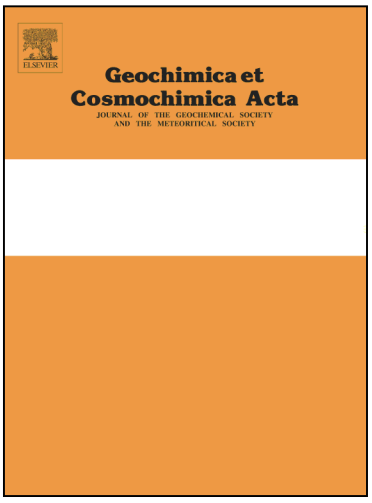


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# Solvent stress-induced changes in membrane fatty acid composition of denitrifying bacteria reduce the extent of nitrogen stable isotope fractionation during denitrification

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**Abstract**

Microcosm experiments with the well-studied denitrifier *Thaurera aromatica* show a link between a higher maximum membrane concentration (MMC) of the toxic organic solvents 1-octanol and 4-chlorophenol and a higher degree of saturation (DoS) of the fatty acids in the cell membrane. This coincides with less pronounced stable isotope fractionation during denitrification. We suggest that the change in cell membrane fluidity and the cell's stress response leads to a decrease in nitrate transport across the cell membrane and/or an increase in the relative ratio of respiratory nitrate reduction rate versus efflux of unreacted nitrate. Both models show that the apparent kinetic isotope effect (AKIE) approach unity and thus reduce the extent of the resulting stable isotope enrichment factor $\varepsilon^{15}N\text{-}NO_3^-$ in

dissolved nitrate during denitrification, as experimentally and mathematically shown in this study. This may lead to an underestimation of nitrate reduction determined by nitrate stable isotope analysis in aquatic habitats where various types of stresses may affect the physiology of the driving microorganisms.

## 1. Introduction

The knowledge about isotopic fractionation effects for key reactions and transformation pathways in biogeochemical processes is a powerful tool to understand the mechanisms of compound transformations and elemental fluxes in the environmental cycles of elements. This is widely used for carbon (C), oxygen (O), sulfur (S), and nitrogen (N). Numerous studies have begun to elucidate the nitrogen cycle in aquatic environments by using stable isotope analysis in the environment or by experiments with labelled substances (e.g. Casciotti and Buchwald (2012); Einsiedl and Mayer (2006); Francis et al. (2007); Füssel et al. (2012); Nunoura et al. (2013); Snider et al. (2009)). In these field studies, however, a wide range of isotope enrichment factors was found for numerous processes. For example, for denitrification a variable range in the enrichment factor $\varepsilon^{15}N\text{-}NO_3^-$ between -36 and 0‰ was published (Barford et al., 1999; Böttcher et al., 1990; Brandes and Devol, 1997; Chen and MacQuarrie, 2005; Cline and Kaplan, 1975; Delwiche and Steyn, 1970; Lehmann et al., 2003; Mariotti et al., 1981; Mariotti et al., 1988). The extent of stable isotope fractionation can be quantitatively described by the Rayleigh equation and calculated with a specific enrichment factor $\varepsilon^{15}N$. Such analyses require, however, a stable and robust $\varepsilon^{15}N$. To elucidate the underlying processes and the origin of the observed variation in enrichment factors, systematic studies on N and O isotope fractionation during nitrate and nitrite reduction were performed in laboratory experiments (Casciotti and Buchwald, 2012; Granger et al., 2004, 2008; Knöller et al., 2011; Mariotti et al., 1981). We already found that the presence of different carbon sources during microbial nitrate reduction in pure cultures had a significant influence on $\varepsilon^{15}N$ and $\varepsilon^{18}O$ associated with nitrate reduction (Wunderlich et al., 2012). The same is true for $\varepsilon^{34}S$ during

bacterial sulfate reduction (BSR) (Kleikemper et al., 2004; Schroth et al., 2001). Kritee et al. (2012) systematically studied changes in the N isotope effects during denitrification on the cellular level relative to isotope fractionation on the enzyme level as a function of changing environmental conditions. In a set of pure culture experiments, they demonstrated that low cell-specific nitrate reduction rates can mask the specific fractionation factor of the nitrate reductase enzyme. They hypothesized that a transport limitation of nitrate into the cell could be responsible for this effect, as nitrate reduction usually is performed in the cytoplasm by a membrane bound nitrate reductase enzyme (NaR). The masking of enzyme-associated isotope fractionation $\varepsilon^{13}C$ due to membrane transport was uniquely demonstrated for phenoxyacids by Qiu et al. (2014). The detection of enantiomer fractionation in the absence of isotope fractionation could pinpoint active membrane transport as rate-determining step in the bacterial herbicide degradation. Cichocka et al. (2007) suggested an effect of membrane transport limitation on carbon isotope fractionation of PCE and TCE. Also, it was reported that stable isotope fractionation during bacterial sulfate reduction respond to variables such as substrate availability and temperature (Canfield et al., 2006). The effect of temperature was also explained by the balance between the transfer rates of sulfate into and out of the cell. Thus several indicators point towards an influence of transport limitations on stable isotope fractionation.

Microorganisms are known to react to changes in the environment by showing a stress response, which leads to physiological modulations reflected in the uptake of substrates and flow of metabolites (Atashgahi et al., 2018). As a result, the balance between the transfer rates of an ionic substance such as nitrate or sulfate into and out of the cell and the exchange between the nitrogen and sulfur pools internal to the organism may change and may probably mask the stable isotope fractionation of metabolic processes. A major reaction to variation of temperature, pressure or the presence of hydrocarbon contaminants is a change in the membrane fatty acid composition, with the latter changing the concentration of the organic compounds in the membrane of the microorganism. Temperature

(Russell, 1997), pressure (DeLong and Yayanos, 1985) and solvents (Duldhardt et al., 2010; Heipieper and de Bont, 1994; Heipieper et al., 1994; Trautwein et al., 2008; Zink and Rabus, 2010) can change the membrane fluidity to which the cell reacts by changing the membrane composition and the degree of saturation of the membrane fatty acids in an effort to decrease potential harmful effects. Such changes in the membrane fluidity and its composition can change the influx of ionic substances such as nitrate into the cell (Nedwell, 1999; Rabus et al., 2014). In this context Rabus et al. (2014) described a decreased uptake of nitrate by bacteria in the presence of BTEX contaminants.

To elucidate the relationship between stress response and isotope effects during denitrification and to advance our understanding of the processes controlling the extent of stable isotope fractionation particularly by nitrate reducers we grew pure cultures of *Thauera aromatica* K172, a gram negative aromatic compound-degrading strain, under controlled anoxic conditions in the laboratory. We used nitrate as electron acceptor and acetate as electron donor, while simultaneously adding 4-chlorophenol and 1-octanol in sublethal concentrations according to Duldhardt et al. (2007) in order to stress the cells. The concentrations used may occur in contaminated sites, which can have dead zones with even higher concentrations at the center, allowing bacterial growth at out experimental conditions at the plume fringes. However our main goal in adding organic solvents to the cultures was to produce cell stress that changes the cell membrane composition. We subsequently compared the composition of the membrane fatty acids - serving as an indicator of cellular stress - with the apparent isotope effect on the residual nitrate during denitrification. The relations found during these experiments may also apply for other substances which need active transport across the cell membrane such as sulfate, ibuprofen or diclofenac as well as other means of applying stress to the cells (e.g. temperature, pressure).

**2. Material and methods**

### 2.1. Isotope Parameters

Isotope measurements are reported using the conventional delta (δ) notion (Eq. 1):

$$\boldsymbol{\delta} = \left(\frac{R_{sample}}{R_{standard}} - \mathbf{1}\right)[‰] \qquad \text{(Eq. 1)}$$

$R_{sample}$ and $R_{standard}$ are the ratios of heavy versus light isotopes ($^{15}N/^{14}N$) in the sample and an international standard respectively. The standard used was AIR for $^{15}N$. The simplified Rayleigh equation for closed systems (Eq. 2) was used to calculate the stable isotope fractionation factor α (Mariotti et al., 1981) and consequently the enrichment factor $\varepsilon^{15}N$ (Eq. 3) (Clark and Fritz, 1997):

$$(\propto -1) \times \ln\left(\frac{C_t}{C_0}\right) = (\propto -1) \times \ln(f) = \ln\left(\frac{R_t}{R_0}\right) \qquad \text{(Eq. 2)}$$

$$\varepsilon = (\alpha - 1)\,[‰] \qquad \text{(Eq. 3)}$$

In this equation, $R_t$ and $R_0$ denote the ratios of heavy versus light isotopes in the remaining nitrate at times t and the start of the experiment. $C_t$ and $C_0$ represent the respective concentrations of the residual nitrate. Since R was smaller than 0.01, we were able to use the approximation described by Mariotti et al. (1981), assuming that C is close to the concentrations of light isotopes alone. Enrichment factors derived from these definitions via determination of the slope of linear regression analysis in double-logarithmic plots are negative for "normal" isotope effects that enrich the residual substance in heavy isotopes.

### 2.2. Experimental procedures

#### 2.2.1. Batch experiments with the pure culture *Thauera aromatica* K172

The gram negative denitrifying bacterium *Thauera aromatica* K172 (DSM-6984) was already available at our laboratory. The cultures were grown in a carbonate-buffered fresh water medium (Widdel and Bak, 1992) with salt concentrations changed to: 100 mg/l NaCl, 40 mg/l $MgCl_2 \times 6H_2O$, 20 mg/l $KH_2PO_4$, 25 mg/l $NH_4Cl$, 50 mg/l KCl, 15 mg/l, $CaCl_2 \times 2H_2O$. Trace elements and vitamins were added in quantities described in the referenced literature (Widdel and Bak, 1992). Ascorbic acid at a concentration of 700 mg/l was added as a reducing agent. Carbonate buffer concentrations were 3 g/l $NaHCO_3$ to reach a pH of 7.2-7.4. The medium was dispensed in 400 ml aliquots into 500 ml glass bottles closed with a butyl stopper. The headspace was flushed with a mixture of 80% $N_2$ and 20% $CO_2$ (BIOGON® C 20). The medium contained 3.5 mM acetate as carbon source and sole electron donor and nitrate at a concentration of 13 mM as the sole electron acceptor. Initial carbon source concentrations were set by electron balance calculations to ensure the presence of sufficient residual nitrate for isotope measurements at the end of the experiment. From experience with previous experiments, we assumed that most of the nitrate would only be reduced to nitrite as long as nitrate was not limiting. This is also supported by the descriptions of Liu et al. (2013) on the successive expression of the enzymes used in full denitrification. During our experiments, we confirmed this by measuring nitrate and nitrite concentrations at each sampling time and calculation of the molar sum of $NO_3^-$ + $NO_2^-$, which did not change significantly throughout the experiments.

| | | |
|---|---|---|
| Reduction: | $NO_3^- + 2\,H^+ + 2\,e^- \rightarrow NO_2^- + H_2O$ | x4 |
| Oxidation: | $CH_3COO^- + 2\,H_2O \rightarrow 2\,CO_2 + 7\,H^+ + 8\,e^-$ | x1 |
| Total: | $CH_3COO^- + 4\,NO_3^- + H^+ \rightarrow 2\,CO_2 + 4\,NO_2^- + 2\,H_2O$ | |

In addition to this, we added non-biodegradable toxic hydrocarbons at sublethal concentrations (Table 1) according to Duldhardt et al. (2007) to induce a stress response in the bacteria, leading to a change in the membrane fatty acid composition. We tested the non-biodegradability of the compounds by

attempting to grow cultures with low concentrations of the compound and no addition of acetate. No growth was observed over several days. 1-octanol was added to target concentration from a 1:10 stock solution with acetone; 4-chlorophenol was added from a stock solution containing 2000 mg/l in pure water. For each set of conditions, we prepared stable pure cultures by growing the bacteria 4 times sequentially under the target conditions. Subsequently, we transferred 40 ml of the prepared viable culture in its stationary phase into each of the glass bottles with 400 ml medium and identical growth conditions. The bottles were incubated at 30°C in an incubation shaker. We sampled the bottles at the beginning of the experiment and at regular intervals after we visually observed turbidity in the bottles, indicating the beginning of the exponential microbial growth phase. The onset and duration of the exponential growth phase was then determined via calculating the nitrate reduction rate to ensure taking cell samples for lipid analysis during that phase.

The maximum membrane concentration (MMC) was estimated according to Neumann et al. (2005) using the calculations of Endo and Goss (2014) made on the basis of the LSER database of Ulrich et al. (Ulrich et al., 2017), referencing several sources. According to the LSER database, $Log\ P_{M/W}$ (the partition coefficient between membrane and water) of the two substances vary between 2.54-2.78 for 1-octanol and between 2.55-2.75 for 4-chlorophenol depending on the cited source therein. We thus assumed a nearly equal $Log\ P_{M/W}$ for both substances and took an average value of $Log\ P_{M/W} \sim 2.7$. From that, the membrane concentration was calculated using Eq. 4.

$$MMC = C_{(toxin\ in\ water)} \times P_{M/W} \qquad \text{(Eq. 4)}$$

Table 1: *Experimental setup of microbial incubations*

| Toxin | No. of repeat | concentration of toxin | maximum concentration of toxin in the |
|---|---|---|---|

| | experiments | in growth medium | | cell membrane (MMC) |
|---|---|---|---|---|
| | | [mg/l] | [mM] | [mM] |
| 4-chlorophenol | 3 | 20 | 0.156 | 78 |
| 4-chlorophenol | 6 | 40 | 0.311 | 156 |
| 4-chlorophenol | 2 | 70 | 0.545 | 273 |
| 1-octanol | 3 | 25 | 0.192 | 96 |
| 1-octanol | 3 | 50 | 0.385 | 193 |
| none (control) | 3 | 0 | 0 | 0 |

**2.2.2. Sampling procedure**

For sampling, the glass bottles were taken from the incubator. 30 ml of BIOGON® C 20 gas (80% $N_2$, 20% $CO_2$) was injected with a sterile syringe to create a slight overpressure and subsequently 30 ml of sample was withdrawn from each bottle with the same syringe. The sample was filtered with a 0.22 µm PES syringe filter (Millipore, Cork, Ireland) to avoid significant biological processes in the stored samples. The first aliquot of 2 ml was discarded to avoid contamination from filter material and oxygen contained in the filters. 900 µl of this filtered sample was pipetted into 1.5 ml centrifuge tubes for analysis of the anion concentrations (chapter 2.2.3). 100 µl of 1 M NaOH was added for preservation. The remainder of the sample in the syringe was injected in $N_2$-filled glass vials for nitrate isotope extraction and analysis (chapter 2.2.5).

We calculated nitrate reduction rates by immediately measuring nitrate content of the samples (chapter 2.2.3) during the running experiments, when we saw a reduction in nitrate of about 20% compared to the measurements at the beginning of the experiments, we assumed that exponential growth had started. During the exponential growth phase, an additional sample of 50 ml was taken for membrane

lipid analysis in a similar fashion. This sample was transferred from the syringe directly into sterile 50ml centrifuge tubes (Falcon®), immediately cooled down to 4°C and centrifuged at a RCF of ~4000 for 10 minutes. The growth medium was then discarded and the cell pellet was frozen at -20°C until further processing (chapter 2.2.4).

**2.2.3. Ion concentration analysis**

The aliquot for ion concentration analysis was diluted 1:10 prior to analysis in a Dionex 1100 Ion chromatograph (Thermo Fisher) with an Ionpac AS4A-SC or AS9-HC column installed, using the Chromeleon 6.8 software. All measurements with this instrument were done twice and averaged to improve precision. We determined nitrate and nitrite concentrations in each sample.

**2.2.4. Determination of fatty acid composition**

The extraction of membrane lipids was performed according to Bligh et al. (1959) and Morrison et al. (1964). Membrane lipids were extracted from cells pellets. The pellets were resuspended into 0.5 mL of water, 1 mL of methanol, and 2 mL of chloroform. After vortexing the mixture for 3 min, an additional 0.5 mL of water was added and mixed again for 30 s. The sample was then centrifuged 10 min at a RCF of ~2000 and room temperature and the chloroform phase was transferred to a new vial. The chloroform was removed completely using a nitrogen gas stream. To methylate the extracted fatty acids to FAME, 0.6 mL boron trifluoride in methanol was added and the sample was incubated at 95 °C for 15 min. Finally, 0.3 mL of water and 0.3 ml of hexane were added and mixed for 60 s and the final hexane phase was transferred into a GC-vial and used for analysis using an Agilent 6890N gas chromatograph with an FID detector (Agilent Technologies, Santa Clara, CA). The CP-Sil 88 capillary column (Chrompack, Middelburg, The Netherlands) was 50 m in length with an internal diameter of 0.25 mm. The oven temperature was set to 40 °C for 2 min followed by a temperature gradient to a final temperature of 220 °C with an increase of 8 °C/min. The injector was at 240 °C, the flow was in splitless mode with helium

gas carrier gas. The detector was set at 270 °C. A qualitative standard of bacterial FAMEs (Supelco; Sigma Aldrich, St. Louis, MO) was used to identify the detected peaks. The analysis was able to show the relative amounts of 6 distinct FAME components (*16:0, 16:1cis, 17cyclo, 18:0, 18:1cis, 19cyclo*), from which the degree of saturation (DoS) was calculated according to Eq 5:

$$DoS = \frac{16{:}0+18{:}0}{16{:}1cis+18{:}1cis} \qquad \text{(Eq. 5)}$$

Only membrane lipid samples that were taken during the exponential growth phase, confirmed by observing significant nitrate reduction at the time of sampling, were used for the calculations, since entering the stationary ("starvation") phase upon electron donor depletion would increase the DoS independent of the previous changes.

**2.2.5. Nitrate isotope analysis**

After sampling (chapter 2.2.2), the filtered aliquots for nitrate N isotope analysis were directly transferred into 40 ml glass vials with silicone septum caps, which were constantly flushed with a stream of $N_2$ gas for 30 minutes before the sampling. They were then treated according to Wunderlich et al. (2012) by reducing the nitrite content to $N_2O$ with 2 ml of 1 M ascorbic acid, using an anion exchange resin to capture nitrate via solid phase extraction, flush the ascorbic acid from the column with 200 ml of 10 mM HCl, then elute the nitrate from the columns with 10% HCl and neutralizing the solution with silver oxide, after which the produced $AgNO_3$ was freeze-dried and analyzed for $\delta^{15}N$ in an Isotope Ratio Mass Spectrometer (IRMS) in continuous flow mode (Thermo Finnigan Electron MAT 253) in conjunction with an elemental analyzer located at Helmholtz Zentrum München. Analytical uncertainty was ±0.5‰ for $\delta^{15}N$. International nitrate salt standards from the IAEA ("N2" and "NO3") and USGS ("#34") were used for calibration. We did not analyze the samples for $\delta^{18}O$, since the literature evidence of $\delta^{15}N$/ $\delta^{18}O$ ratio during denitrification in batch experiments under various conditions (e.g. Kritee et al. (2012))

overwhelmingly show a ratio of nearly 1 so that this additional parameter would not yield additional information.

## 3. Results

### 3.1. Effects of membrane contaminant concentration

The degree of saturation was analyzed for a sample taken during the exponential growth phase of each of the incubations and averaged over multiple incubations with identical growth conditions (Table 1). A positive correlation ($R^2$=0.79) can be observed between the calculated membrane concentration of the contaminant and the degree of saturation of the membrane fatty acids (Fig 1a). In the presence of increased concentrations of the tested contaminants the cells synthesize a higher percentage of saturated fatty acids in their membrane phospholipids. This increase in the DoS leads to a higher membrane rigidity counteracting the fluidizing effects of the toxic solvents. This is in accordance with previous studies which showed that an exposure to stressors like hydrocarbon contaminants will cause the cells to change the membrane composition as a protective mechanism (e.g. Duldhardt et al. (2010)).

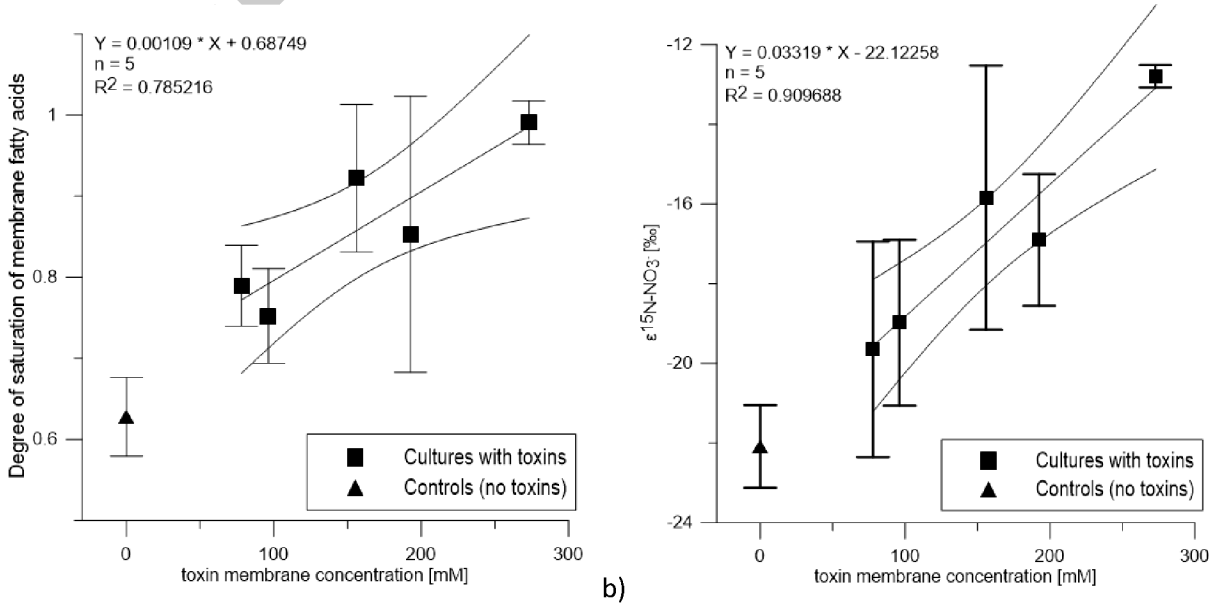


**Figure 1.** *Correlation of membrane contaminant concentration (MMC) with a) degree of saturation of the cell membrane fatty acids and b) isotope enrichment factor of nitrogen in residual dissolved nitrate. Error bars reflect variation between the duplicate cultures including analytical errors. 95% confidence intervals are depicted as curved lines bordering the regression line.*

All samples taken from each incubation bottle were utilized to calculate an isotope enrichment factor $\varepsilon^{15}N\text{-}NO_3^-$ for nitrate reduction using the Rayleigh equation. Enrichment factors for multiple incubations with identical growth conditions were averaged. As with the degree of saturation of the membrane fatty acids, a positive correlation ($R^2$=0.91) can be seen between membrane concentrations of the contaminant and the stable isotope enrichment factor $\varepsilon^{15}N$ during nitrate reduction (Fig 1b). A higher membrane concentration of contaminants resulted in a less negative enrichment factor $\varepsilon^{15}N$ during bacterial nitrate reduction, which is a novel result and in accordance with our expectations.

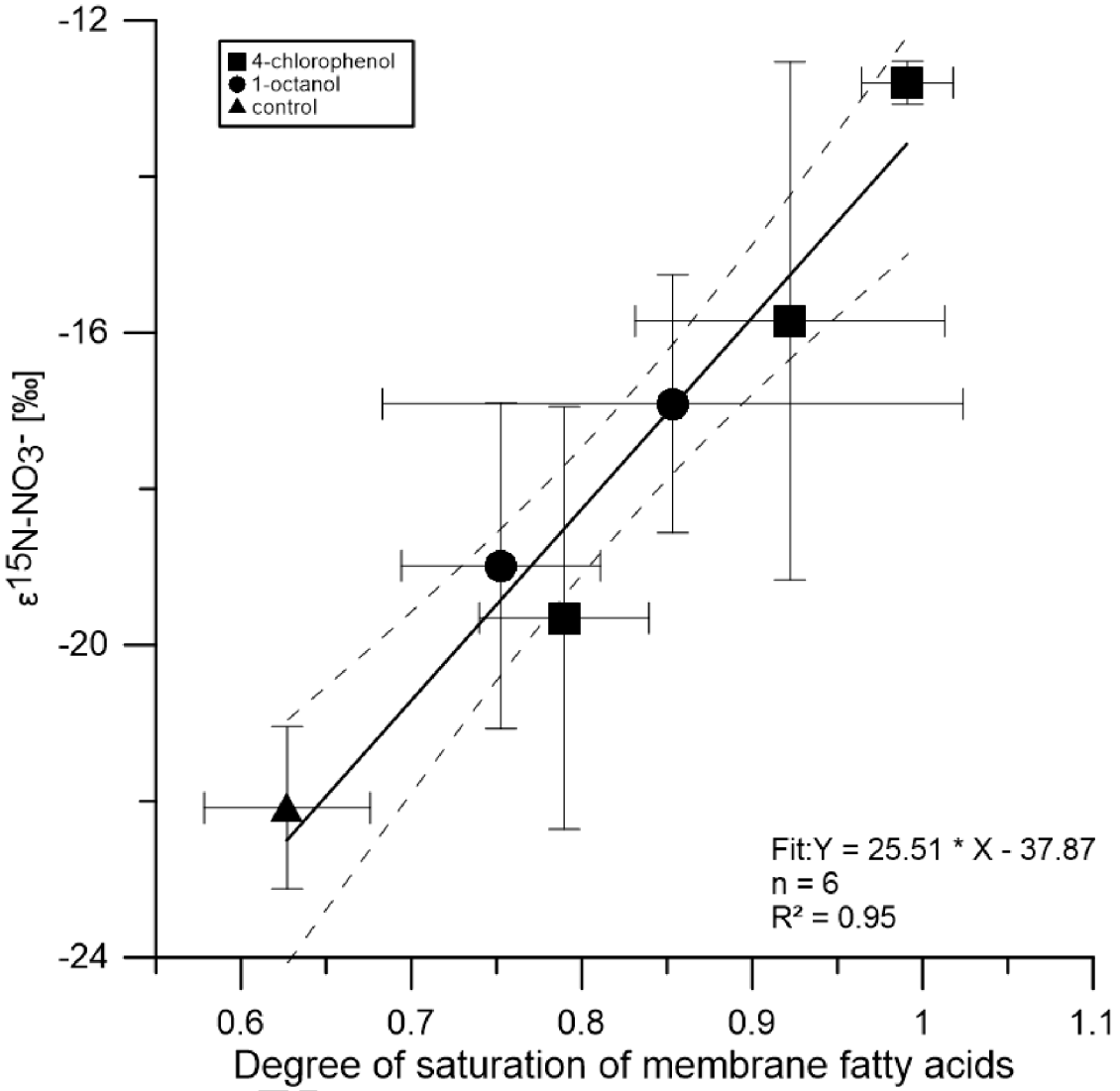



**Figure 2.** *Correlation of the degree of saturation of membrane fatty acids as an indicator of cell stress to the isotope enrichment factors during nitrate reduction for various concentrations and contaminants. A linear regression (solid line) with confidence intervals (dotted lines) is depicted as well.*

**3.2. Degree of saturation as indicator of isotope enrichment factor**

We found a significant correlation ($R^2$=0.84) of the degree of saturation of the membrane fatty acids - which is an adaptive response of the cells to exposure to solvent stress (Duldhardt et al., 2010; Heipieper

et al., 2007) - with a change in the nitrogen stable isotope enrichment factor during denitrification by *Thauera aromatica* (Fig 2).

**4 Discussion**

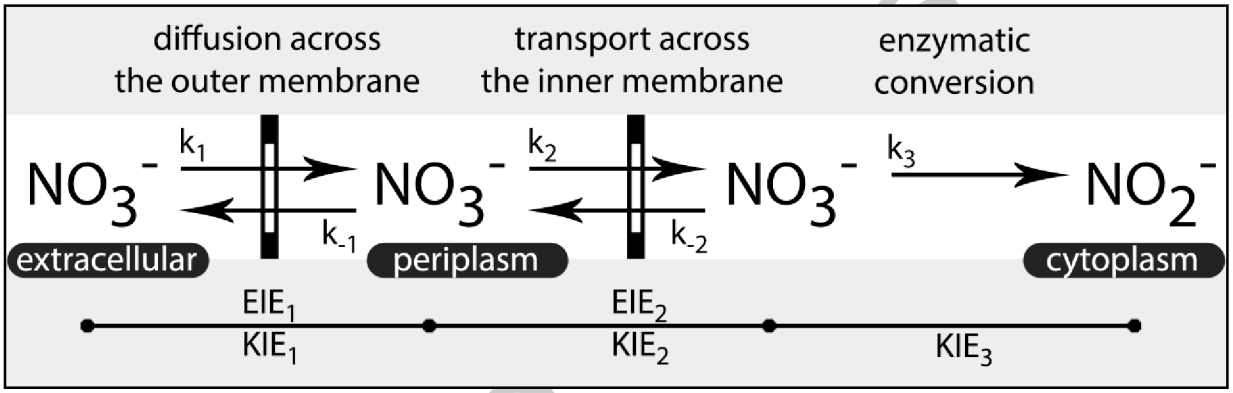
In denitrifying bacteria, nitrate generally is reduced inside the cytoplasm by the respiratory NaR enzyme (Simon and Klotz, 2013). Alternatively the periplasmic NaP enzyme is able to reduce nitrate, however, it does not generate a proton motive force and thus is unlikely to play a major role in nitrate respiration (Palmer et al., 2009; Simon and Klotz, 2013). Nitrate is, therefore, transferred across the periplasmic and the cytoplasmic membrane and then respired inside the cell. Each of these three steps including the irreversible enzymatic conversion of nitrate has a different isotope effect which is potentially measureable in the residual nitrate outside the cell due to an efflux of unused nitrate.

Two ways of nitrate efflux have been hypothesized. One possible mechanism is that the transporter proteins may work in a dynamic chemical equilibrium, thus facilitating a forward and backward transport with the equilibrium, trending strongly towards the influx of nitrate but allowing some efflux out of the cell, as well. Alternatively, passive diffusion of nitrate across the cell membrane regulates the outflow, creating an efflux of nitrate which depends on the chemical gradient and the properties of the membrane (as also described by Kritee et al. (2012)). The rapid bidirectional transport across the outer membrane into the periplasm is attributed to openings in the membrane (porins). The extent of the apparent kinetic isotope effect (AKIE) of denitrification, measured by isotopic analysis of the residual nitrate outside the cell, is determined by the forward and backward kinetics ($k_x$ /$k_{-x}$) of all steps involved as well as the kinetic ($KIE_x$) and equilibrium isotope effects ($EIE_x$) these steps inherently produce. It is assumed those isotope effects are identical in net value regardless of the direction of the process. A mathematical description of this model is represented by Eqs. 6 & 7 and described in great detail by

Elsner (2010). The model is depicted graphically in Scheme 1 (based in part on descriptions in Granger et al. (2008)).

$$AKIE = \frac{\left(KIE_1 \times \frac{k_2}{k_{-1}} \times \frac{k_3}{k_{-2}}\right) + \left(EIE_1 KIE_2 \times \frac{k_3}{k_{-2}}\right) + \left(EIE_1 EIE_2 KIE_3\right)}{1 + \frac{k_3}{k_{-2}} + \frac{k_2}{k_{-1}} \times \frac{k_3}{k_{-2}}} \qquad \text{(Eq. 6)}$$

$$\varepsilon = (1/AKIE - 1)[‰] \qquad \text{(Eq. 7)}$$




Scheme 1*: Nitrate is transferred from the water into the periplasm by diffusion and then transported across the cytoplasmic inner membrane, where it is converted to nitrite. Each step has a different kinetic and a different equilibrium and kinetic isotope effect.*

A decrease in the kinetics of the efflux processes ($k_{-1}$ and $k_{-2}$) in relation to the enzymatic conversion process ($k_3$) will shift the AKIE towards a combination of the transport isotope effects ($EIE_1$, $EIE_2$, $KIE_1$, $KIE_2$). Conversely, an increase of efflux of nitrate out of the cell would allow for the isotope effect of the nitrate reduction step ($KIE_3$) to dominate the AKIE. The transport-related isotope effects ($EIE_1$, $EIE_2$, $KIE_1$, $KIE_2$) are generally described as small (EIE/KIE ≈ 1, $\varepsilon^{15}N \approx 0$), while the isotope effects of the nitrate reduction reaction ($KIE_3$) are generally pronounced (KIE >> 1, $\varepsilon^{15}N$ up to -36‰) (Brandes and Devol,

1997; Granger et al., 2008; Lehmann et al., 2003; Mariotti et al., 1981; Mariotti et al., 1988). As a result, a partial transport limitation in relation to nitrate reduction would shift the AKIE towards a value of unity ($\varepsilon^{15}N = 0$), while rapid transport of nitrate into and out of the cell would create a significant isotope fractionation (AKIE > 1, $\varepsilon^{15}N < 0$).

We consider two scenarios leading to such a change in the ratio of $k_3/k_{-2} \cdot k_{-1}$. Both can be described as the relative ratio of cytoplasmic nitrate used in nitrate reduction versus outflow across the two membranes.

Generally, our main hypothesis is that the changes in the cell membrane cause a decreased efflux of nitrate out of the cell. This can either be explained by an impediment of the transporter proteins by the increased membrane fatty acid saturation (Nedwell, 1999; Tsukagoshi and Fox, 1973) or by a limitation of the diffusive transport of nitrate across the cytoplasmic (and possibly periplasmic) membrane due to its lessened permeability for $HNO_3$. In this context it is worth mentioning that the reversibility of the nitrate transport in cells was also observed in our previous experiments with $^{18}O$-isotope labelled nitrite (Wunderlich et al., 2013). An impediment of the partially reversible (and thus "bidirectional") transporter proteins may lead to a lessened reverse flow of nitrate out of the cell and contribute to the outflow of nitrate out of the cell in the fashion of a chemical equilibrium. Possible explanations of this phenomenon may include a reduced formation of nitrate transporters due to stress or a reduced activity of the transporters in the affected cytoplasmic membrane due to membrane viscosity effects. Another highly likely pathway is a diffusive transport of protonated nitrate as $HNO_3$ due to a steep chemical gradient from the inside of the cell, which is enriched in nitrate, to the outside of the cell which is controlled by environmental nitrate concentrations. Kritee et al. (2012) describe the outflow of nitrate as most likely driven by diffusion. An outflow across the lipid membrane can, however, not occur while nitrate carries a negative charge. In contrast, its protonated form $HNO_3$ would be able to cross the membrane (Gutknecht and Walter, 1981). If the degree of saturation of the lipid membrane increases, its permeability decreases and a higher degree of saturation would thus limit the outflow of nitrate out of the cell ($k_{-2}$). In

both scenarios, the ratio of $k_3/(k_{-2}\cdot k_{-1})$ would increase and the AKIE would be closer to unity ($\varepsilon^{15}N$ being closer to 0).

Kritee et al. (2012) describe the possibility of an isotope effect linked to the cell-specific nitrate reduction rate (CSNR). They found a correlation of low CSNR, which translates to a low $k_3$, with less pronounced isotope fractionation. Their hypothesis states that as $k_3$ increases, so does $k_{-2}$, but if $k_3$ decreases, $k_{-2}$ paradoxically decreases proportionally more. The effect would be that an increase of the ratio of $k_3/(k_{-2}\cdot k_{-1})$ occurs concurrently with lower CSNR, correlating to an AKIE closer to unity ($\varepsilon^{15}N$ less negative). Since the toxins can also cause a decreased growth rate (Duldhardt et al., 2007), it cannot be excluded that the ratio of efflux to nitrate reduction is also affected by lower CSNR. However, a transport-independent decrease of the nitrate reduction rate alone due to an influence of the lipid membrane changes on the activity of the NaR enzyme would decrease $k_3$ and thus $k_3/k_{-2}\cdot k_{-1}$, leading to a more pronounced isotope fractionation, which was not observed in our experiments. Consequently an influence of decreased CSNR due to the application of toxins on the extent of stable isotope fractionation during denitrification is unlikely. However we cannot entirely exclude from our experiments the possibility of this influence, since there is no data on the CSNR available.

**4 Conclusions**

In accordance with former isotope fractionation models focusing on nitrate reduction, the effect of the toxin addition to the growth medium would thus lead to an increase in the ratio of $k_3/k_{-2}\cdot k_{-1}$ and consequently result in an isotope fractionation that is less pronounced than observed in our experiments. Since other stress factors like pH, temperature or pressure can also cause changes in the cell membranes, it stands to reason that other environmental conditions also can have an influence on the stable isotope fractionation during denitrification.

If quantification of the extent of biodegradation/denitrification B (Eq. 8) is performed, using $\varepsilon^{15}N$ values from laboratory experiments or environmental experiments under low stress conditions, the calculations would underestimate the real amount of denitrification in environments associated with various types of stress (organic contaminant stress, temperature and pressure stress) because a more strongly negative $\varepsilon^{15}N$ is assumed in the calculations usually done with a formula similar to Eq. 8. (f is the fraction of the compound/nitrate remaining)

$$B = (1 - f) = 1 - \left(\frac{\delta^{15}N + 1}{\delta^{15}N_0 + 1}\right)^{1/\varepsilon} \qquad \text{(Eq. 8) (modified from Elsner (2010))}$$

It can now be speculated, that the previously suggested apparent imbalance between global ocean N inputs and outputs by N isotope budgeting as discussed by Kritee et al. (2012) could also be addressed in case that increasing water pressure in the deep ocean causes changes in the membrane permeability. Further studies would be needed on the effects of pressure on isotope fractionation during denitrification, though. A similar effect could hypothetically also bias quantifications of other processes, involving different compounds and isotopes such as sulfate during bacterial sulfate reduction if these processes also involve a transport of a substance across the cytoplasmic membrane before it is converted or consumed. These transport processes might be influenced by the stress response of the cells in a similar way, and this phenomenon may be detected by a change in DoS of the phospholipids, when looking at the same species of organisms in comparison. However, different species would have different DoS readings in any case, so a determination of the DoS of bulk samples of a local ecosystem would not be applicable for calculating enrichment factors for a specific process.

The knowledge of this effect could eventually allow us to estimate isotope enrichment factors for a process in less than ideal growth conditions by analyzing the membrane composition of the bacteria responsible for the process (key players) and by performing realistic laboratory determinations of

enrichment factors by mimicking the stresses of the natural environment to be studied in the laboratory more closely.

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