

Influence of changes in microbial cell membrane composition on isotopic fractionation of nitrate during denitrification

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Abstract. Pure cultures of *Thauera aromatica* were stressed by the addition of the toxic solvents 1-octanol and 4-chlorophenol causing adaptive modifications in their cell-membrane fatty acid composition. At the same time, we investigated the isotopic fractionation in $\delta^{15}\text{N-NO}_3^-$ during denitrification. We observed a change in the degree of saturation (DoS) of the bacteria as a reaction to solvent stress and a higher degree of saturation was directly correlated to the concentration of solvents in the cell membrane. The enrichment factor $\epsilon^{15}\text{N-NO}_3^-$ during denitrification showed a linear dependency to the DoS as well as to the membrane solvent concentration. Denitrifying bacteria thus may express less negative $\epsilon^{15}\text{N-NO}_3^-$ when they are exposed to environmental stress that changes their cell membrane composition.

1 Introduction

Isotope enrichment factors $\epsilon^{15}\text{N}$ and $\epsilon^{18}\text{O}$ of nitrate during denitrification are used to determine the presence of this process in anoxic environments. More detailed knowledge of the enrichment factors under environmental conditions could be used to quantify denitrification or to model processes like coupled denitrification with anammox or other processes affecting nitrate pools in the water. So far, enrichment factors for both isotopes have been found to vary greatly in laboratory and field experiments. In a previous study, we found that the presence of bacteria with a NXR nitrate reductase enzyme can foster an isotope exchange of $^{18}\text{O-NO}_3^-$ with the oxygen atoms in water, under anoxic conditions thus providing one possible explanation for the varied $\epsilon^{18}\text{O-NO}_3^-$ values observed during denitrification, particularly the difference between field observations and laboratory experiments [1]. The $\epsilon^{15}\text{N-NO}_3^-$ seems to depend to some extent on the nitrate reaction rate and it was hypothesized that transport limitation would also influence the enrichment factor [2]. In a previous study, we found an effect of the presence of organic solvents on the

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$\epsilon^{15}\text{N}$ and $\epsilon^{18}\text{O}$ during denitrification [3]. In the experiments presented here, we deliberately changed the composition of the cell membrane of denitrifiers to influence its ability to transport nitrate. The aim was to provoke changes in the nitrate transport across the cell membrane and thus a change in the $\epsilon^{15}\text{N}\text{-NO}_3^-$ during denitrification which is a combination of the different enrichment factors of nitrate transport into the cell and nitrate conversion inside the cell. To change the permeability of the cell membranes we use the well documented method of exposing the bacteria to nonlethal dosages of organic solvents [4-7].

2 Methods

Pure culture experiments with the solvent-tolerant gram negative bacterium *Thauera aromatica* were performed in 500ml glass bottles under anoxic conditions with nitrate as electron acceptor and acetate as electron donor. Organic solvents 1-octanol and 4-chlorophenol were added at different nonlethal concentrations according to [4] (see Table 1). Each experiment was carried out at least in duplicates. Cultures were preconditioned to the solvent at the respective concentration during 3 previous incubations before being used to start the experimental incubations. During regular intervals after initiating the growth, samples were taken for analysis. We performed ion chromatographic measurements of the nitrate and nitrite concentrations, nitrate $\delta^{15}\text{N}$ isotope analysis according to the protocol used by [1, 2] and centrifuged cell pellets for the extraction of membrane phospholipid fatty acids. Those were then converted to fatty acid methyl esters (FAME) and analysed with gas chromatography for 6 distinct components, from which the degree of saturation (DoS) was calculated. FAME data was only used for samples during the exponential growth phase. Isotope data is given in the conventional delta-notation and was used together with nitrate concentrations to determine enrichment factors $\epsilon^{15}\text{N}$ using the Rayleigh equation. Organic solvents were added at defined concentrations and the maximum membrane concentration (MMC) of the solvent in the cell wall was calculated using known membrane-water partition coefficients.

Table 1. Experimental setups listing solvent concentrations in water and calculated maximum membrane concentrations (MMC) of the solvent in the cell membrane [11][†]

| toxin | n | concentration [mg/l] | MMC [mM] |
|----------------|---|----------------------|----------|
| 4-chlorophenol | 3 | 20 | 78 |
| 4-chlorophenol | 6 | 40 | 156 |
| 4-chlorophenol | 2 | 70 | 273 |
| 1-octanol | 3 | 25 | 96 |
| 1-octanol | 3 | 50 | 193 |
| none (control) | 3 | 0 | 0 |

3 Results and Discussion

A positive linear relationship was observed between the calculated membrane concentration MMC of solvent in the cell and the calculated DoS of the cell membrane fatty acids. This was to be expected according to [4], since the cells compensate for the loss in membrane fluidity caused by the solvents dissolved in the membrane lipids with an increase in the DoS. Both of these parameters however also had a positive and linear relationship to the observed $\epsilon^{15}\text{N-NO}_3^-$ during denitrification (Fig. 1). The correlation of the DoS of the cells in an incubation with the $\epsilon^{15}\text{N-NO}_3^-$ during denitrification was high ($R^2 = 0.95$).

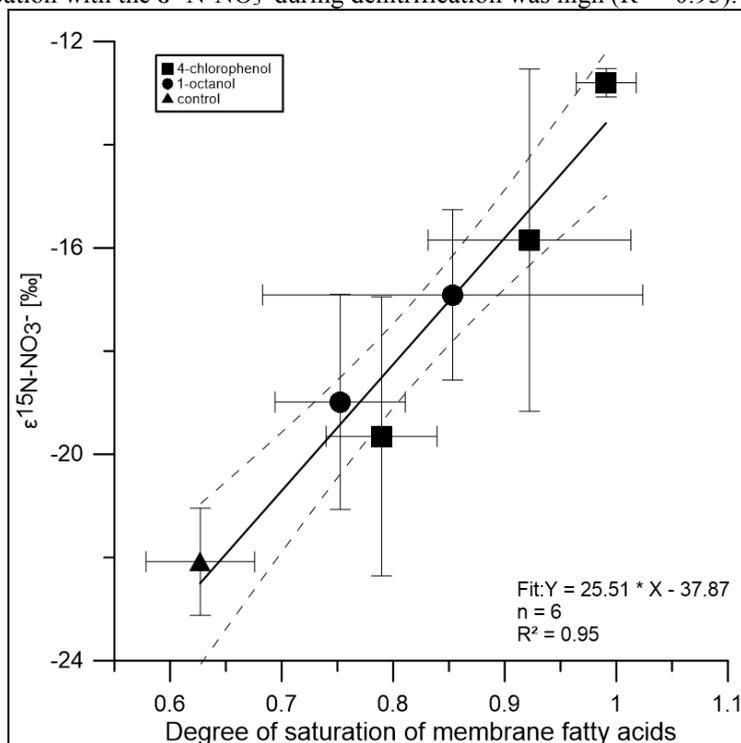


Fig. 1. Correlation of the degree of saturation of membrane fatty acids as an indicator of cell stress to the isotope enrichment factors during nitrate reduction for various concentrations and contaminants. A linear regression (solid line) with confidence intervals (dotted lines) is depicted as well [11]^{†,*}.

A higher level of stress to the cell, resulting from a higher membrane concentration of organic solvents in the cell membrane resulted in a higher DoS of the membrane phospholipid fatty acids as well as a less negative isotope enrichment factor during denitrification.

In the Gram-negative bacteria utilized in these experiments, nitrate is transported with a transporter protein across the cytoplasmic membrane and then converted to nitrite inside the cell. Both steps could possibly cause an isotope effect on the nitrate with the transport process generally being assumed to infer a very small isotope effect, while the chemical conversion step causes a strong isotope effect. Nitrate from inside the cell must leave the cell to a certain degree, either by a reversal of the transport by the transporter proteins, or

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by diffusion - otherwise no isotope effect could be observed in the water. We propose a decrease in outflow of nitrate from the cells interior to the outside, which would cause a lessened observed isotope effect since the ratio of nitrate reduced to the nitrate efflux is increased. This conclusion follows from the AKIE (apparent kinetic isotope effect) model by [8] when applied to this scenario (Eq. 1+2). In this model, the relative fluxes of nitrate into and out of the cell determine which isotope effect dominates the observation outside the cell – either the small isotope effect of the transport across the cell membrane or the strong isotope effect of the nitrate reduction. Thus an increase in nitrate outflux would result in an increase of the dominance of the isotope effect of the nitrate reduction step and vice versa.

$$\text{AKIE} = \frac{1 + \frac{k_2^{-1}}{k_3} + \frac{k_2^{-1}}{k_3} \cdot \frac{k_3}{k_2}}{\left(\text{KIE}^1 \cdot \frac{k_2^{-1}}{k_3} \cdot \frac{k_3}{k_2} \right) + \left(\text{EIE}^1 \cdot \text{KIE}^2 \cdot \frac{k_3}{k_2} \right) + \left(\text{EIE}^1 \cdot \text{EIE}^2 \cdot \text{KIE}^3 \right)} \quad (1)$$

$$\varepsilon = (1/\text{AKIE} - 1) [1] \quad (2)$$

In this model, step 1 is the transport of nitrate to (1) and away from (-1) the cell, step 2 is the transfer of nitrate into (2) and out of the cell (-2) and step 3 is the (irreversible) chemical conversion of nitrate to nitrite inside the cell (3). EIE represents equilibrium isotope effects, KIE represents kinetic isotope effects and k_x represents the relative kinetics of the various processes.

We propose two possible mechanisms for the reduced outflow of nitrate in stressed cells with an increased content of organic solvents. Either the nitrate transport via the transporter protein is impaired in the outward direction [9, 10], or the diffusive outflow of nitrate is impaired. Diffusive nitrate outflow can only occur in its protonated form HNO_3 , but cell wall permeability decreases with increasing degree of saturation of the phospholipid membrane, which essentially is a natural reaction of the cell to protect itself from an environment containing toxic organic compounds. In both cases k_2 would decrease in comparison to k_3 and thus the influence of the weak isotope effect of the first two steps would dominate the total AKIE, resulting in an overall less pronounced isotope effect and a less negative isotope enrichment factor.

4 Conclusion

The extent of transport of nitrate out of a cell (“leaking”) during microbial denitrification in comparison to the extent of nitrate reduction to nitrite within the cell can be a determining factor for the variability of observed $\varepsilon^{15}\text{N-NO}_3^-$ during microbial denitrification. Changes in the cell physiology, particularly the cell membrane composition, may change the relative fluxes of nitrate into and out of the cell. As a result we observed a dependency of $\varepsilon^{15}\text{N}$ in residual nitrate to the composition of the cell membrane and the extent of solvent stress put onto the cell. We hypothesize that denitrifiers will react to different stresses by changing the aforementioned relative fluxes of nitrate and thus displaying a wide variety of $\varepsilon^{15}\text{N}$ observed in residual dissolved nitrate.

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