducing the nitrate content in aquifers is critical for the quality of water systems. Among nitrate biological removal methods, heterotrophic and autotrophic denitrifying microorganisms are important mediators in the denitrification process of nitrate-contaminated ground and surface waters. Biological denitrification including both heterotrophic denitrification (HD) and autotrophic denitrification (AD), is the anaerobic process which allows the breakdown of nitrates by bacteria, resulting in the release of gaseous products $N_2O$ and $N_2$ into atmosphere (Korom, 1992).

HD process requires an organic carbon source, which can be supplied as soluble (glucose, citrate, methanol, ethanol, etc.) (e.g., Akunna et al., 1993; Christensson et al., 1994; Nyberg et al., 1996; Soares, 2000) or insoluble carbon compounds (cellulose based materials such as newspaper, wheat straw, cotton, etc.) (e.g., Volokita et al., 1996a; Volokita et al., 1996b; Schipper et al., 2004; Aslan and Turkman, 2005). In the case of trisodium citrate as a sole carbon source, the denitrifying reaction may be simplified as:

$$Na_3C_6H_5O_7(s) + 3NO_3^{-}(aq) \rightarrow 3/2N_2(g) + 3NaHCO_3(s) + 3CO_2(g) + H_2O$$  (1)

On the other hand, AD can be carried out using zero-valent iron (e.g., Choe et al., 2004; Biswas and Bose, 2005), ferrous ions (e.g., Benz et al., 1998), elemental sulphur (e.g., Batchelor and Lawwrence, 1986; Soares, 2002; Mohammadi et al., 2011) or reduced sulfur compounds such as FeS$_2$ (pyrite) (e.g., Haaijer et al., 2007; Jorgensen et al., 2009; Torrentó et al., 2010), typically supplied as granular material, as an electron donor and reduces nitrate or nitrite to nitrogen gas. Pyrite is the stable iron sulfide phase in anoxic low-temperature sedimentary or aquatic environments (Rickard and Luther, 2007) and has been proposed as an abundant and inexpensive material, which can be easily decomposed by bacteria action especially when the pyrite is provided in fine crystals and dust (Rickard and Luther, 2007). For the FeS$_2$ case the following denitrification overall reaction is expected (Torrentó et al., 2010):

$$15NO_3^{-}(aq) + 5FeS_2(s) + 10H_2O \rightarrow 15/2N_2(g) + 10SO_4^{2-}(aq) + 5Fe(OH)_3(s) + 5H^+$$  (2)

The coupled N and O isotope behavior during in HD and AD reactions has been studied in laboratory-scale using pure cultures of heterotrophic and autotrophic bacteria
and is very well acknowledged (e.g., Wellman et al., 1968; Delwicche and Steyn, 1970; Barford et al., 1999; Granger et al., 2008; Knöeller et al., 2011; Wunderlich et al., 2012; Torrentó et al., 2010). In both cases, HD and AD, a decrease in nitrate concentration is coupled with an increase in the $\delta^{15}$N and $\delta^{18}$O of residual nitrate and it has been commonly thought that the fractionation ratio of $\delta^{15}$N$_{NO_3}$ and $\delta^{18}$O$_{NO_3}$ during denitrification ranges within 1.3-2.1 (Aravena and Robertson, 1998; Mengis et al., 1999; Böttcher et al., 1990; Fukada et al., 2003).

Carbon dioxide is one of the by-products of HD reaction (Eq. (1)). Since the organic source carbon is isotopically more depleted in $^{13}$C compared to that of the carbonate/bicarbonate pools (i.e., alkalinity) (Nascimento et al., 1997), the $\delta^{13}$C values of the DIC containing this carbon will be more negative than the DIC which either does not contain this carbon or the DIC whose $\delta^{13}$C has been modified significantly by carbonate dissolution (Schulte et al., 2011). Therefore, the carbon isotope ratios of DIC can serve as a useful tool in monitoring HD.

Similarly, sulfate is one of the productions of AD reaction through pyrite oxidation (Eq. (2)). During sulfide oxidation a moderate isotopic fractionation, ranging from $-4\%\_o$ to $3.5\%\_o$, was reported between sulfide metals and dissolved sulfate (Balci et al., 2007, 2012; Pisapia et al., 2007; Thurston et al., 2010; Heidel and Tichomirowa, 2011; Brabec et al., 2012; Heidel et al., 2013). Since sulfur in sulfide minerals is typically more depleted in $\delta^{34}$S compared to that of sulfate pools in earth surface environments (i.e., evaporate) (Krouse and Grinenko, 1991; Bottrell and Newton, 2006), a depression of $\delta^{34}$S values of sulfur in produced sulfate might be expected with the progress of AD reaction.

A coupled use of chemical data with the $\delta^{15}$N and $\delta^{18}$O of dissolved nitrate and the isotopic compositions of the ions involved in denitrification reactions, as, for example, the $\delta^{34}$S of dissolved sulfate, and/or the $\delta^{13}$C of dissolved inorganic carbon, can be applied to determine the relative role of heterotrophic and autotrophic processes in natural denitrification. In fact, the use of combined $\delta^{15}$N$_{NO_3}$, $\delta^{13}$C$_{DIC}$, and $\delta^{34}$S$_{SO_4}$ for natural aquifer systems have been proposed to distinguish the kind of denitrification occurring in aquifer (Aravena and Robertson, 1998; Otero et al., 2009; Carrey et al., 2013; Puig et al., 2013; Hosono et al., 2014). For instance, in a field study in a shallow aquifer Aravena and Robertson (1998) found that as denitrification proceeds $\delta^{15}$N$_{NO_3}$ showed a drastic increasement (6.4\% to 58.3\%) and at the same time $\delta^{13}$C$_{DIC}$ values decreased (-1.9\% to -8.6\%). In this study, the authors suggested that this decline in $\delta^{13}$C$_{DIC}$ could be regarded as an indicator of heterotrophic denitrification. Otero et al. (2009) found also a distinct trend in $\delta^{34}$S$_{SO_4}$ as denitrification occurred in a deep aquifer.
In this case of study $\delta^{34}\text{S}_{\text{SO}_4}$ decreased (10‰ to -20‰) while an enrichment of $\delta^{15}\text{N}_{\text{NO}_3}$ (10‰ to 35‰) was observed. The authors suggested that this declining in $\delta^{34}\text{S}_{\text{SO}_4}$ is an indication that the denitrification here proceeded autotrophically. However, the behavior of $\delta^{13}\text{C}_{\text{DIC}}$ and $\delta^{34}\text{S}_{\text{SO}_4}$ during HD and AD has rarely been reported in batch experiments and the utility of this multi-isotopic approach has not been fully evaluated and evidenced at laboratory scale yet.

The purpose of this study was to find out the extent to which $\delta^{15}\text{N}_{\text{NO}_3}$, $\delta^{13}\text{C}_{\text{DIC}}$, and $\delta^{34}\text{S}_{\text{SO}_4}$ isotopes are discriminated during HD and AD in anaerobic microbial cultures. In this study, we used two pure denitrifier cultures: Pseudomonas aerofaciens and Thiobacillus denitrificans capable of heterotrophic and autotrophic denitrification, respectively. In order to evaluate the isotopic response in HD and AD processes two different experiments were carried out: (1) 120 hours batch experiment with P. aerofaciens reacting with a trisodium citrate medium with a defined amount of NO$_3^-$ and (2) 70 days batch experiment of T. denitrificans reacting with pyrite and a SO$_4^{2-}$ free medium. The obtained data allow evaluating the magnitude of the isotopic fractionation expected in nitrate-contaminated water systems.

2. Materials and methods

2.1. Pyrite preparation

Cuboid pyrite crystals of more than 99% of purity were obtained from sedimentary deposits in Navajún, La Rioja (Spain). The minor impurities are Ni and Co. These impurities do not affect the basic cubic structure. To generate a clean pyrite surface, the experimental procedure suggested by Sasaki et al. (1995) was followed. This procedure would create a condition that the chemical species on the surface of the pyrite are only iron (Fe) and sulfur (S) (Sasaki et al., 1995). Briefly, the crystals were pulverized in a tungsten carbide vessel using a Herzog HSM-F36 (Germany) in order to obtain a particle size of approximately 5 μm. The pyrite powder was washed in Milli-Q pure water and ultrasonicated for 1 h to remove fine pyrite particles adhering to the surface. Subsequently, it was shaken in a 10% hydrochloric acid solution at 90 rpm for 1 hour to remove possible impurities on the pyrite surface. At this point, the powder was rinsed with Milli-Q pure water and ultrasonicated three times to eliminate the acid from the surface. Then, the pyrite powder was sterilized and dehydrated with pure ethanol and finally was vacuum-dried in an oven (EYELA VOS-301SD, Tokyo Rikakikai Co. Ltd, Japan) at 60°C for 1 day. The final material was stored in a N$_2$-purged desiccator for
batch culture experiments.

2.2. Growth and maintenance of bacterial cultures

For the heterotrophic denitrification experiments, cells of *P. aerofaciens* (strain ATCC No. 13985) were obtained from the German Collection of Microorganisms and Cell Culture (GCMCC) and were grown under sterile conditions in a citrate minimal medium (CMM) used previously by Anderson et al. (1993), containing 53.3 mM of K₂HPO₄ and 13.3 mM of KH₂PO₄ in 1 liter of distilled water adjusted to pH 7.5 and autoclaved prior to addition of the following filter-sterilized (pore size, 0.2 μm) reagents: 4.24 mM of (NH₄)₂SO₄, 0.1 mM of Na₂MoO₄⋅2H₂O, 0.02 mM of FeSO₄⋅7H₂O, 0.005 mM of MnCl₂⋅4H₂O, 0.35 mM of CaCl₂⋅2H₂O, 0.81 mM of MgSO₄⋅7H₂O, and 4.6 mM of trisodium citrate (Na₃C₆H₅O₇). Exponentially growing *P. aerofaciens* preparations were produced by growing cultures in CMM for two days at 25°C and unshaken. *T. denitrificans* (strain ATCC No. 23644) a classic autotrophic denitrifying bacteria was purchased from GCMCC. Cells of *T. denitrificans* were grown in batch culture in a synthetic medium (100 ml) containing the following reagents: Na₂HPO₄ (3.35 mM), KH₂PO₄ (13.24 mM), MgSO₄⋅7H₂O (0.41 mM), (NH₄)₂SO₄ (0.76 mM), CaCl₂⋅2H₂O (0.20 mM), FeCl₃⋅6H₂O (0.074 mM), MnSO₄⋅H₂O (0.12 mM), Na₂S₂O₃⋅5H₂O (40.32 mM), NaHCO₃ (5.95 mM), and KNO₃ (5 mM). All chemicals were of analytical grade. *T. denitrificans* cultures were incubated for two weeks, maintained under anaerobic conditions at 25°C and unshaken.

2.3. Experimental setting for HD

After cultures were grown in CMM medium to late exponential growth phase, 150 ml cultures were combined and concentrated by centrifugation at 3287 × g for 10 min at 0°C. The supernatant was discarded and the cell pellets were resuspended in 60 ml CMM medium with KNO₃ (sole nitrate source) to a final concentration of 10 mM NO₃⁻. Batch cultures experiments were started by diluting in anaerobic conditions 3 mL of resuspended culture into 100 mL of fresh CMM medium with 10 mM NO₃⁻ in a 100 ml bottle purged with N₂ for 1 hour. In total, 19 bottles of 100 ml containing bacterial cells and CMM medium with 10 mM NO₃⁻ were incubated static for 120 hours inside a nitrogen gas glove box at 25°C. As a blank (regarded as starting composition at time 0) 1 bottle containing CMM medium with 10 mM NO₃⁻ only was stored for the same period of time as an uninoculated control under the same conditions. The biological
reaction was stopped by the addition of 2 ml of 10N NaOH every 4 hours. This period was chosen after pilot experiments done and taking into account the growth rate of *P. aerofaciens*. At the end of the experiment, the water samples in the bottles were filtered (0.2 μm) and 20 ml aliquots were taken for carbon and nitrate isotope measurements, this water samples were kept tightly sealed and frozen until the concentration and C and N isotope measurements were done. For δ³⁴S isotope analysis the remaining of the filtrate of each bottle was preserved acidified to pH<2 with 20% HCl solution in order to remove HCO₃⁻ and CO₃²⁻ species. Water soluble sulfate was precipitated as barite (BaSO₄) by adding a solution of BaCl₂. The precipitates were filtered with a 0.45 μm pore size filter, were rinsed with deionized water twice and finally oven-dried at 100°C for 3 hours.

2.4. Experimental setting for AD

After cultures were grown for two weeks to late exponential phase, 600 ml cultures were combined and concentrated by centrifugation at 3287 × g for 10 min at 0°C. The supernatant was discarded and the cell pellets were resuspended in 150 ml of modified synthetic medium without SO₄²⁻ (modified synthetic medium: NH₄Cl (18.7 mM), KH₂PO₄ (14.7 mM), NaHCO₃ (30 mM), MgCl₂·6H₂O (3.25 mM), CaCl₂·2H₂O (0.05 mM), KNO₃ (5 mM)) to minimize the sulfate blank. The pH of the final modified synthetic medium was adjusted to 7.2 with a K₂HPO₄ - KH₂PO₄ buffer. The autoclaved modified medium solution was purged with N₂ for 1 hour before inoculation with bacteria to remove the dissolved oxygen. The bottles of the modified medium containing the pyrite powder were equilibrated inside the anaerobic chamber for 2 days prior to the start of the experiments. The experimental cultures were prepared by inoculating, in anaerobic conditions, 5 ml of resuspended cultures into 200 ml of the modified synthetic medium containing 4 g of pyrite powder, in a 250 ml pre-sterilized polyethylene bottle. The 5 ml cell suspensions were added to each bottle before the bottles were sealed with a gas headspace inside the anaerobic chamber. Under these conditions pyrite will be the only electron donor available for the cultured cells. The 250 ml bottles containing modified medium, pyrite and bacterial cells were incubated for 70 days. This period was chosen after pilot experiments done and taking into account the growth rate of *T. denitrificans*. For each sampling time point the pH of the solutions was measured, the liquid in the bottle was filtered (0.2 μm) to separate the pyrite from the modified medium containing the cells and the possible bacterial activity in the filtrate was stopped by the injection of 5 ml of 10 N NaOH. At the beginning of
the experiment, this process was performed every day. After the day sixteen the interval of time to stop the reaction was changed from 1 to every 3 days since the NO$_3^-$ concentration of the samples didn’t change very much, and for the last three intervals of time the reaction was stopped after 7 days. In total, 30 bottles for each time-point containing modified medium + pyrite + bacterial cells and 1 blank consisting of medium and pyrite only (no cells, regarded as starting composition at time 0) were produced. The 31 bottles were stored static the seventy days inside a nitrogen gas glove box. Table 1 summarizes the conditions varied for denitrifiers in the HD and AD denitrification experiments. Since *T. denitrificans* activity is very pH sensitive, at each sampling time point it was confirmed that the pH values of the liquid inside the bottles was between 7 and 8 throughout the incubations.

2.5. Chemical and isotope analyses

Samples were analyzed for chemical and isotopic composition, nitrate concentration varied during denitrification; after nitrate concentration became constant the samples were not analyzed for isotope ratios.

2.5.1 Concentrations of SO$_4^{2-}$ and NO$_3^-$

The content of [SO$_4^{2-}$] and [NO$_3^-$] fractions of samples solutions were determined by ion chromatography (IC) using a 761 Compact IC System, equipped with an anion-separator column (Dual 2) and a conductivity detector (Metrohm, Switzerland). For the heterotrophic and autotrophic cultures the samples for IC were collected by taking an aliquot of 1 ml of the supernatant from each of the bottles and were diluted by a factor of 100 with de-ionized water. Diluted samples were measured after filtration through a 0.2 μm syringe filter.

2.5.2. Isotope ratios of nitrate and oxygen in NO$_3^-$

The $\delta^{15}$N$_{NO_3}$ and $\delta^{18}$O$_{NO_3}$ were analyzed using an isotopic ratio mass spectrometer (IRMS) (Finnigan Delta V Advantage, Thermo Fisher Scientific, USA), coupled with a headspace gas sampler (GasBench II, Thermo Fisher Scientific, USA). The samples preparation follows the bacterial denitrifier method developed by Sigman et al. (2001) and Casciotti et al. (2002), which is outlined briefly here. *Pseudomonas aerofaciens* (*P. aerofaciens*) cultures grown for 3 days are concentrated 5-fold by centrifugation and then split into 4.5 ml aliquots in 20 ml glass vials. The vials are crimp-sealed with Teflon-backed silicone septa and purged for 2 h with high purity (99.9995%) N$_2$ gas.
The NO$_3$ concentration of the samples for nitrate isotope analysis is calculated using ion chromatography, so the dissolved nitrate is then added to the sample glass vials and is incubated for 12 hours to allow the complete conversion of nitrate to N$_2$O before the addition of 0.1 ml of 10 N NaOH to stop bacterial activity. Nitrogen and oxygen isotope ratios were measured relative to Air-N$_2$ and VSMOW, respectively. For calibration of nitrogen and oxygen isotope values of the samples the reference nitrates IAEA-N3, USGS 34 and USGS 35 were used. Standards were reacted and analyzed in parallel to samples, with an aliquot of each standard analyzed initially and after every five experimental samples. Based on replicate measurements of standards and samples ($n = 40$), the analytical precisions for $\delta^{15}$N$_{NO_3}$ and $\delta^{18}$O$_{NO_3}$ were better than $\pm 0.2\%$ and $\pm 0.3\%$, respectively, during the analytical period.

2.5.3 Carbon isotope ratio and concentration of DIC

Water samples were analyzed for carbon isotope and concentration of DIC using a headspace gas sampler (GasBench II, Thermo Fisher Scientific, USA) coupled to an IRMS (Finnigan Delta V Advantage; Thermo Fisher Scientific, USA). In brief, 1 ml of liquid sample was taken and treated with 100% H$_3$PO$_4$ previously flushed with high purity (99.9996%) He in a glass vial at 25°C. The CO$_2$ produced in the headspace of the vial is transferred into the mass spectrometer and $\delta^{13}$C values were measured. Reproducibility of $\delta^{13}$C values is reported in per mil relative to Vienna PeeDee Belemnite (VPDB). Final $\delta^{13}$C values are obtained after adjusting the provisional values such that correct $\delta^{13}$C values for IAEA-CO-9 standard and laboratory standards. Based on replicate measurements of standards and samples ($n = 41$), the analytical precision of $\delta^{13}$C values was better than $\pm 0.1\%$. For DIC concentration four laboratory standards of CaCO$_3$ at four DIC concentrations (2, 3, 4, 5 mM) were prepared as calibration standards and were analyzed with every 5 samples. The CaCO$_3$ powder was flushed with high purity helium gas for 2 hours and treated with an excess of 100% H$_3$PO$_4$. The carbon isotope ratio of powdered Na$_3$C$_6$H$_5$O$_7$ was determined by mass spectrometry interfaced with an elemental analyzer (Flash 2000, Thermo Fisher Scientific, USA) (Table 2). The isotope values were calibrated using the IAEA-CO-9 and several interlaboratory standards. Analytical precision of the analysis is generally better than $\pm 0.15\%$.

2.5.4 Sulfur isotope ratio

Water samples were filtered through 0.2 μm cellulose acetate filters. Filtered samples were acidified to pH 2 with distilled 6 M HCl. The SO$_4^{2-}$ in acidified samples
was collected as BaSO₄ compounds by adding 10% BaCl₂ (aq). Afterwards, the samples were dried at 60 °C for 72 h. Samples were combusted with O₂ and V₂O₅ at 1030 °C using an elemental analyzer (Flash 2000, Thermo Fisher Scientific, USA) coupled to IRMS (Finnigan Delta V Advantage; Thermo Fisher Scientific, USA). Isotopic results are expressed in per mil basis (‰) with respect to the Vienna Canyon Diablo Troilite (VCDT) standard. International reference materials IAEA-S2 and NBS 127 and some laboratory standards were used for calibration and quality check. The analytical precision for δ³⁴S_SO₄ was better than ±0.2‰ (n = 35). The sulfur isotope ratio of powdered samples of FeS₂ was also determined by IRMS interfaced with the same elemental analyzer (Table 2). Analytical precision of the analysis was better than ±0.2‰.

2.6. Enrichment factors

In addition to the isotope changes as an indicator of denitrification, empirical isotope enrichment factors ε can be calculated using differences in the isotopic composition and concentration of nitrate of samples. According to Mariotti et al. (1981) a simplified linearization of the Rayleigh model was used, in which the exponential decrease in nitrate concentration with isotopic enrichment produced by denitrification defines the isotopic enrichment factor (ε). Using the linearized form of the Rayleigh equation (Eq. (3)), the slope of the line calculated from a linear regression analysis, approximates to the nitrogen isotopic enrichment factor (εN) (Mariotti et al., 1981):

\[ \delta^{15}N = \delta^{15}N_{\text{initial}} + \varepsilonN(\ln[\text{NO}_3^-]) \] (3)

Here, \( \delta^{15}N \) and \( \delta^{15}N_{\text{initial}} \) are the isotopic signatures of residual and initial nitrate, respectively, and \([\text{NO}_3^-] \) represent the concentration of the residual nitrate.

Similarly, equations of Rayleigh model for \( \delta^{18}O \) and \( \delta^{13}C \) can be defined as follows:

\[ \delta^{18}O = \delta^{18}O_{\text{initial}} + \varepsilonO(\ln[\text{NO}_3^-]) \] (4)
\[ \delta^{13}C = \delta^{13}C_{\text{initial}} + \varepsilonC(\ln[HCO_3^-]) \] (5)

The use of this Rayleigh model-based approach for the quantification of biodegradation, requires the existence of a constant isotopic fraction factor, and depends on the nature of the transport processes taking place during denitrification (Thullner et
al., 2008). In our experiments, systematic degradation of NO$_3$ by the previously mentioned anaerobic microorganisms was observed, and for simplicity, and to have a rough estimate of the enrichments factors the Rayleigh model was employed. However, microscale transport processes around the microbial cells may occur even in closed batch systems (Thullner et al., 2008), such as the diffusion of the substrate toward a bacterial cell (Harms, 1996) and adsorption across a cell membrane (Buttom, 1991; Buttom, 1998). Moreover, isotopic effects can be additionally influenced by other physical processes, e.g., an inhomogeneous substrate dispersion, not perfect anaerobic environment (or variations with time of other environmental conditions), and spatial and temporal heterogeneity of reaction rates (Well et al., 2012, Lewicka-Szczechak et al., 2014), which can occur, for example, due to the continuous bacterial growth during denitrification. Nevertheless, according to Meckenstock et al. (2004), values for enrichment factors determined using the Rayleigh approach provide a conservative estimate of biodegradation occurring in laboratory scale experiments.

3. Results and discussion

3.1. Factors controlling composition and isotopic changes

It is of considerable interest to determine the concentration and $\delta^{15}$N, $\delta^{13}$C, and $\delta^{34}$S characteristics changes with progress of HD and AD reaction, respectively. Denitrification by oxidation of organic matter (HD) should result in a decrease of nitrate concentration together with an increase in HCO$_3^-$ concentration in near neutral pH condition. Fig. 1a shows the nitrate concentration variation with time. Concentrations of nitrate decreased systematically and nearly complete reduction of nitrate was obtained after approximately 50 hours, the rapid consumption of the electron acceptor species corresponds to a fast increase of the bacteria population. After that, the nitrates are completely consumed and the number of bacteria is stationary in time (Fig. 1a). Finally, a plateau region that was clearly observed at the end of the experiment indicating that the system reached a steady state (Fig. 1a). Accordingly, bicarbonate concentration tended to increase with time as nitrate is being consumed (Fig. 1b). It was observed that nitrate depletion and bicarbonate production occurred in a 1:1 proportion, as can be seen in Figs. 1a,b. Since Pseudomonas aerofaciens is an incomplete denitrifier strain that reduce NO$_3^-$ to nitrous oxide (N$_2$O), we propose the following corrected version of equation (1):
\[
C_{\text{source}} + 2\text{NO}_3^-(aq) + H_2O \rightarrow \text{N}_2\text{O}(g) + 2\text{HCO}_3^-(s) + C_{\text{cell tissue}} \tag{6}
\]

In our case, we used as a carbon source \(\text{Na}_3\text{C}_6\text{H}_5\text{O}_7(s)\) and we suggest that carbon will accumulate into the biomass at the end of the reaction forming a biosolid. As shown in Fig. 1c sulfate concentration remained constant with time, as sulfate does not interfere in the HD reaction.

In general, nitrate elimination by denitrification produces an increase in \(\delta^{15}\text{NNO}_3\) and \(\delta^{18}\text{ONO}_3\). Several field studies (e.g., Aravena and Robertson, 1998; Mengis et al., 1999; Fukada et al., 2003) and laboratory studies with bacterial cultures (e.g., Granger et al., 2008) have demonstrated that fractionation during denitrification causes that \(\delta^{15}\text{NNO}_3\) and \(\delta^{18}\text{ONO}_3\) become progressively more enriched as NO\(_3^\) depletion proceeds. Fig. 1d shows a clear trend of \(\delta^{15}\text{NNO}_3\) enrichment with time and correlate with the decreasing nitrate levels observed in Fig. 1a, suggesting strong evidence of

**Fig. 1** Time depending change of concentration and isotope ratios during heterotrophic denitrification: (a) Nitrate, (b) bicarbonate, (c) sulfate, (d) \(\delta^{15}\text{NNO}_3\), (e) \(\delta^{13}\text{CDIC}\), and (f) \(\delta^{34}\text{SSO}_4\). The solid and dotted lines represent the measured isotope values for the trisodium citrate and CMM medium, respectively.
denitrification. In this experiment $^{18}$O$_{NO_3}$ could not be measured accurately; however, the behavior of $^{18}$O$_{NO_3}$ is out of scope of this study. Fig. 1e shows that as citrate is being consumed $^{13}$C$_{DIC}$ tend to approximate to its value (Table 2). No fractionation occurred for $^{34}$S$_{SO_4}$ as can be observed in Fig. 1f.

Fig. 2a shows the nitrate concentration variation with time for the AD reaction. Nitrate concentration decreased from approximately 5 mM to 2 mM in 70 days that was the duration of the experiment. The degree of denitrification reached 60%, much lower than the one achieved with HD. Autotrophic microorganisms are relatively slow growing as compared to heterotrophs (Komor and Fox, 2002) resulting in small growth during the experiment. In the AD experiment, for every time point steep changes in the NO$_3^-$, HCO$_3^-$ and SO$_4^{2-}$ concentrations occurred during the first thirteen days of the experiment (Figs. 2a to 2c). The pH of the cell culture medium (which contained HCO$_3^-$) was adjusted to be near 7-7.5; therefore, DIC can be expected to be only in the predominant form of HCO$_3^-$. All autotrophic bacteria utilize DIC concentrations in their host environment as carbon source to support various physiological functions involving DIC (Kusian et al., 2002). In other words, CO$_2$ can be used as a sole source of carbon for growth. Due to this fact, the concentration of CO$_2$ expressed as HCO$_3^-$ decreased with time as the experiment proceeded (Fig. 2b). According to our results, the molar ratio of

![Fig. 2 Time depending change of concentration and isotope ratios during autotrophic denitrification](image)

the reactants NO$_3^-$ and produced HCO$_3^-$ seems to be approximately 1:1.

Sulfate concentration increased with time as denitrification continues due to the
oxidation of pyrite forming sulfate (Fig. 2c). However, we observed that the amount of SO$_4^{2-}$ formed is more than the expected stoichiometrical according to reaction (2). In our experimental conditions, we made an effort to avoid the chemical oxidation of pyrite; however, at the very beginning of the experiment during the equilibration time in the glove box, pyrite may be oxidized by dissolved molecular oxygen according to the following reaction (Singer and Stumm, 1970):

$$\text{FeS}_2 + 3.5\text{O}_2 + \text{H}_2\text{O} \rightarrow \text{Fe}^{2+} + \text{SO}_4^{2-} + 2\text{H}^+ \quad (7)$$

Reaction (7) is a general reaction because other sulfur species, such as elemental sulfur, could also form as oxidation products (Schippers et al., 1996). Field studies clearly demonstrate pyrite oxidation in neutral and slightly alkaline groundwater (e.g., Postma et al., 1991; Kinniburg et al., 1994; Schreiber et al., 2000). Microbial examination of groundwater in a zone with ongoing pyrite oxidation suggests that the oxidation is abiotic (Kinniburg et al., 1994). The rate of reaction (7) is limited by the availability of dissolved oxygen, which may come from either atmospheric oxygen, water-derived oxygen and/or adsorbed molecular oxygen onto the pyrite surface, especially on ultrafine pyrite grains (Heidel and Tichomirowa, 2011). During the first thirteen days of the experiment the SO$_4^{2-}$ generation can be simultaneously attributed to Eq. (2) and Eq. (7), taking into account that in waters with high nitrate concentrations $T.\text{denitrificans}$ microbially catalyze reaction (2) (Welch et al., 2000). Nevertheless, little is known about the kinetics, mechanisms, the limiting factors and the degree of involvement of $T.\text{denitrificans}$ like bacteria in reaction (2) (Torrentó et al., 2010). From 14 days on, the sulfate production was less steeply as the time increases and this behavior could be attributed to the decrease of the bacterial activity, which should catalyze reaction (2).

On Figs. 2d-f $\delta^{15}\text{N}_{\text{NO}_3}$ and $\delta^{18}\text{O}_{\text{NO}_3}$, $\delta^{13}\text{C}_{\text{DIC}}$, and $\delta^{34}\text{S}_{\text{SO}_4}$ are plotted vs. time. As a clear evidence of denitrification, it was seen that the isotopic fractionation of nitrogen and oxygen increased with time, especially during the first thirteen days of the experiment (Fig. 2d), which is coincident with the concentration changes observed in Figs. 2a-c. AD resulted in a decrease in HCO$_3^-$ concentration together with an increase in $\delta^{13}\text{C}_{\text{DIC}}$. In Fig. 2e during the first 13 days and after the day 25 fractionation of $\delta^{13}\text{C}_{\text{DIC}}$ can be observed, meaning that the $T.\text{denitrificans}$ bacteria prefer to uptake the light isotope $^{12}\text{C}$ leaving the remaining carbon progressively enriched in $^{13}\text{C}$. The $\delta^{13}\text{C}_{\text{DIC}}$ for AD range from 6.1‰, that is the DIC present in the synthetic medium (Table 2, $n = 5$), to 13.7‰. We still don’t have clear evidence to explain why less fractionated
signature in $\delta^{13}C_{\text{DIC}}$ with high HCO$_3^-$ concentration was observed during days 13 and 25 (Fig. 2e). Since the pH was maintained stable during all the experiment, we can infer that bacterial physiological changes and/or variation in the denitrifying bacterial activity during this period may be occurred producing this phenomenon. Since in our batch system the bacterial are continuously growing, the microbial activity is somehow variable.

In Fig. 2f, in the first time point, the sulfate concentration was sufficiently high to recover a measurable quantity of barium sulfate (day 0) and yielded a $\delta^{34}S_{\text{SO}_4}$ value of -1.1‰. It is possible that in the initial superficial preparation of FeS$_2$ crystal a certain amount of S impurities remained. Lasaga (1998) has reported S release probably due to the dissolution of an outer layer of the reacting material or to dissolution of microparticles. Moreover, S-rich secondary phases or elemental S as an intermediate phase could have been formed at the beginning of the experiment, as suggested by Torrentó et al. (2010). The possible subsequent oxidation of this S$^0$ product cannot be discarded also. Therefore, the $\delta^{34}S_{\text{SO}_4}$ value obtained at day 0 could be the sum of these factors. In correspondence with the increasing tendency of sulfate concentration, $\delta^{34}S$ decreased from -1.1‰ toward the value of pyrite (-2.7‰), strongly suggesting the occurrence of pyrite oxidation through AD reaction. Significant decline of $\delta^{34}S_{\text{SO}_4}$ was observed (-4.8‰ in maximum) during first 13 days and after 14 days this trend gradually weaken (Fig. 2f). This tendency corresponds well with SO$_4^{2-}$ concentration changing behavior (Fig. 2c) as described previously.

3.2. Evaluation of enrichments factors

Using equations 3-5, we calculated the enrichment factors for HD and AD. For the HD experiment, the enrichment factor calculated by the linear regression line of $\delta^{15}N_{\text{NO}_3}$ vs. ln[NO$_3^-$] is shown in Fig. 3a. The value of $\varepsilon_N$ based on the slope of the regression line was -4.7‰. Granger et al. (2008) measured $\varepsilon_N$ in heterotrophic cultures of different denitrifying bacteria and the obtained values spanned a broad range, between -5‰ and -25‰. Sutka et al. (2006) measured $\varepsilon_N$ in a batch experiment using *P. aerofaciens*, citrate electron donor and 0.01 mM NaNO$_3$ (conditions very similar to our experimental conditions except for the cell concentration and the initial concentration of NO$_3^-$) and the $\varepsilon_N$ obtained was -36.7‰. Enrichment factors in the range of -5‰ to -10‰ indicate rapid denitrification reactions (Mariotti et al., 1988). The regression coefficient of the plot of Fig. 3(a) was $r^2=0.82$. As was mentioned before, a lack of perfect linearity can be due to a variety of microscale biological transport processes and other physical
processes that are involved in the microbial denitrification.

Fig. 3 (a) $\delta^{15}$N$_{NO_3}$ vs. ln[NO$_3^-$] during heterotrophic denitrification (the last point corresponding to the NO$_3^-$ concentration of almost 0 mM was excluded in the regression analysis), (b) $\delta^{15}$N$_{NO_3}$ vs. ln[NO$_3^-$] during autotrophic denitrification, (c) $\delta^{18}$O$_{NO_3}$ vs. ln[NO$_3^-$] during autotrophic denitrification, and (d) $\delta^{13}$C$_{DIC}$ vs. ln[HCO$_3^-$] during autotrophic denitrification.

Figs. 3b,c showed the correlation between $\delta^{15}$N$_{NO_3}$ vs. ln[NO$_3^-$] and $\delta^{18}$O$_{NO_3}$ vs. ln[NO$_3^-$] for the AD experiment. The initial [NO$_3^-$] was around 5 mM and we found enrichment factors of $\varepsilon$N = -12.6‰ and $\varepsilon$O = -8.8‰, respectively. Although, the achievement of denitrification in our AD experiment was 60%, the enrichment factors calculated using the data of Figs. 3b,c resulted to be similar to the ones reported by Torrentó et al. (2010) ($\varepsilon$N = -15.01‰ and $\varepsilon$O = -13.55‰), which until date is the only study that measured $\varepsilon$N and $\varepsilon$O in an autotrophic denitrification experiment using pyrite as an electron donor.

Fig. 3d depicts the enrichment factors of carbon during AD reaction. The obtained value for $\varepsilon$C was -7.8‰. The $\varepsilon$S could not be calculated since a linear relationship between $\delta^{34}$S and ln[SO$_4^-$] was not obtained; however, significant sulfur isotope
fractionation was observed during the first thirteen days of the experiment, decreasing
down to -4.8‰ in maximum (Fig. 2f), which value is around 2‰ lower than that of
pyrite (-2.7‰). This negative shift of $\delta^{34}$S value should account for the occurrence of
pyrite oxidation through Eqs. (2) and (7) simultaneously. Observed degree of
fractionation corresponds well with the values reported in the sulfide oxidation by O$_2$
(coming from atmospheric oxygen) or water molecule oxygen (O in H$_2$O) (Balci et al.,
2007).

Denitrification reaction can produce a distinctive isotope signature on a cross-plot
of $\delta^{15}$N$_{NO_3}$ vs. $\delta^{18}$O$_{NO_3}$. For AD, values of $\delta^{15}$N$_{NO_3}$ and $\delta^{18}$O$_{NO_3}$ tended to be linearly
correlated (Fig. 4). A best-fit regression line allowed determining the ratio of isotopic
enrichment factor $\varepsilon_N/\varepsilon_O$. The slope of the line indicates that $\delta^{15}$N fractionates by a
factor of 1.04 greater than $\delta^{18}$O. For the HD experiment, the $\varepsilon_N/\varepsilon_O$ ratio could not be
calculated, however, for the AD experiment, the isotopic enrichment factor $\varepsilon_N/\varepsilon_O$ ratio
resulted to be in agreement with the ratio obtained by Torrentó et al. (2010), which is a
same study of AD using pyrite (with a different grain size, which is bigger than the one

![Fig. 4 Isotopic signatures ($\delta^{18}$O$_{NO_3}$ vs. $\delta^{15}$N$_{NO_3}$) during autotrophic denitrification
used in our experiment) and Thiothrix denitrificans ($\varepsilon_N/\varepsilon_O = 1.13$-1.18). These
values are also comparable to the ratios obtained from field studies of denitrification in
groundwater in natural environments (Otero et al., 2009).
3.3. Potential applications in the groundwater environmental studies

The different compartments for HD and AD presented in δ\(^{15}\)N\(_{\text{NO}_3}\) vs. δ\(^{13}\)C\(_{\text{DIC}}\) and δ\(^{15}\)N\(_{\text{NO}_3}\) vs. δ\(^{34}\)S\(_{\text{SO}_4}\) diagrams are very important, because, comparing across the multi-isotope behavior allow us ideally to assess the type of denitrification that is taking place in subsurface environments (Aravena and Robertson, 1998; Otero et al., 2009; Carrey et al., 2013; Puig et al., 2013; Hosono et al., 2014). Figs. 5a,b displays the relationship between δ\(^{15}\)N\(_{\text{NO}_3}\) and δ\(^{13}\)C\(_{\text{DIC}}\) and δ\(^{15}\)N\(_{\text{NO}_3}\) and δ\(^{34}\)S\(_{\text{SO}_4}\), respectively, for HD and AD. For HD, as nitrate is being consumed a decrease of δ\(^{13}\)C\(_{\text{DIC}}\) was observed to the value of the trisodium citrate (-12.4‰), while denitrification is taking place no fractionation of δ\(^{34}\)S\(_{\text{SO}_4}\), was observed (Fig. 5b). The variation of δ\(^{13}\)C\(_{\text{DIC}}\) and δ\(^{34}\)S\(_{\text{SO}_4}\) with δ\(^{15}\)N\(_{\text{NO}_3}\) for HD was in agreement with the obtained by the scenario suggested by Aravena and Robertson (1998).

In contrast, for AD, as denitrification proceeded, clear isotopic fractionation of δ\(^{13}\)C\(_{\text{DIC}}\) (from 6 to 14‰) with εC of -7.8‰ and an enrichment factor ratio εN/εC of 0.3

---

![Graph](image-url)  
**Fig. 5** Isotopic comparison between heterotrophic and autotrophic denitrification on (a) δ\(^{13}\)C\(_{\text{DIC}}\) vs. δ\(^{15}\)N\(_{\text{NO}_3}\) and (b) δ\(^{34}\)S\(_{\text{SO}_4}\) vs. δ\(^{15}\)N\(_{\text{NO}_3}\) diagram.
were observed. In addition, $\delta^{34}\text{S}_{\text{SO}_4}$ decreased with the progress of denitrification reaction and shifts towards more negative values than $\delta^{34}\text{S}_{\text{SO}_4}$ of the pyrite. In other words, a slight but clear $\delta^{34}\text{S}$ fractionation (around -2‰ in maximum) during AD reaction in addition to low $\delta^{34}\text{S}$ signature of pyrite (-2.7‰) was observed. In natural nitrate-rich aquifers, zones with imperfectly anaerobic conditions are present (suboxic environment) and under this conditions the simultaneous occurrence of reaction (7) and (8) could be present. Otero et al. (2009) found a decrease in $\delta^{34}\text{S}_{\text{SO}_4}$ accompanied by an enrichment of $\delta^{15}\text{N}_{\text{NO}_3}$. Their results are partially similar to those of our results; however, in this study we found a fractionation of approximately -2‰ for $\delta^{34}\text{S}_{\text{SO}_4}$ and an increase in $\delta^{13}\text{C}_{\text{DIC}}$ with the increase of $\delta^{15}\text{N}_{\text{NO}_3}$.

In the field, dissolved species of organic and inorganic carbon take part in microbial reactions and alter the $\delta^{13}\text{C}_{\text{DIC}}$ value. In fact, in the aquifer systems including carbonate units, Otero et al. (2009) could not detect a clear trend between carbon concentration and $\delta^{13}\text{C}_{\text{DIC}}$ during AD. However, we suppose that in different types of aquifers, such as shale aquifer with defined pyrite zone where significant occurrence of AD has been confirmed (Molénat and Gascuel-Odoux, 2002), the carbon isotopic signature might be a sensitive indicator of AD process with combination use of $\delta^{34}\text{S}_{\text{SO}_4}$ tracer. Detailed behavior of $\delta^{13}\text{C}_{\text{DIC}}$ and $\delta^{34}\text{S}_{\text{SO}_4}$ versus $\delta^{15}\text{N}_{\text{NO}_3}$ found in our batch culture experimental study (Fig. 5) would be helpful to understand which type of denitrification is occurring in particular aquifer systems of natural environments.

4. Concluding remarks

The results of our batch experimental study demonstrated the usefulness of N-C-S isotopic combination to understand the type of anaerobic bacterial denitrification processes (autotrophic or heterotrophic) in contaminated groundwater where a carbon source and/or pyrite are present. However, in natural aquifers, mixing with another S and C sources such through dissolution of carbonate materials and marine evaporites, and other anaerobic microbial activities such as sulfate reduction and methanogenesis would take place after or in the middle of the progress of the denitrification reaction, which play a decisive role changing isotope ratios of carbon and sulfur (Hosono et al., 2014). Nevertheless, obtained results can be applicable in environments where complex simultaneous anaerobic reactions would not occur after, in the middle of the denitrification reaction, or at organic poor land that prevent further heterotrophic bacterial reactions to proceed. Although this is a batch experiment study, our results using could be qualitatively applied to natural environments to better understand HD
and pyrite-driven AD in aquifers.

Acknowledgement
This study was funded by a Grant-in-Aid for Young Scientist (A) (No. 24681007) and by the CREST Project (JST: Japan Science and Technology Agency).

References


investigations of the oxidation of sulfide mixtures containing pyrite, galena, and sphalerite. Chem. Geol. 342:29-43


denitrification process in groundwater: Application to the chalk aquifer of northern

analysis as a tool to monitor biodegradation in contaminated aquifers. J. Contam.
Hydrol. 75:215-255

Multiple geochemical and isotopic approaches for assessing ground water NO$_3$
elimination in a riparian zone. Ground Water 37:448-457

with autotrophic denitrifying bacteria in a fluidized bed bioreactor (FBBR).
Fresenius Environ. Bull. 20:2427-2434

groundwater for the prediction of water travel times and of consequences of land

Letters 24:1511-1514


nitrate attenuation in a regional system coupling hydrogeology with multi-isotopic
methods: The case of Plana de Vic (Osona, Spain). Agric. Ecosyst. Environ. 133:103-113

dissolved and attached oxidation products of pyrite by Acidithiobacillus
71:2474-2490

sandy aquifer: water chemistry, reduction processes, and geochemical modeling.
Water Resour. Res. 27:2027-2045

Puig R., Folch A, Menció A, Soler A, Mas-Pla, J (2013) Multi-isotopic study ($^{15}$N, $^{34}$S,
$^{18}$O, $^{13}$C) to identify processes affecting nitrate and sulfate in response to local and
regional groundwater mixing in a large-scale flow system. Appl. Geochem.
32:129-141
Figure legends

**Fig. 1** Time depending change of concentration and isotope ratios during heterotrophic denitrification: (a) Nitrate, (b) bicarbonate, (c) sulfate, (d) δ¹⁵N\textsubscript{NO₃}, (e) δ¹³C\textsubscript{DIC}, and (f) δ³⁴S\textsubscript{SO₄}. The solid and dotted lines represent the measured isotope values for the trisodium citrate and CMM medium, respectively.

**Fig. 2** Time depending change of concentration and isotope ratios during autotrophic denitrification. (a) Nitrate, (b) bicarbonate, (c) sulfate, (d) δ¹⁵N\textsubscript{NO₃} and δ¹⁸O\textsubscript{NO₃}, (e) δ¹³C\textsubscript{DIC}, and (f) δ³⁴S\textsubscript{SO₄}. The solid and dotted lines represent the measured isotope values for the pyrite and synthetic medium, respectively.

**Fig. 3** (a) δ¹⁵N\textsubscript{NO₃} vs. ln[NO₃⁻] during heterotrophic denitrification (the last point corresponding to the NO₃ concentration almost 0 mM was excluded in the regression analysis), (b) δ¹⁵N\textsubscript{NO₃} vs. ln[NO₃⁻] during autotrophic denitrification, (c) δ¹⁸O\textsubscript{NO₃} vs. ln[NO₃⁻] during autotrophic denitrification, and (d) δ¹³C\textsubscript{DIC} vs. ln[HCO₃⁻] during autotrophic denitrification.

**Fig. 4** Isotopic signatures (δ¹⁸O\textsubscript{NO₃} vs. δ¹⁵N\textsubscript{NO₃}) during autotrophic denitrification.

**Fig. 5** Isotopic comparison between heterotrophic and autotrophic denitrification on (a) δ¹³C\textsubscript{DIC} vs. δ¹⁵N\textsubscript{NO₃} and (b) δ³⁴S\textsubscript{SO₄} vs. δ¹⁵N\textsubscript{NO₃} diagram.
Table 1 Conditions varied for denitrifiers in the denitrification experiments

<table>
<thead>
<tr>
<th>Conditions</th>
<th>P. aerofaciens</th>
<th>T. denitrificans</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium</td>
<td>CMM*</td>
<td>Synthetic*</td>
</tr>
<tr>
<td>Electron donor</td>
<td>Na\textsubscript{3}C\textsubscript{6}H\textsubscript{5}O\textsubscript{7}</td>
<td>FeS\textsubscript{2}</td>
</tr>
<tr>
<td>Initial NO\textsubscript{3}\textsuperscript{-} concentration</td>
<td>10 mM</td>
<td>5 mM</td>
</tr>
<tr>
<td>Duration of the experiment</td>
<td>120 h</td>
<td>70 days</td>
</tr>
<tr>
<td>Sampling interval</td>
<td>Every 4 h</td>
<td>For days 1-16 every day, days 17-49 every 3 days, days 50-70 every 7 days</td>
</tr>
<tr>
<td>Mechanical stirring</td>
<td>No</td>
<td>No</td>
</tr>
</tbody>
</table>

*See text for details

Table 2 δ\textsuperscript{13}C and δ\textsuperscript{34}S of the source materials used in the denitrification experiments.

<table>
<thead>
<tr>
<th>Denitification type</th>
<th>Source material</th>
<th>δ\textsuperscript{13}C (‰)</th>
<th>δ\textsuperscript{34}S (‰)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HD</td>
<td>CMM medium (time = 0)</td>
<td>-7.7</td>
<td>-1.5</td>
</tr>
<tr>
<td></td>
<td>Na\textsubscript{3}C\textsubscript{6}H\textsubscript{5}O\textsubscript{7} (electron donor)</td>
<td>-12.4</td>
<td>no data</td>
</tr>
<tr>
<td>AD</td>
<td>Modified synthetic medium (time = 0)*</td>
<td>6.1</td>
<td>-1.1</td>
</tr>
<tr>
<td></td>
<td>Pyrite (FeS\textsubscript{2}) (electron donor)</td>
<td>no data</td>
<td>-2.7</td>
</tr>
</tbody>
</table>

Note: the results are the average values of 5 repeated measurements.

* At time = 0 the modified synthetic medium contained FeS\textsubscript{2}