Geochemical and isotopic study of abiotic nitrite reduction coupled to biologically produced Fe(II) oxidation in marine environments

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Estuarine sediments are often characterized by abundant iron oxides, organic matter, and anthropogenic nitrogen compounds (e.g., nitrate and nitrite). Anoxic dissimilatory iron reducing bacteria (e.g., *Shewanella loihica*) are ubiquitous in these environments where they can catalyze the reduction of Fe(III) (oxyhydr)oxides, thereby releasing aqueous Fe(II). The biologically produced Fe(II) can later reduce nitrite to form nitrous oxide.

The effect on nitrite reduction by both biologically produced and artificially amended Fe(II) was examined experimentally. Ferrihydrite was reduced by *Shewanella loihica* in a batch reaction with an anoxic synthetic sea water medium. Some of the Fe(II) released by S. loihica adsorbed onto ferrihydrite, which was involved in the transformation of ferrihydrite to magnetite. In a second set of experiments with identical medium, no microorganism was present, instead, Fe(II) was amended. The amount of solid-bound Fe(II) in the experiments with bioproduced Fe(II) increased the rate of abiotic NO$_2^-$ reduction with respect to that with synthetic Fe(II), yielding half-lives of 0.07 and 0.47 d, respectively.

The $\delta^{18}$O and $\delta^{15}$N of NO$_2^-$ was measured through time for both the abiotic and inoculated experiments. The ratio of $\varepsilon^{18}$O/$\varepsilon^{15}$N was 0.6 for the abiotic experiments and 3.1 when NO$_2^-$ was reduced by S. *loihica*, thus indicating two different mechanisms for the NO$_2^-$ reduction. Notably, there is a wide range of the $\varepsilon^{18}$O/$\varepsilon^{15}$N values in the literature for abiotic and biotic NO$_2^-$ reduction, as such, the use of this ratio to distinguish between reduction mechanisms in natural systems should be taken with caution. Therefore, we suggest an additional constraint to identify the mechanisms (i.e. abiotic/biotic) controlling NO$_2^-$ reduction in natural settings through the correlation of $\delta^{15}$N-NO$_2^-$ and the aqueous Fe(II) concentration.
Keywords: iron reducing bacteria, chemodenitrification, nitrite reduction, Fe(II) oxidation, nitrite isotope
1 Introduction

Sediments in estuarine and coastal areas often contain terrigenous organic matter and other constituents such as iron and nitrogen compounds (e.g., NO\textsubscript{3}^-), which arrive via rivers and submarine groundwater inputs [1]. Currently, the intensive use of nitrogen-based fertilizers and the systematic release of domestic and industrial waste account for the majority of nitrogen input to these systems [2]. When oxygen is limited in these environments, dissimilatory iron reducing bacteria (e.g., *Shewanella loihica*) are able to reduce Fe(III) (oxyhydr)oxides minerals [3] producing Fe(II) (Eq. 1) [4]. Further, the biologically produced Fe(II) can reduce available nitrite (NO\textsubscript{2}^-) to form nitrous oxide (N\textsubscript{2}O) (Eq. 2) [5].

\[
\text{CH}_3\text{CHOHCOO}^- + 4\text{Fe(OH)}_3 + 7\text{H}^+ \rightarrow \text{CH}_3\text{COO}^- + \text{HCO}_3^- + 4\text{Fe}^{2+} + 10\text{H}_2\text{O}
\] (1)

\[
4\text{Fe}^{2+} + 2\text{NO}_2^- + 5\text{H}_2\text{O} \rightarrow 4\text{FeOOH} + \text{N}_2\text{O} + 6\text{H}^+
\] (2)

Nitrous oxide is a potent greenhouse gas and the single greatest ozone-depleting substance [6]. In recent years, nitrite reduction by Fe(II) oxidation (i.e. chemodenitrification) has been the subject of much research given its environmental relevance [5, 7-10].

Both iron and nitrogen cycles are related in anaerobic environments where bioreduction of hydrous ferric oxides (HFO), such as ferrihydrite, leads to nitrite reduction coupled with Fe(II) oxidation [3, 11, 12]. Since nitrite reduction occurs in the presence of aqueous Fe(II) and in the absence of HFO [13, 14], higher abiotic NO\textsubscript{2}^- reduction rates have been observed in the presence of solid iron phases [5, 15, 16]. Tai and Dempsey (2009) observed higher NO\textsubscript{2}^- reduction rates when the initial aqueous Fe(II)/HFO ratio was 0.3. They demonstrated that ratio values higher than 0.3 indicate a halt of the reduction even in
the presence of mineral-associated Fe(II). Furthermore, they showed that the abiotic NO$_2^-$ reduction was negligible in the absence of HFO. In experiments with aqueous Fe(II) and nitrite, precipitation of HFO or mixed valence (Fe(II), Fe(III)) iron minerals, such as green rust [17], will probably occur due to the oxidation of aqueous Fe(II) [8, 18].

Solid Fe(II) (also referred to as structural or solid-bound Fe(II)) may be involved in nitrite reduction [19] together with the dissolved Fe(II). Dhakal et al. (2013) [16] studied the ability of magnetite to reduce nitrite and showed that abiotic NO$_2^-$ reduction by magnetite had a greater impact on nitrite removal than microbially mediated denitrification. However, Lu et al. (2017) [8] showed that magnetite was not able to reduce nitrite in a wide NO$_2^-$ concentration range (30-280 mg L$^{-1}$) in the absence of solid-bound Fe(II). Few studies on abiotic nitrite reduction in experiments with fresh biogenic magnetite in marine conditions are available to date [20].

Currently, the evaluation of abiotic nitrogen reduction coupled with oxidation of Fe(II) in heterogenous systems at laboratory scales has been performed by the addition of synthetic Fe(II) (e.g., FeCl$_2$) to aqueous solutions with different iron minerals [8, 13, 21]. However, in natural settings Fe(II) can derive from microbial reduction of Fe(III)-minerals. Dissimilatory Fe(III) reduction could alter the properties of the iron mineral surface or result in the formation of secondary iron mineral phases such as magnetite or siderite [22]. The evaluation of abiotic nitrite reduction therefore requires that experiments be carried out under conditions more comparable to natural settings (e.g., marine environment).

In this study, ferrihydrite was the Fe(III) mineral used in biotic and abiotic nitrite reduction experiments with synthetic seawater at pH 8.2 because it is abundant in marine sediments [23] and therefore comparable to natural systems. Fe(II) was either added as
FeSO$_4$ or biologically produced by *Shewanella loihica* (strain PV-4) at similar Fe (II) aqueous concentrations. This strain of *S. loihica* is known to reduce Fe(III) (oxyhydr)oxides in seawater under anoxic conditions [24]. Given its thermodynamic instability and large surface area, ferrihydrite has a high reactivity in the presence of aqueous Fe(II), which may lead to a mineral transformation made up of more crystalline phases containing Fe(II) such as magnetite [25-30].

Isotopic analysis is a useful tool for tracing NOx transformation processes. The enzymatic NO$_3^-$ reduction provokes an enrichment in the heavy isotopes of the unreacted substrate [31-34] unlike processes such as dilution that lead to a decrease in concentration without influencing the isotopic ratios. The same pattern is expected for the biotic reduction of all N intermediate products (e.g., NO$_2^-$ or N$_2$O), which will be initially depleted in $^{15}$N and $^{18}$O with respect to the substrate. However, data on the dual N-O isotope systematics during the biotic reduction of intermediate compounds such as NO$_2^-$ remain scarce[35, 36]. Moreover, two recent isotopic studies on the abiotic NO$_2^-$ reduction by Fe(II) found results similar to what is expected from the biotic reaction[7, 9]. Essentially, it is unclear to what degree the isotopic characterization might help in distinguishing biotic and abiotic NO$_2^-$ reduction. Further studies on the application of isotopic data to elucidate the process controlling the fate of nitrite in natural systems are therefore warranted.

In the present study, biotic and abiotic NO$_2^-$ reduction experiments using synthetic and biologically produced Fe(II) were performed with anoxic synthetic seawater to (1) shed light on the kinetics of NO$_2^-$ reduction in marine environments and (2) evaluate the possible use of isotopic analysis to distinguish between abiotic and biotic (heterotrophic) NO$_2^-$
reduction. In addition, the reductive dissolution of ferrihydrite by *Shewanella loihica* and
the fate of bioproduced Fe(II) was investigated.

### 2 Materials and methods

#### 2.1 Solutions

Synthetic sea water (SSW) was prepared to simulate marine sediment conditions following
the standard protocol D1141-98 (ATSM International). In addition to this basal medium,
10.0 mM of sodium lactate as both a carbon source and electron donor, and 10.0 mM of
TRIS-HCl (Tris) as a buffer (pH ≈ 8.2) were added. Hereafter, this medium will be referred
to as M-SSW.

Stock solutions of Fe(II) at pH 1 (HCl) and NO$_3^-$ (230.0 mM 60.0 mM, respectively) were
prepared in an anoxic glove box dissolving suitable amounts of FeSO$_4$ and KNO$_2$ into
nitrogen degassed ultrapure (18.1 MΩ) Milli-Q water. Both solutions were subsequently
filtered with a 0.22 µm membrane and stored in sterile bottles.

All solutions used in this study were sterilized by autoclave (121 °C, 20 min) unless stated
otherwise. Dissolved oxygen concentrations were measured by luminescent dissolved
oxygen (LDO) probe (detection limit 0.01 mgL$^{-1}$).

#### 2.2 Bacterial culture

*Shewanella loihica* strain PV-4 was purchased from the German Collection of
Microorganisms and Cell Cultures (DSMZ 17748). Bacteria were recovered and cultivated
in M1 medium [37] with 10.0 mM of lactate as the electron donor and carbon source and 10.0 mM of Fe(III) citrate as the electron acceptor. To obtain bacterial suspensions, cells were cultivated for 24 h and then harvested by centrifugation (5000 rpm for 10 min). The pellet was then re-suspended in SSW. This step was repeated three times as a washing protocol. *S. loihica* was inoculated with a concentration of 1·10^7 colony-forming units (cfu) mL⁻¹.

### 2.3 Ferrihydrite: synthesis and characterization

2L-ferrihydrite was synthesized according to a modified protocol of Schwertmann and Cornell (2008) [38] (see supporting information (SI) for more details). The specific surface area was measured by the Brunauer-Emmett-Teller (BET) method [39] with a Gemini 2370 surface area analyzer using 5-point N₂ adsorption isotherms. Sample degassing with nitrogen lasted for 2 h at 137 °C. The BET specific surface area measured for unreacted samples varied between 140 and 180 m²g⁻¹, and for the bioreacted samples it was between 144 and 152 m²g⁻¹.

The reacted and unreacted samples were examined by three techniques: (1) scanning electron microscopy (SEM) using a Hitachi H-4100FE instrument under a 15–20 kV potential in a high vacuum and utilizing the backscattered electron detector (BSD) in field emission (FE) and coating the samples with carbon, (2) X-ray diffraction (XRD) using a *PANalytical X’Pert PRO MPD θθ* Bragg-Brentano powder diffractometer of 240 mm in radius and Cu Kα radiation (λ = 1.5418 Å) together with Rietveld analysis to quantify the amount of phases, and (3) Fourier transform infrared spectrometry (FTIR) utilizing a Perkin
Elmer frontier / ATR diamond / detector DTGS, accumulation at 16 scans, spectral resolution 4 cm\(^{-1}\), spectral range 4000 - 225 cm\(^{-1}\).

2.4 Experimental setup and sampling procedure

Table 1 lists the initial experimental conditions. Most of the batch experiments were run in the dark (bottles wrapped with aluminum foil) and in triplicate at 22 ± 2 °C. Bottles (reactors) were placed in an anoxic glove box purged with N\(_2\) and equipped with UV germicidal light for periodic sterilization. Glassware, septa, caps, tips, and media solutions were sterilized by autoclave at 121 °C for 20 min before the experiments.

2.4.1 Abiotic nitrite reduction experiments with biologically produced Fe(II)

Batch experiments consisted of two stages. In the first stage, no nitrate was amended while Fe(II) was produced biologically (experiment Ferr; Table 1). The anaerobic reductive dissolution of ferrihydrite mediated by *S. loihica* was performed in cultures prepared with the M-SSW medium described above. Bottles of 500 mL were sealed with a screw cap, silicone O-ring and blue butyl rubber stopper before being wrapped in aluminum foil to avoid exposure to light. Autoclaved ferrihydrite powder was put into the bottles (1:100 w/v ratio). Each reactor consisted of a multi-point batch experiment in which the butyl rubber stopper allowed for multiple collection of samples with a syringe over time. Before sampling, the reactors were thoroughly shaken for liquid-solid homogenization. Aliquots of 5 mL were extracted about every 48 h, filtered through a 0.22 μm membrane,
and acidified with 200 µL of 6 M HCl solution. One mL was used for immediate Fe(II) analysis, and the remaining 4 mL were stored in the dark at 4 °C for further lactate/acetate measurements.

In the second stage, nitrite was amended to the reactors and reduced by the biologically produced Fe(II) (NFerr experiment in Table 1). In other words, the initial conditions of stage two correspond to the final conditions of stage one, in which lactate was consumed and ferrihydrite bioreduction ended. The concentrations of bioproduced Fe(II) and acetate were 1.15 and 8.1 mM, respectively, for at least 10 days. On the tenth day, 4.81 mL of a 60.0 mM NO$_2^-$ stock solution were injected into the reactors under anoxic conditions, resulting in a NO$_2^-$ concentration of 0.76 mM. NFerr experiment was performed in duplicate to ensure reproducibility.

Three sample aliquots were extracted at each sampling interval: a 5 mL aliquot for aqueous Fe(II) and Fe(III) concentration measurements, another 5 mL aliquot to measure the nitrite isotopic composition ($\delta^{15}$N-NO$_2^-$ and $\delta^{18}$O-NO$_2^-$), and a 1 mL aliquot to measure the NO$_2^-$ concentration. Concentrations of dissolved iron and nitrite were analyzed immediately to prevent measurement error due to subsequent iron oxidation/nitrite reduction. The aliquots taken for isotope analysis were immediately frozen and later defrosted before measurement preparation (Section 2.6).

### 2.4.2 Abiotic nitrite reduction experiments with synthetic Fe(II)

To investigate the role of solid and aqueous Fe(II) in nitrite reduction, three abiotic experiments were performed with synthetic Fe(II) in the presence and the absence of
ferrihydrite. The $\delta^{15}$N and $\delta^{18}$O of nitrite were monitored through time. In the experiments containing ferrihydrite, the liquid/solid ratio was the same as in the NFerr experiment. Three distinct experimental conditions were employed: (1) dissolved Fe(II) + NO$_2^-$ without ferrihydrite, (2) ferrihydrite + synthetic Fe(II) (totally solid-bound on by ferrihydrite) + NO$_2^-$ in the absence of aqueous Fe(II) and (3) ferrihydrite + both solid-bound and dissolved Fe(II) + NO$_2^-$, which are labeled A1, A2, and A3, respectively (Table 1). Three replicates were performed for these experiments. All experiments consisted of a basal medium of SSW supplemented with 10.0 mM acetate and 10.0 mM Tris-HCl buffer. Acetate was added to match the initial conditions in the NFerr experiment (8.1 mM of acetate final concentration; Table 1). Control experiments with autoclaved culture of *Shewanella loihica* were carried out to examine an effect of dead cells on the overall process, and no residual nitrite reduction was observed.

In experiment A1, the abiotic reduction of NO$_2^-$ (0.65 mM concentration) by aqueous Fe(II) (1.20 mM concentration) took place in batch reactors with 250 mL of SSW basal solution. The decrease in aqueous Fe(II) and NO$_2^-$ was monitored to evaluate the nitrite reduction rate by implementing a multi-point approach. In multi-point batch experiment A2, reactors contained 2.5 g of ferrihydrite and 250 mL of SSW basal solution amended with Fe (II) (1.20 mM concentration). The aqueous Fe(II) was consumed in 400 min due to its uptake on ferrihydrite (see SI and Fig. S1). Once aqueous Fe(II) was depleted, 3.16 mL of 60.0 mM nitrite (0.76 mM concentration) were added to the reactor to promote nitrite reduction by solid-bound Fe(II).

The multi-point batch experiment A3 contained 2.5 g of ferrihydrite and significantly more synthetic Fe(II) (2.60 mM final concentration; Table 1) than A2 experiments. Similar to
experiment A2, a fast uptake of approximately 1.40 mM Fe(II) occurred, yielding a fairly constant aqueous Fe(II) concentration of approximately 1.20 mM for 8 days. Subsequently, 3.16 mL of 60.0 mM of nitrite (0.76 mM final concentration) were injected into the reactor to promote nitrite reduction by oxidation of both solid bound and aqueous Fe(II). Note that the aqueous Fe(II) concentration in the experiments A1, A2, A3 and in the NFerr experiment, previous to the addition of nitrite, were approximately the same (i.e., 1.20 mM). The identical sample collection and preservation method used for NFerr was also implemented in experiments A1, A2 and A3 (Section 2.4.1).

2.4.3 Biotic nitrite reduction experiments with S. loihica

Bio1 and Bio2 experiments were performed to investigate the heterotrophic nitrite reduction mediated by S. loihica in the absence of ferrihydrite and aqueous Fe(II) (Table 1). Each reactor was amended with SSW and adjusted to 10.0 mM of either lactate or acetate as electron donor and carbon source, 10.0 mM of Tris-HCl buffer, and 0.65 nM of nitrite. This enabled the comparison of the biological and abiotic denitrification rates to further characterize of the isotopic signature for each mechanism. Moreover, these experiments allowed an evaluation of the potential contribution of the heterotrophic nitrite reduction in the abiotic experiments with biologically produced Fe(II).

2.4.4 Control and adsorption experiments
Control reactors with SSW were performed to examine any potential interference between acetate and Fe(II), nitrite and acetate or buffer, acetate and Fe(II) and only nitrite or Fe(II) in SSW (details in SI). Adsorption experiments were carried out to quantify the amount of Fe(II) adsorbed during reductive dissolution of synthetized ferrihydrite (see SI). A Fe(II) adsorption isotherm was performed with increasing concentrations of aqueous Fe(II) in anoxic SSW, acetate and TRIS pH buffer to investigate the mechanisms responsible for the Fe(II) uptake on ferrihydrite (Fig. S2 in SI).

2.5 Chemical analyses

Concentrations of dissolved iron and nitrite were both measured by spectrophotometry (SP-830 PLUS, Metertech Inc.) at wavelengths of 510 nm and 540 nm, respectively. Fe(II) and total iron concentrations were measured immediately after sampling by the phenanthroline method [40]. Nitrite concentration was measured following the method defined by Garcia-Robledo et al. (2004) [41]. The total iron dissolved was also measured using a Perkin-Elmer 3000 inductively coupled plasma optical emission spectrometer (ICP-OES) to confirm that all dissolved iron was in fact Fe(II). Differences in Fe concentrations measured by the phenanthroline method and ICP-OES were smaller than 5%. Concentrations of lactate and acetate were measured by high performance liquid chromatography (Waters 600 HPLC pump controller equipped with an Aminex HPX-87H column (300 x 7.8 mm), BioRad column, and a Waters 717plus autoinjector). The associated uncertainty was less than 3%. The pH of the initial medium was measured in a glove box using a Thermo Orion
pH electrode (± 0.02 pH units) and periodically calibrated with standard solutions of pH 2, 4 and 7.

2.6 Isotopic analyses

δ^{15}\text{N-NO}_2^- and δ^{18}\text{O-NO}_2^- were determined following the azide reduction method [42, 43]. N\textsubscript{2}O was analyzed using a Pre-Con (Thermo Scientific) coupled with a Finnigan MAT 253 Isotope Ratio Mass Spectrometer (IRMS, Thermo Scientific). Notation is expressed in terms of delta per mil (δ‰) (i.e., δ = (R\text{sample} - R\text{standard})/R\text{standard}, where R is the ratio between the heavy (\textsuperscript{15}\text{N, 18}\text{O}) and the light (\textsuperscript{14}\text{N, 16}\text{O}) isotopes) [44]. The δ^{15}\text{N} and δ^{18}\text{S} values are reported against international atmospheric N\textsubscript{2} (AIR) and Vienna Standard Mean Oceanic Water (V-SMOW). According to Coplen (2011) [44], several international and laboratory (in-house) standards were interspersed among samples for normalization of analyses. Two international standards (USGS 34 and 35) and two internal laboratory standards (UB-NaNO\textsubscript{3} (δ\textsuperscript{15}\text{N} = +16.9 ‰ and δ\textsuperscript{18}\text{O} = +28.5 ‰) and UB-KNO\textsubscript{2} (δ\textsuperscript{15}\text{N} = -28.5 ‰)) were employed to calibrate the δ^{15}\text{N-NO}_2^- and δ^{18}\text{O-NO}_2^- raw values to the international scales. The reproducibility (1σ) of the samples, calculated from the standards systematically interspersed in the analytical batches, was ±1.0 ‰ for δ^{15}\text{N-NO}_2^- and ±1.5 ‰ for δ^{18}\text{O-NO}_2^-.

Under closed system conditions, the isotopic fractionation values (ε\textsuperscript{15}\text{NNO}_2 and ε\textsuperscript{18}\text{ONO}_2) are calculated according to the Rayleigh distillation equation:

\[
\ln \left( \frac{R\text{residual}}{R\text{initial}} \right) = \varepsilon \times \ln \left( \frac{C\text{residual}}{C\text{initial}} \right) \quad (3)
\]
where $\varepsilon$ is the slope of the linear regression between the natural logarithms of the substrate remaining fraction ($\ln(C_{\text{residual}}/C_{\text{initial}})$, where $C$ refers to the analyte concentration, and the determined isotope ratios ($\ln(R_{\text{residual}}/R_{\text{initial}})$, where $R = \delta + 1$.

Given that the use of NO$_3^-$ (and NO$_2^-$) standards to correct $\delta^{18}$O-NO$_2^-$ may cause a bias on their values for the loss of one O atom during NO$_3^-$ to NO$_2^-$ reduction, the results were interpreted according to the changes in the NO$_2^-$ isotopic composition with respect to the initial one.

### Results and discussion

#### 3.1 Bioreduction of ferrihydrite

Figure 1 shows the three distinct stages of the bioreduction experiment. In the first stage (approximately 10 days), a significant drop in the initial concentration of lactate (from 10.8 to 3.9 mM) was accompanied by a sharp increase in acetate concentration (up to 3.8 mM). However, aqueous iron was not detected during this interval. In the second stage (from 10 to 30 days), a gradual decrease in lactate and a progressive increase in acetate were observed together with a significant increase in dissolved iron. In the third stage, lactate was totally depleted after about 60 days, and acetate and Fe(II) concentrations stabilized at 8.1 and 1.15 mM, respectively. The total consumption of lactate (i.e. the electron donor) effectively halted Fe(III)-bioreduction and, therefore, the acetate and aqueous Fe(II) concentrations remained constant.
Referring to the bioreduction reaction (Eq. 1), the molar ratio of [acetate]/[lactate] is 320 1. Nevertheless, based on the measured lactate consumption, a 20 % deficit of acetate was observed throughout the experiments (Fig. 1). This non-stoichiometric behavior was mainly attributed to the use of lactate as a carbon source for biomass formation during microbial growth [45]. Further, since the stoichiometric [Fe(II)/[acetate]] ratio is 4 (Eq. 1) and the highest measured concentrations of aqueous Fe(II) and acetate were 1.15 and 8.1 mM, respectively, only a minor fraction of Fe(II) produced (i.e. ≈ 3.5 %) was found in solution. This Fe(II) deficit could be explained by a large Fe(II) adsorption on ferrihydrite. For instance, Dzomback and Morel (1990) [46] demonstrated that at relatively high pH (e.g. pH ≈ 8.2), ferrihydrite that has a large surface area combined with a poor crystalline organization can cause an exceptionally large sorption capacity of cations. In order to evaluate the Fe(II) adsorption process under the investigated conditions, several Fe(II)-adsorption assays were performed to obtain a Fe(II) adsorption isotherm (Figs. S1 and S2 in SI). The results confirmed a maximum uptake of Fe(II) on ferrihydrite of ≈ 1.20 mM (Fig. S1 in SI) and revealed that, in addition to adsorption, an additional process (ferrihydrite transformation) was responsible for the Fe(II) uptake on ferrihydrite (Fig. S2 in SI).

Earlier studies indicated that re-adsorption of Fe(II) on ferrihydrite can result in ferrihydrite transformation to goethite, magnetite or lepidocrocite [27, 29, 30, 47-49]. In addition, the thermodynamic properties of the minerals involved, the aqueous Fe(II) concentration, the biological and physical settings, the presence of humic substances or the design of the experimental setup can play a role in ferrihydrite transformation [49, 50]. SEM images (Fig. 2a) show that the surface of the reacted ferrihydrite grains is rougher than that of the unreacted ones. XRD and FTIR analyses of the solid samples before and
after the Fe(III) bioreduction process show that ferrihydrite indeed transformed into magnetite (Fe\(^{2+}\)Fe\(^{3+}\)O\(_4\)) (Fig. 2b,c). Yang et al. (2010) [27] pointed out that this transformation is caused by the inclusion of the biologically produced Fe(II) into the mineral lattice. Figure 2b compares two XRD patterns after performing high statistic wide range scans of pristine and bioreduced samples. In addition to initial ferrihydrite, two new phases (nanocrystalline magnetite and microcrystalline hematite) were determined to be present in the reacted sample (NFerr experiment) with estimated amounts of 96 wt% (magnetite) and 4 wt% (hematite). The much smaller content of the latter was formed during the ferrihydrite autoclave process [51].

3.2 \(\text{NO}_2^-\) reduction coupled with Fe(II) oxidation

Figure 3 shows the evolution through time of the concentrations of nitrite and Fe(II) during abiotic (Fig. 3a-3c) and biotic (Fig. 3d) nitrite reduction. Figure 3a shows the variation in Fe(II) and \(\text{NO}_2^-\) in a representative A1 experiment with an initial aqueous Fe(II) concentration of \(\approx 1.0 \text{ mM}\) in the absence of ferrihydrite. After a week, Fe(II) depletion was approximately 50% of the initial concentration and 35% of nitrite was reduced. After a month, the Fe(II) depletion was 70% of the initial concentration and nitrite concentration fell to 65% of the initial concentration. The average nitrite reduction rate constant (\(k_{\text{obs}}\)) was estimated to be 0.059 mM\(^{-1}\) d\(^{-1}\) with a half-life value (\(t_{1/2}\)) of 18.7 d (second-order rate equation (Eq. (S1)) and parameters in Table S2 in SI).

Figure 3b depicts the variation in Fe(II) and nitrite concentration in a representative A2 experiment in the presence of solid-bound Fe(II) with (i) product magnetite and (ii) Fe(II)
adsorbed on the remaining ferrihydrite. About 27% of the initial NO$_2^-$ was reduced within 2 days, indicating that in the absence of aqueous Fe(II), Fe(II) in the solid phase was able to reduce some NO$_2^-$. After 2 days, the reaction stopped, and nitrite concentration remained constant. An average nitrite reduction rate of 0.22 mM$^{-1}$ d$^{-1}$ was calculated for all replicates (Eq. (S1)) and Table S2 in SI). Figure 3c shows the variation in Fe(II) and nitrite concentration in a representative A3 experiment in the presence of both aqueous Fe(II) and solid bound Fe(II). NO$_2^-$ and aqueous Fe(II) concentrations dropped 13% and 62% from the initial value, respectively, within about 2 d, yielding an average nitrite reduction rate of 0.74 mM$^{-1}$ d$^{-1}$ ($t_{1/2} = 0.47$ d) (Fig. S4 Table S2 in SI).

Figure 3d shows the evolution of bioproduced Fe(II) after the cessation of the Fe(III) reduction in the Ferr experiment (Fig. 1), along with the nitrite concentration added in a representative NFerr experiment. To ensure comparability of the results, the experiment NFerr (Fig. 3d) was selected for its high initial concentration of aqueous bioproduced Fe(II), which was similar to those of the experiments with synthetic Fe(II). Considering the reductive dissolution reaction (Eq. 1) and acetate production, the total concentration of bioproduced Fe(II) was estimated to be 32.0 mM. Nevertheless, the initial concentration of aqueous Fe(II) in the NFerr experiment was 1.20 mM because most of the bioproduced Fe(II) was adsorbed on ferrihydrite and incorporated in to form magnetite (see section 3.1). During the first 2 h, both nitrite and aqueous Fe(II) fell to about 50% and 30% of their initial concentrations, respectively. After 10 h, 87% of the initial nitrite and 38% of the initial aqueous Fe(II) were removed. The nitrite calculated reduction rate was 6.47 mM$^{-1}$ d$^{-1}$ ($t_{1/2} = 0.07$ d) (Fig. S4 in SI). In the NFerr experiment with lower concentrations of
Fe(II) and nitrite, the rate calculated are within the same range of that from A3 experiment (Table S2 in SI).

The *S. loihica* used for the bioproduction of Fe(II) in the Ferr experiment (prior to nitrite addition in the NFerr experiment) could not be eliminated because both autoclave and antibiotics interfered with dissolved Fe(II) (Table S2 in SI). However, as explained in Sections 3.3 and 3.4, the evidence resulting from (i) the isotopic data from the NFerr experiment (Fig. S5 in SI) and (ii) the observed biotic nitrite reduction by *S. loihica* in the Bio1 and Bio2 experiments ruled out any microbial reduction of nitrite.

The fastest abiotic nitrite reduction rate was observed in the NFerr experiment where bioproduced Fe(II) was the electron donor. In experiments with synthetic Fe(II), the rate was slower, despite both experiments having similar aqueous Fe(II) concentrations. In experiments with synthetic Fe(II), the nitrite reduction rate was highest in the presence of both aqueous and solid Fe(II) (e.g. A3 experiment), slower in the presence only of solid-bound Fe(II) (e.g. A2 experiment), and slowest in the experiment with only aqueous Fe(II) (e.g. A1 experiment). The highest nitrite reduction rate in the NFerr experiments compared to A3 experiment, both with aqueous and solid-bound Fe(II), suggests that the larger amount of solid-bound Fe(II) obtained in the NFerr experiments could play a crucial role on the nitrite reduction rate. Previous studies suggested that solid-bound Fe(II) is able to reduce nitrite \([5, 19, 52]\), and that an enhanced Fe(II)-rich surface (e.g. magnetite) of bioreduced Fe(III) (oxyhydr)oxides is able to consume electron acceptors (e.g., toxic hexavalent chromium).

The highest nitrite reduction rates were observed in the presence of both aqueous and solid-bound Fe(II). This is in accordance with Gorski and Scherrer (2011) \([53]\) who showed that aqueous Fe(II) removal by iron oxide could affect the reduction potential of the
oxide, as a decrease in its oxidation grade leads to an increase in the reducing capacity of the oxide. The difference between the reduction rates calculated in experiments with only solid-phase Fe(II) and experiments containing both solid-phase Fe(II) and dissolved Fe(II) is similar to that calculated in reductive dechlorination by Fe(II)-associated with goethite [54].

3.3 Biotic (heterotrophic) NO$_2^-$ reduction by *S. loihica*

Biotic experiments showed a lag in microbial activity before nitrite reduction commenced. In the reactors amended with lactate, nitrate reduction began after a 1-day lag period. For reactors amended with acetate, nitrite reduction began after a 10-day lag period (Fig. S3 in SI). Yoon et al. (2013) [55] reported a similar behavior for *Shewanella* spp. In contrast, abiotic experiments with bioproduced Fe(II) and acetate, nitrite was consumed in only 10 h (Fig. 3d). These results suggest an absence of microbial nitrite reduction in the abiotic experiments with bioproduced Fe(II). As explained further in Sections 3.4 and 3.5, the isotopic data confirmed that the microbial nitrite reduction can be ruled out in the abiotic nitrite reduction experiments (NFerr experiment).

3.4 Isotopic fractionation during abiotic NO$_2^-$ reduction owing to dissolved or solid-bound Fe(II)

As is commonly observed for denitrification (sources), the unreacted NO$_2^-$ became enriched in the heavy isotopes of N and O ($^{15}$N and $^{18}$O) during abiotic nitrate reduction. Table 2 lists
the values determined for $\varepsilon^{15}$N\textsubscript{NO\textsubscript{2}}, $\varepsilon^{18}$O\textsubscript{NO\textsubscript{2}} and $\varepsilon^{18}$O/$\varepsilon^{15}$N (calculations shown in Fig. S5 in SI). These values are within the range reported in the literature for both the biotic (heterotrophic) and abiotic NO\textsubscript{2}\textsuperscript{-} reductions (Table 3).

In the experiments to test the abiotic NO\textsubscript{2}\textsuperscript{-} reduction, differences in NO\textsubscript{2}\textsuperscript{-} isotopic fractionation were not observed (i) when using Fe(II) from biotic or synthetic sources (NFerr and A3 experiments, respectively) nor (ii) when using both aqueous and solid-bound Fe(II) or only aqueous Fe(II) (A1 and A3 experiments, respectively; Table 2). By contrast, in the experiments with solid-bound Fe(II) in the absence of aqueous Fe(II) (A2 experiment), the $\varepsilon^{15}$N\textsubscript{NO\textsubscript{2}} and $\varepsilon^{18}$O\textsubscript{NO\textsubscript{2}} determined were higher (Table 2).

In these abiotic NO\textsubscript{2}\textsuperscript{-} reduction experiments, the observed variability of $\varepsilon^{15}$N\textsubscript{NO\textsubscript{2}} and $\varepsilon^{18}$O\textsubscript{NO\textsubscript{2}} could be caused by the different NO\textsubscript{2}\textsuperscript{-} reduction rates or by a different reaction mechanism during oxidation of dissolved or solid-bound Fe(II). In earlier studies, lower $\varepsilon$ values have been associated with higher NO\textsubscript{2}\textsuperscript{-} reduction rates [9, 35]. Buchwald et al. (2016) [9] observed differences in $\varepsilon$ and NO\textsubscript{2}\textsuperscript{-} removal rates using aqueous Fe(II) as electron donor or Fe(II) associated with the oxide surface. However, our results do not show a correlation between the NO\textsubscript{2}\textsuperscript{-} reduction rates and the isotopic fractionation values (Table 2). For instance, $\varepsilon^{15}$N\textsubscript{NO\textsubscript{2}} and $\varepsilon^{18}$O\textsubscript{NO\textsubscript{2}} were similar in the A3 and NFerr experiments with highly dissimilar NO\textsubscript{2}\textsuperscript{-} reduction rates (0.75 and 6.47 mM\textsuperscript{-1} d\textsuperscript{-1}, respectively).

The kinetics of the abiotic NO\textsubscript{2}\textsuperscript{-} reduction could be affected by the initial concentration and proportion of the reactants (NO\textsubscript{2}\textsuperscript{-} and Fe(II)), solution pH, and the presence of minerals that were added externally or those precipitated during the reaction [7, 9]. In the latter case, the amount, composition (including the Fe oxidation state) and the mineral specific surface
area could have influenced the reaction. In the present study, the formation of secondary
magnetite during the Fe(II) oxidation in the Ferr experiment complicates a comparison
between the effect of the conditions investigated in this study and earlier studies.

Therefore, it is difficult to determine whether the ε variability observed is only due to
differences in the reduction rates or to the differences in mechanisms (oxidation of aqueous
or solid-bound Fe(II) coupled with NO\textsubscript{2}\textsuperscript{-} reduction).

A dual element isotope approach was used to further investigate the differences in the ε
values in the different experiments (Fig. 4). The different slopes (i.e., \( \Delta\delta^{18}\text{O}/\Delta\delta^{15}\text{N} \approx \varepsilon^{18}\text{O}/\varepsilon^{15}\text{N} \)) suggest the occurrence of different nitrite reduction mechanisms. The higher ε
values determined in the experiment A2 (solid-bound Fe(II)) compared with the similar
values in the NFerr and A3 experiments (aqueous and solid-bound Fe (II)) and the A1
experiment (aqueous Fe (II)) suggest that nitrite reduction is controlled by a different
mechanism in the presence of only solid-bound Fe(II). Nevertheless, the similar slopes in
the dual N-O plot for A1, A2, A3 and NFerr (\( \Delta\delta^{18}\text{O}/\Delta\delta^{15}\text{N} = 0.60 \pm 0.02 \)) indicates a
common nitrite reduction mechanism in the abiotic experiments. Further research is needed
to elucidate the process controlling the magnitude of ε values during nitrite reduction by
solid-bound Fe(II).

Another consideration in the abiotic NO\textsubscript{2}\textsuperscript{-} reduction experiments is the possible effect of
\( \delta^{18}\text{O}-\text{NO}_2^- \) equilibration with \( \delta^{18}\text{O}-\text{H}_2\text{O} \) on the \( \varepsilon^{18}\text{O}/\varepsilon^{15}\text{N} \) ratio. The magnitude of this effect
depends on solution salinity, temperature and/or pH [56]. Buchwald et al. (2016) [9]
demonstrated that NO accumulated in a reversible reaction could re-oxidize to NO\textsubscript{2}\textsuperscript{-} by
incorporating an O atom from water, which could also influence the \( \varepsilon^{18}\text{O}/\varepsilon^{15}\text{N} \) ratio.
Nevertheless, Martin and Casciotti (2016) [36] have shown a negligible effect (0.0035‰) due to equilibrium isotopic exchange at room temperature and pH 7.6 over 2 h between sampling and the azide reaction. Given that our nitrite samples in synthetic seawater were retrieved at pH between 7.8 and 8.2, an oxygen equilibration effect was ruled out. The slopes obtained in the abiotic NO$_2^-$ reduction experiments for relatively short (NFerr experiment) and long (A3 experiment) incubation periods (Table 2 and Fig. 4) reinforce the lack of $\delta^{18}$O-NO$_2^-$ equilibration with $\delta^{18}$O-H$_2$O.

3.5 Use of isotopic tools to distinguish between abiotic and biotic NO$_2^-$ reduction in the field

As in the abiotic reduction, a decrease in concentration resulted in an enrichment in the heavy isotopes ($^{15}$N and $^{18}$O) of the unreacted substrate during biotic NO$_2^-$ reduction. The isotopic fractionation results are listed in Table 2 (see calculations in Fig. S5 in SI). NO$_2^-$ reduction by *S. loihica* using lactate as electron donor yielded $\varepsilon^{15}$N$_{NO2} = -1.6$ ‰, $\varepsilon^{18}$O$_{NO2} = -5.3$ ‰ and $\varepsilon^{18}$O/$\varepsilon^{15}$N = 3.1. The $\varepsilon^{15}$N$_{NO2}$ and $\varepsilon^{18}$O$_{NO2}$ obtained are within the range of the values reported in the literature for both the biotic (heterotrophic) and abiotic NO$_2^-$ reduction (Table 3). Nevertheless, under the conditions of these experiments, the value of the isotopic fractionation of nitrogen ($\varepsilon^{15}$N$_{NO2}$) was smaller than those from our abiotic experiments. As a consequence, the value of the $\varepsilon^{18}$O/$\varepsilon^{15}$N ratio obtained differs from those calculated for the abiotic experiments (Fig. 4 and Table 2) and becomes higher than prior values reported (Table 3).

In the biotic NO$_2^-$ reduction, the magnitude of the $\varepsilon^{15}$N$_{NO2}$ and $\varepsilon^{18}$O$_{NO2}$ values could depend on the enzymes involved, on the NO$_2^-$ transport across the cell and on the NO$_2^-$ reduction rate. However, it is unknown whether the effect of pH or salinity could be negligible on the
biotite nitrite reduction as it occurs in the biotic nitrate reduction [57-59]. Bacterial NO$_2^-$ reduction can be catalyzed by two enzymes located in the periplasm (Cu containing NO$_2^-$ reductase encoded as nirK (Cu-NIR) and Fe-containing NO$_2^-$ reductase encoded as nirS (Fe-NIR) ([60] and references therein). The $^{18}$O/$^{15}$N ratio of 3.1 obtained for the biotic NO$_2^-$ reduction by *S. loihica* bears no resemblance to those reported in a study on NO$_2^-$ reduction with different bacterial species. Martin and Casciotti (2016) [36] attributed the variations in the $^{18}$O/$^{15}$N ratio to the use of different enzymes since the species with Fe-NIR yielded higher $^{18}$O/$^{15}$N ratios (from 0.4 to 1.2) than the species containing Cu-NIR (from 0.05 to 0.2). These authors suggested that Fe-NIR could produce a higher NO$_2^-$-O isotopic fractionation because it allows cleavage of both N-O bonds since the Fe-NIR catalytic site might bind NO$_2^-$-N [61, 62]. By contrast, the Cu-NIR catalytic site might bind both the NO$_2^-$-O atoms and the N-O bond closest to the Asp98 residue, which is cleaved [63, 64], independently of the isotopic composition. The NO$_2$ reductase associated with *S. loihica* is Cu-NIR [65]. However, our results are not indicative of this hypothesis. Our study showed an $^{18}$O$_{NO2}$ higher than $^{15}$N$_{NO2}$ in contrast to a lower $^{18}$O$_{NO2}$ associated with microorganisms containing Cu-NIR [36].

The $^{18}$O/$^{15}$N of 3.1 ratio determined for the NO$_2^-$ reduction by *S. loihica* differs from the range obtained for the abiotic experiments (0.6 – 0.7; Fig. 4). Thus, given that *S. loihica* is the only NO$_2^-$ reducing microorganism in our experiments, the $^{18}$O/$^{15}$N values calculated in the present study could allow us to distinguish the contribution of the biotic (heterotrophic) and abiotic NO$_2^-$ reductions at the laboratory. However, considering the large variability of the $^{18}$O/$^{15}$N ratio (from 0.05 to 3.1) in this study and in the literature for the biotic NO$_2^-$ reduction (Table 2 and Table 3), it would be difficult to distinguish
between biotic and abiotic reactions in natural marine environments using this technique. One reason for this is the existence of complex bacterial communities with various \( \text{NO}_2^- \) reducing enzymes. Another reason is the overlap of biotic \( \varepsilon^{18}\text{O}/\varepsilon^{15}\text{N} \) values with the ones attributed to the abiotic reduction (0.6-2.0; Table 2 and Table 3).

Alternatively, the correlation between changes in nitrite isotopic composition (\( \Delta \delta^{15}\text{N}_{\text{NO}_2} \) or \( \Delta \delta^{18}\text{O}_{\text{NO}_2} \)) and dissolved Fe(II) iron concentration (\( \ln[\text{Fe(II)}] \)) during the abiotic nitrite reduction, could be useful to investigate the process controlling \( \text{NO}_2^- \) reduction under field conditions. A good correlation between \( \delta^{(15}\text{N} \) or \( ^{18}\text{O} \))-\( \text{NO}_2^- \) and \( \ln[\text{Fe(II)}] \) in field samples suggests \( \text{NO}_2^- \) reduction by Fe(II) oxidation, either abiotically or biotically (chemolithotrophically). By contrast, no correlation is expected for heterotrophic \( \text{NO}_2^- \) reduction. A decrease in Fe(II) concentration coupled with an increase in \( \delta^{15}\text{N}_{\text{NO}_2} \) and \( \delta^{18}\text{O}_{\text{NO}_2} \) was observed (Fig. 5). In the A1 experiment, the slopes for \( \delta^{15}\text{N}_{\text{NO}_2} \) and \( \delta^{18}\text{O}_{\text{NO}_2} \) (-5.4 and -3.8, respectively) were lower than those in the A3 (-32.2 and -20.3, respectively) and NFerr experiments (-32.6 and -19.0, respectively). This was due to the higher decrease in aqueous Fe(II) concentrations during the A1 experiment. In contrast to A3 and NFerr, which also contained solid-bound Fe(II) and the total amount of Fe(II) was thus higher than in A1, in the A1 experiment only aqueous Fe(II) was available for nitrite reduction (Table 1).

Given that the equilibration between \( \delta^{18}\text{O}_{\text{NO}_2} \) and \( \delta^{18}\text{O}_{\text{H}_2\text{O}} \) could affect \( \delta^{18}\text{O}_{\text{NO}_2} \) under natural conditions, only the variation of \( \delta^{15}\text{N}_{\text{NO}_2} \) versus Fe(II) concentration could provide reliability of the \( \text{NO}_2^- \) fate in the environment. However, a possible effect of other N cycling processes (e.g. \( \text{NO}_2^- \) oxidation to \( \text{NO}_3^- \), \( \text{NO}_2^- \) reduction to \( \text{NH}_4^+ \) or \( \text{NH}_4^+ \) oxidation to \( \text{NO}_2^- \)) on \( \delta^{15}\text{N}_{\text{NO}_2} \) should also be considered.
4 Conclusions

Experiments simulating an anoxic marine medium were carried out to study nitrite reduction coupled with (bioproduced and synthetic) Fe(II) oxidation. Fe(II) bioproduction was driven by ferrihydrite reduction mediated by S. loihica. Fe(II) released was partially re-incorporated into ferrihydrite, which transformed to nanocrystalline magnetite, producing solid Fe(II). Both the bioproduced aqueous Fe(II) and solid Fe(II) played a role in nitrite reduction.

Experiments with bioproduced or synthetic Fe(II) (aqueous and solid-bound Fe(II)) revealed that abiotic NO$_2^-$ reduction is faster in a system with bioproduced Fe(II). The newly formed nano-crystalline magnetite with a high content of solid Fe(II) showed a significant reactivity in the presence of nitrite. Results obtained from the laboratory nitrite reduction experiments using synthetic Fe(II) suggest that with similar concentrations of aqueous Fe(II), nitrite reduction in natural systems could be stronger given the higher amounts of solid-bound Fe(II) obtained in the experiments with bioproduced Fe(II).

Experiments with only synthetic Fe(II) (aqueous, solid-bound Fe(II) or both) revealed that in the presence of Fe(II) in both aqueous and solid-bound forms, abiotic NO$_2^-$ reduction is faster and more effective in terms of nitrite removal than in the ones with only aqueous Fe(II) or only solid-bound Fe(II).

No differences in the $\varepsilon^{15}$N$_{NO2}$ and $\varepsilon^{18}$O$_{NO2}$ were found for the abiotic NO$_2^-$ reduction regardless of whether the source of Fe(II) was biotic or synthetic. Differences in $\varepsilon^{15}$N$_{NO2}$ and $\varepsilon^{18}$O$_{NO2}$ were neither found for the abiotic NO$_2^-$ reduction by (i) aqueous Fe(II) or (ii)
aqueous and solid-bound Fe(II). By contrast, the isotopic fractionation was higher in the experiments with only solid-bound Fe(II). The similar slopes derived in the dual N-O isotope plot ($\varepsilon^{18}O/\varepsilon^{15}N = 0.6$) suggest a sole mechanism controlling the NO$_2^-$ reduction in the abiotic experiments. The higher slope related to the biotic (heterotrophic) experiment ($\varepsilon^{18}O/\varepsilon^{15}N = 3.1$) contrasts with those of the abiotic experiments, becoming one of the highest values reported in the literature.

Hence, in laboratory microcosms, which mimic marine environments with *S. loihica* as the only existing NO$_2^-$-reducing microorganism, the value of the $\varepsilon^{18}O/\varepsilon^{15}N$ ratio allows us to distinguish between the biotic and abiotic NO$_2^-$ reduction. Given the wide range of $\varepsilon^{18}O/\varepsilon^{15}N$ values reported in the literature for the biotic and abiotic NO$_2^-$ reduction by other heterotrophic bacteria, the use of the $\varepsilon^{18}O/\varepsilon^{15}N$ ratio to distinguish different NO$_2^-$ reduction processes in field-scale studies should be discretionally applied.

Moreover, the correlation between $\delta^{15}N_{NO2}$ and the natural logarithm of the Fe(II) concentration observed could be used as an additional line of evidence to distinguish between NO$_2^-$ reduction by Fe(II) oxidation, either abiotically or biotically (chemolithotrophically), and heterotrophic bacteria. This observation can improve the prospect of using isotopic data to investigate nitrite reduction processes in the field.

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References


Figure 1
**Figure 2**

*a*) Unreacted vs. reacted samples showing differences in the microstructure.

*b*) Graph showing wavenumber (cm$^{-1}$) vs. absorbance with peaks labeled for reacted and unreacted magnetite and hematite.

**c**) Absorbance vs. wavenumber (cm$^{-1}$) graph with absorbance values for synthetic magnetite, Nferr, and Unreacted ferrihydrite.
Figure 3

A1 Experiment

Fe(II), NO$_2^-$ (mM)

A2 Experiment

Fe(II), NO$_2^-$ (mM)

A3 Experiment

Fe(II), NO$_2^-$ (mM)

Nferr Experiment

Fe(II), NO$_2^-$ (mM)

NO$_2^-$ (mM)

t (d)
Figure 4

- A1: $0.69 \pm 0.20$
- A2: $0.62 \pm 0.10$
- A3: $0.62 \pm 0.04$
- Bio1: $3.12 \pm 0.43$
- NFerr: $0.58 \pm 0.04$

$R^2 = 0.96$ for A1, A2, and A3.
$R^2 = 0.97$ for Bio1 and NFerr.
Figure 5

(a) 

NFe: -18.9 ± 5.6  
A3: -20.3 ± 1.8  
R² = 0.85  

A1: -3.8 ± 2.3  
R² = 0.90

(b) 

A3: -32.2 ± 3.1  
R² = 0.94  

NFe: -32.6 ± 8.8  
R² = 0.87  

A1: -5.4 ± 3.2  
R² = 0.91