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RESEARCH ARTICLE
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Comparison of Denitrification Induced by Various Organic Substances—Reaction Rates, Microbiology, and Temperature Effect

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Key Points:

- Higher denitrification rate with ethanol at 10°C, consequently, reaction kinetics does not generally increase with rising temperature
- Addition of organic substances and temperature strongly modify the denitrifying microbial community
- Electron donor selection for induced nitrate reduction depends on the groundwater temperature of the region

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Abstract Widespread groundwater pollution with nitrate (NO_3^-) and the finite and decreasing geogenic NO_3^- degradation capacity in aquifers require a better understanding of potential treatment methods. This project aimed at exploring and comparing the efficiency of four organic substances as electron donors for heterotrophic denitrification. Circulation column experiments using sediment without NO_3^- degradation capacity and high agricultural NO_3^- groundwater were conducted. Acetate, glucose, ascorbic acid, and ethanol were added to these columns in three concentration steps to induce biological denitrification, whereby also temperature dependence of denitrification rates (room temperature and typical groundwater temperature of 10°C) was taken into account. Results show denitrification with all four carbon (C) sources with intensities varying considerably between electron donors. Comparison of the two temperature approaches shows substantial differences between applied organic substances and indicates *T* as an important variable for denitrification. Ethanol is clearly the most effective electron donor for biodenitrification in groundwater investigated in this study, with a stronger and more effective NO_3^- degradation at 10°C than at room temperature. In contrast, much higher reaction rates are achieved with glucose at room temperature, compared to 10°C. Denitrification with ascorbic acid is very low at both temperatures; its addition produces biomass which repeatedly led to column clogging. In the entire test series, nitrite (NO_2^-) accumulation occurred more frequently and in higher concentrations at 10°C. Analysis of microorganisms shows a strong modification in microbial community in reaction to the addition of different organic C as well as between the two temperature approaches.

1. Introduction

Nitrate pollution of water resources is a global problem (Almasri, 2007). In some regions, the guideline values of groundwater and drinking water regulations are considerably exceeded in surface waters, but also in groundwater (Carrey et al., 2014). Several European Union member states, including Germany, were penalized by the European Court of Justice for non-compliance with the Nitrate Directive (European Court of Justice, 2010, 2018). However, further infringements and sanctions are to be expected. This contamination results mainly from anthropogenic N fertilizers used to increase agricultural productivity (Hosono et al., 2013). Excess N enters groundwater as NO_3^-. Due to a geogenic NO_3^- degradation capacity of most aquifers (sulfide minerals and organic C), part of the NO_3^- can be degraded (Rivett et al., 2008). Based on sulfide-S and organic C contents of the aquifer, groundwater recharge and NO_3^- concentration, the remaining time until a NO_3^- breakthrough to drinking water production wells may be calculated (e.g., Ortmeyer, Volkova, et al., 2021). Overall, a link between hydrologic and water quality models is important because including transit times can improve understanding of NO_3^- transport in aquifers (Hrachowitz et al., 2016). In the future, considerable increases in NO_3^- concentrations and NO_3^- breakthroughs to raw water wells are expected, as this degradation capacity is decreasing and finite (Knowles, 1982; Schwientek et al., 2017). Climate change is expected to enhance this deterioration of water resource quality and quantity (Fleck et al., 2017; Ortmeyer, Mas-Pla, et al., 2021). In addition to a decrease in groundwater recharge and a drop in water levels, Stuart et al. (2011) point to possible increasing rates of NO_3^- leaching under future climate scenarios. The thickness of the unsaturated zone is crucial for reaching NO_3^- peaks at the groundwater table (Wang et al., 2012). Nitrate storage in the vadose zone is also important, but often not considered in

model calculations (Ascott et al., 2017) although long-term observational data remain crucial for predictive models (Howden & Burt, 2008).

Many measures have already been implemented to reduce NO_3^- concentrations in groundwater. These include cooperation with farmers to reduce the amount of fertilizer, to fertilize only during certain periods, and to increase the efficiency of N uptake by crops (Cameron et al., 2013; Eulenstein et al., 2017). Alternatively, several treatment processes for NO_3^- removal have been investigated, such as adsorption, membrane separation and electrochemical processes (Gao et al., 2019; Gouran-Orimi et al., 2018; Kalaruban et al., 2017). However, these measures are either very expensive or prove moderately successful. Besides ion exchange (Kapoor & Viraraghavan, 1997), biological denitrification enhanced by organic C is one of the best known and most effective treatment methods (e.g., Khan & Spalding, 2004; Vidal-Gavilan et al., 2013). An advantage relative to the other methods is the selective NO_3^- removal. Akunna et al. (1993) investigated denitrification in digested sludge with addition of different pure organic C compounds in batch tests. In a flow-through experiment, Carrey et al. (2014) investigated NO_3^- degradation by glucose at temperatures between 18°C and 27°C. Ge et al. (2012) analyzed NO_3^- reduction with methanol, acetate, and glucose at 28°C. Likewise at 28°C, Schroeder et al. (2020) investigated the denitrification of different mixing ratios of glycerol and ethanol.

While not all studies have used glucose, the highest denitrification rates were observed in experiments with this electron donor at room temperatures (Akunna et al., 1993; Ge et al., 2012). Enhanced denitrification is also possible with complex organic substances like pine bark, sawdust or bamboo biomass (Costa et al., 2018; Schipper & Vojvodic, 2000; Trois et al., 2010). However, carbons in liquid aggregate state seem to be more promising for groundwater remediation as they can be injected into the aquifer.

Denitrification takes place in the environment when an electron donor and denitrifying bacteria (for catalysis) are present at low oxygen concentrations (Korom, 1992). Therefore, microbial communities were investigated in many studies. In the deep vadose zone, denitrification is limited by organic C availability, not a lack of denitrifiers (Chen et al., 2018). Hellman et al. (2019) report that external C addition influences the bacterial community composition. Selection of the specific electron donor and feeding strategy play an important role in enhanced denitrification (Vidal-Gavilan et al., 2014). Evaluating further effects, Ebrahimi et al. (2015) describe the relation between salt concentration and temperature on NO_3^- degradation and observe a lower microbial tolerance to high salinity at low temperature.

This study aims at investigating the denitrification potential of pure organic C compounds (acetate, glucose, ascorbic acid, and ethanol) under realistic aquifer conditions at laboratory scale. For this purpose, sediments from a German aquifer important for regional drinking water production (without geogenic degradation capacity) and natural groundwater with high NO_3^- concentrations were used. We conducted a comparison of NO_3^- reduction, reaction rate, stoichiometric degradation, and response of microbial community between the different applied organic substances. Thereby, different water temperatures (room temperature and groundwater temperature) were taken into account to study *T* influence on denitrification which can be substantial (Rivett et al., 2008). Little is known about the complex interplay of the mentioned parameters (especially *T* influence), their combined effects on N dynamics, and the transferability of respective laboratory results to the field scale, which gave rise to the present study.

2. Materials and Methods

2.1. Experimental Setup

For investigating, the denitrification potential of various organic substrates in groundwater, column tests were carried out to simulate aquifer conditions on a laboratory scale. Columns consisting of two sections were used: the lower part (height: 35 cm, diameter: 18.3 cm Figure 1a) has a volume of 9.2 L. The upper part is the water reservoir (height: 16.2 cm, diameter: 11 cm) with a volume of 1.54 L. The experiment was conducted in a circulation system with the water flowing through the column from bottom to top. The sediment is relatively pure and little consolidated sands from the Haltern Fm. (Upper Cretaceous) almost without geogenic degradation capacity (sulphide-S <0.01 wt%, C_{org} 0.06 wt%, CS, G4 Icarus, Bruker, Ruhr-University Bochum), they are almost completely oxidized. And have likewise a low degradation potential due to iron Fe: water: <0.1 mg/L, sediment: 1.14 Wt.%, in oxidized Haltern Fm. Fe is present as (hydr)oxide (Banning

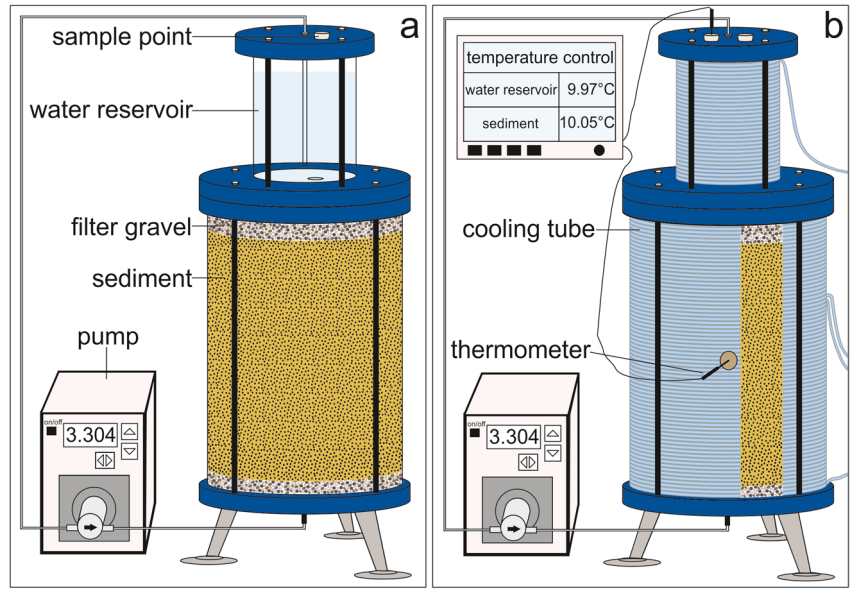


Figure 1. Experimental setup of column tests, (a) Basic setup for experiments at room temperature, (b) Setup with cooling device for experiments at 10°C.

et al., 2009, 2013) and manganese (Mn: water: <0,1 mg/L, sediment: 0.0054 Wt.%) (Bulk geochemical analyses: INAA (Instrumental Neutron Activation Analysis, Activation Laboratories Ltd. Ontario, Canada). It is a medium sand in which sufficient pore volume for microbial growth is available. Otherwise, denitrification could be inhibited by too small pore sizes (West & Chilton, 1997).

To prevent the pump from clogging, a 2–3 cm thick filter gravel layer was installed before and after the sediment. The groundwater used in the column experiments was obtained from a piezometer screened in the Haltern Fm., so the water hydrochemically corresponds to the sediment. Nitrate concentration in the Haltern Fm. is partly >200 mg/L, probably due to intensive agricultural activity in the region (here and throughout the manuscript, presented NO_3^- concentrations always refer to NO_3^-, not NO_3-N). When the sediment was placed in the column, the maximum total water volume in pores and reservoir was determined as 3.74 L. The flow rate was maintained at 1.7×10^{-4} L/s using a piston pump (ISMATEC). Sampling and physico-chemical measurements were performed in situ, at an inlet of the reservoir, which was otherwise closed airtight. After each sampling, argon was injected into the reservoir to remove any oxygen which may have entered. When the water in the reservoir was depleted due to successive sampling, new water was added.

To simulate the aquifer even more realistically, experiments were conducted at 10°C, corresponding to the average groundwater temperature in Germany. Subsequently, reaction rates between experiments at room (approx. 21.5°C) and groundwater temperature (10°C) were compared.

A cooling tube in direct contact with the column was used to accomplish cooling to 10°C (Figure 1b). The column was additionally insulated with aluminum foil to save energy and to avoid cooling fluctuations. Temperature in the sediment and in the water reservoir was monitored.

2.2. Addition of Various Organic Substances

To initiate heterotrophic denitrification, various pure organic C compounds (acetate, glucose, ascorbic acid [manufacturer: CHEMSOLUTE], ethanol [manufacturer: VWR]) were added to the column through the reservoir inlet in three concentration steps (2.5, 5, and 10 mmol). Subsequently, NO_3^- concentration decrease was observed until it was constant again. For each C compound, two new columns (room temperature and groundwater temperature) were set up to avoid microbial cross-contamination between experiments.

2.3. Analytical Methods

At each sampling, pH, Eh, EC, O_2 and temperature were measured once a week. As explained in 2.1, temperature of the cooled columns was permanently monitored. Water samples were filtered (0.2 μm) and analyzed for anions (NO_3^-, NO_2^-, SO_4^{2-}, Cl^-, and F^-) by IC (Compact pro, Metrosep column, Metrohm). Major cation concentrations were quantified using ICP-OES (Optima 8300, Perkin Elmer, Fraunhofer Institute Bochum), but were analyzed at larger intervals to avoid removing large water quantities from the system. Acid and base capacities were determined by titration, DOC with TNM-L (Shimadzu). For comparison, 1-D transport modeling is performed using the computer program PHREEQC 3.5 (Parkhurst & Appelo, 2013) in which C_{org} is added for NO_3^- degradation. The modeling provides additional information about reaction products such as N_2 and investigates whether NH_4^+ is formed. Samples from acetate experiments were additionally analyzed for acetate concentrations by IC (except cations, all measurements conducted at Ruhr-University Bochum). Isotope characterization of N and O in dissolved NO_3^- (Delta V Plus isotope ratio mass spectrometer, TU Dresden) was conducted for the acetate column tests only, whereby samples were stored until analysis at −20°C. The analysis was performed with microbial transformation from NO_3^- to N_2O according to the denitrification method of Zhu et al. (2018), which was further developed by Stock et al. (2020). Notation is expressed in δ‰ ($\delta = ([R_{sample} - R_{standard}]/R_{standard})$, where R is the ratio between the heavy and the light isotopes). International calibration standards are USGS 34, EA-Lab. 13 and IAEA NO3 (−1.8‰–9.8‰). The $\varepsilon^{15}N/\varepsilon^{18}O$ ratio was calculated according to Nikolenko et al. (2018).

2.4. Microbiology

Shaking tests were conducted to analyze microbial communities resulting from the addition of the selected organic carbons for the catalysis of denitrification and influenced by the two temperature approaches.

15 mL falcon tubes were filled with 6.2 g sediment and then water (with different C_{org} concentrations) was added without air inclusion. Concentrations were adjusted to the amount of substance added in the column tests. Again, the same sediment and groundwater as described in 2.1 were used. For each organic C (four), each concentration (three) and both temperature approaches, one shaking test was performed. As a control, one shaking test using the groundwater without C_{org} was also carried out. All shaking tests were repeated three times to minimize any error which results in a total of 78 shaking tests. The overhead shaker was set to the lowest speed in order to simulate a flow. The experiments ran for one week, since this time span was identified in this study as the most important period for denitrification in the column tests.

For the characterization of bacterial communities in the water samples, 7 mL of the supernatant water was centrifuged in several steps in a 500 μL, 0.2 μm filter mini-column for 2 min at 13,000 rpm. The filters containing microbial biomass were transferred to lysis tubes E (MP Biomedicals) and 400 μL SLS lysis buffer were added, followed by mechanical disruption (3 × 6 m s^{-1}, 45 s) with a FastPrep-24™ device (MP Biomedicals). Afterward, 20 μL Proteinase K were added and the reaction was thoroughly mixed. The microbial DNA was then extracted using the my-Budget DNA Mini Kit (BioBudget Technologies, Krefeld), according to Graupner et al. (2017) and the manufacturer's instructions. After extraction, the isolated DNA of all samples was stored at −20°C. The 16S amplicon libraries were prepared by the Deutsches Institut für Mykologie (Bayreuth, Germany), and sequenced by the sequencing service of the Faculty of Biology at LMU Munich, using an Illumina MiSeq® sequencer (2 × 250 bp paired-end sequencing). The sequence reads were processed according to Röhl et al. (2017) and are available from the European Nucleotide Archive (http://www.ebi.ac.uk/ena/) under study PRJEB42532. Taxonomic affiliation for the representative sequence of each operational taxonomic unit (OTU) was assigned using the SILVA database version 138.1 and RDP classifier version 2.11 (16S rRNA training setNo 18 07/2020).

3. Results

3.1. Denitrification Initiated With Organic Substances

Denitrification was initiated with the addition of all four C compounds (acetate, glucose, ascorbic acid, ethanol, Figure 2). Nevertheless, NO_3^- degradation intensity differs between all electron donors and shows considerable variation between room and groundwater temperature (10°C).

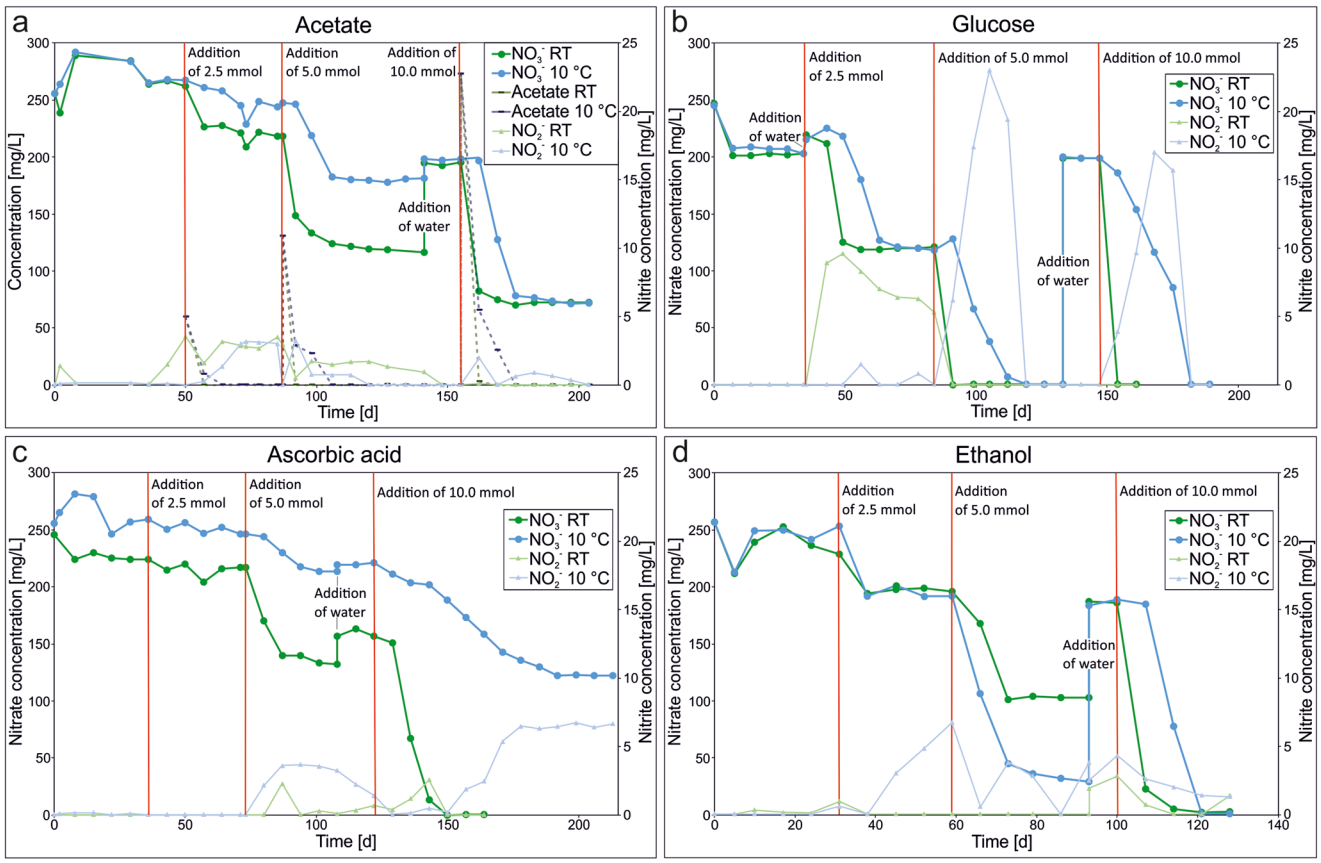


Figure 2. Evolution of NO_3^- and NO_2^- concentration during stimulated denitrification by (a) Acetate, (b) Glucose, (c) Ascorbic acid, and (d) Ethanol, injection of electron donor in 3 steps (2.5, 5, and 10 mmol) marked by red vertical lines, concentration curves at room temperature (RT): green, at 10°C: blue.

Addition of organic C is done in three steps (2.5, 5, and 10 mmol) and is marked by red vertical lines in Figure 2. Prior to the first addition, a stable NO_3^- concentration (ca. 250 mg/L) was obtained as a start condition for all experiments. Initial concentration fluctuations are caused by water mixing in the circulation column test.

With acetate (Figure 2a), NO_3^- concentration was reduced by 43.8 mg/L when adding 2.5 mmol at room temperature. At 10°C, the concentration was reduced by 19.7 mg/L. A larger difference in NO_3^- degradation was observed when 5 mmol was added: while the concentration at 10°C decreased by 66.3 mg/L, it is 101 mg/L at room temperature. After the first week, the concentration has already decreased strongly and then decreases in smaller steps in the following weeks. At 10°C only a very slight denitrification was observed in the first week, and a much greater degradation in the following two weeks. Afterward, NO_3^- concentration is stable, indicating the termination of denitrification. Addition of 10 mmol reduces the concentration at room and groundwater temperature by 201 mg/L each. Consequently, there is no difference in the amount of removed NO_3^- when large quantities are added. When acetate is added in lower concentrations, a greater NO_3^- degradation occurs at room temperature.

Nitrite (approx. 3 mg/L) is formed mainly at low electron donor concentrations. When acetate is present in higher concentrations, nitrite is directly degraded (probably to $N_{2(g)}$) and is formed only in low concentrations (1.97 mg/L) at 10°C.

At least 98% of the dissolved acetate is consumed in all addition steps at room temperature after one week, whereas it is still present between 17% and 27% at 10°C. However, denitrification continues at room temperature for a considerably longer period of time (up to 6 weeks) with lower decreases in NO_3^- concentration. Denitrification seems to be completed at 10°C when no more acetate was detected in solution (Figure 2a).

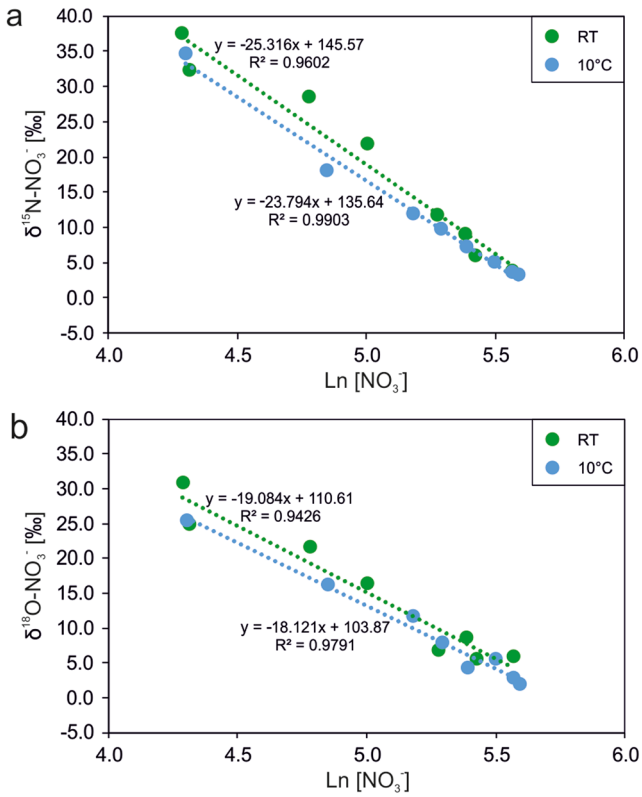


Figure 3. Isotopic results of initiated denitrification by acetate: (a) δ^{15}N-NO_3^-, (b) δ^{18}O-NO_3^-, room temperature (RT): green, 10°C: blue.

Results of isotope analyses show a linear trend between both δ^{15}N-NO_3^- and δ^{18}O-NO_3^- and the natural logarithm of the remaining NO_3^- concentration (Figure 3) which is typical for a biological NO_3^- reduction (Margalef-Marti et al., 2019). Less enriched signatures of δ^{15}N-NO_3^- and δ^{18}O-NO_3^- are observed at 10°C, due to the temperature-dependent degradation differences. Calculated $\varepsilon^{15}N/\varepsilon^{18}O$ ratios are >1, except for the addition of 2.5 mmol acetates at 10°C (Table 1).

The pH is slightly acidic (4.1–6.0) as there is no buffer capacity in the sediment (C_{inorg} < 0.01 wt%). Eh fluctuates between +614 and −523 mV, indicating that the additions of electron donors promote the necessary reducing conditions for denitrification.

Addition of glucose (Figure 2b) reduces NO_3^- concentrations in the individual addition steps at both room- and groundwater temperature, but more intense with larger addition (97.3, 118, 199 mg/L). At 10°C, NO_3^- concentration increases slightly at first and denitrification requires much more time. Nitrite is only formed at room temperature when 2.5 mmol glucose is added (max. 9.6 mg/L) and is not completely degraded by the end of the denitrification step whereas this degradation occurs directly when 5 mmol is added. At 10°C, nitrite is formed in small peaks of max. 1.5 mg/L by addition of 2.5 mmol. However, 5 mmol glucose forms up to 23 mg/L and 10 mmol max. 17 mg/L NO_2^-. By the end of the denitrification steps, NO_2^- is completely degraded. Consequently, the same amount of NO_3^- is reduced in total, but the reaction proceeds much slower at 10°C. A detailed interpretation of the reaction rate is therefore performed in the next section (Figure 5). Addition of 10 mmol glucose at room temperature causes NO_3^- concentration to decrease from 200 mg/L to below the detection limit (0.5 mg/L) in only 6 days and the formation of H_2S was observed. Thus, addition of 10 mmol glucose at room temperature leads to sulfate reducing conditions.

Addition of 2.5 mmol ascorbic acid (Figure 2c) decreases the NO_3^- concentration very slightly at room temperature (7.1 mg/L) and at 10°C (12.9 mg/L). However, no NO_2^- is formed. When 5 mmol is added, the temperature dependence of denitrification rates becomes more obvious: concentrations are reduced by 84.7 mg/L at room temperature and by 32.5 mg/L at 10°C. Reaction time of NO_3^- degradation is the same for this addition step. At room temperature, the reaction is faster when adding 10 mmol: all of the present NO_3^- (156.7 mg/L) was reduced after four weeks. Nitrate (98.5 mg/L) reduction at 10°C takes much longer (10 weeks). Nitrite is formed in the addition steps 5 and 10 mmol in both temperature approaches. At room temperature, a maximum of 2.6 mg/L NO_2^- is released in both addition steps, but then immediately degraded. At 10°C, 3.7 mg/L NO_2^- is formed by adding 5 mmol ascorbic acid, which is completely reduced after adding 10 mmol. After four weeks, an accumulation of 6.7 mg/L NO_2^- was observed. Addition of ascorbic acid produces a lot of biomass, especially at 10°C, resulting in repeated clogging of the tubes with slimy biomass in the experimental setup.

Table 1
Calculated $\varepsilon^{15}N/\varepsilon^{18}O$ Ratios Classified by Acetate Concentration and Temperature

	Acetate concentration (mmol)	$\varepsilon^{15}N/\varepsilon^{18}O$
Room temp.	2.5	1.92
	5	1.49
	10	1.06
10°C	2.5	0.49
	5	1.09
	10	1.40

Results for the electron donor ethanol differ from the findings described above. When adding 2.5 mmol ethanol (Figure 2d), NO_3^- concentrations are reduced by 42.8 mg/L at room temperature and by 49.4 mg/L at 10°C. Nitrite (max. 6.7 mg/L) is formed at 10°C when NO_3^- concentration no longer decreases. With 5 mmol, temperature-dependent differences in denitrification become even clearer: NO_3^- concentration decreases by 93.0 and by 163 mg/L at room temperature and 10°C, respectively. Moreover, denitrification lasts considerably longer (3 weeks more) at 10°C as compared to room temperature. Nitrite, generated in the addition step before, drops at first, but then rises back to 3.8 mg/L. The more efficient NO_3^- degradation at 10°C is no longer visible with 10 mmol ethanol

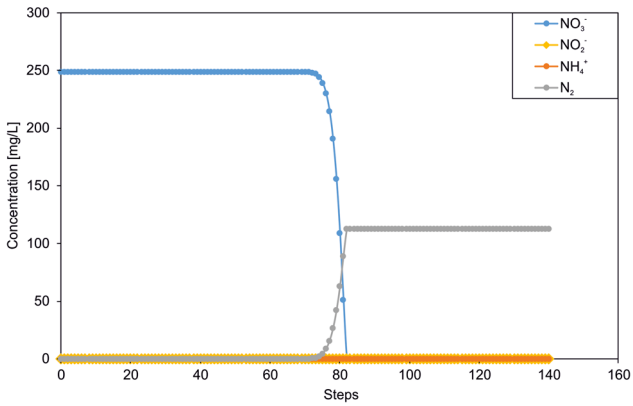


Figure 4. Transport modeling with development of NO_3^-, NO_2^-, NH_4^+, and N_2.

addition. Overall, the concentration is reduced below the detection limit (0.5 mg/L) at both temperatures. Nevertheless, it is noticeable that the larger the amount of added ethanol, the stronger the denitrification takes place within the first week at room temperature. At 10°C, only a small amount of NO_3^- is degraded during this time span when 10 mmol ethanol is added (only 2.5% of degradation at room temperature).

The applied PHREEQC 1-D transport model, in which organic C is added for NO_3^- degradation, shows a decrease of the NO_3^- concentration with all four applied C. Figure 4 shows the concentration development of this transport modeling. Simultaneously with the degradation of NO_3^-, N_2 gas is formed which is dissolved in sample water. Nitrogen gas is formed up to a maximum of 112.5 mg/L and then remains constant. Compared to the circulating column experiments, degassing of N_2 after flowing through the sediment in the water reservoir can be assumed and the N_2 concentration in water is therefore lower. The transport modeling shows a very low accumulation of NO_2^- and additionally that no NH_4^+ is formed.

3.2. Reaction Rate

The reaction rate of denitrification induced by the four C sources acetate, glucose, ascorbic acid, and ethanol in three addition steps (2.5, 5, and 10 mmol) is shown in Figure 5.

Acetate (Figure 5a) indicates a low reaction rate in the first addition step at both temperatures (0.02 and 0.01 mmol L^{-1} d^{-1} at room temperature and 10°C, respectively). When adding 5 mmol, this rate increases considerably and differs by 31% between room- and groundwater temperature. Reaction with 10 mmol further increases the denitrification rate to similar values (i.e., 0.1 and 0.09 mmol L^{-1} d^{-1}) despite the temperature difference.

When glucose is added (Figure 5b), the highest reaction rate is achieved at room temperature among all four C sources. The reaction rate with glucose is 5 (5 mmol) to 7 (10 mmol) times higher at room temperature than at 10°C. At groundwater temperature, rates are very similar to those of acetate.

Lowest reaction rates were obtained with ascorbic acid (Figure 5c) at both tested temperatures. When adding 2.5 mmol at room temperature, its reaction rate of <0.01 mmol L^{-1} d^{-1} is 5.6 times smaller than that of acetate. In higher addition steps, reaction rates successively approach those of acetate (factor 0.6 at 5 mmol, negligible difference at 10 mmol). At 10°C, rates increase proportionally with increasing ascorbic acid addition, but are lower than all other observed rates (max. 0.02 mmol L^{-1} d^{-1} at 10 mmol).

In accordance to the observations on NO_3^- concentration, the reaction rate shows a more efficient NO_3^- degradation with ethanol (Figure 5d) at groundwater temperature compared to the other C compounds. Nevertheless, the reaction rate at room temperature is also higher than those of acetate and ascorbic acid, but is still surpassed by those of glucose (2.5 times for 5 mmol and 3 times for 10 mmol). Reaction rates increase proportionally with increasing amount of ethanol added at room temperature. However, highest reaction rates are achieved at 10°C (0.17 mmol L^{-1} d^{-1}) with an addition of 5 mmol ethanol (threefold higher than acetate and glucose). Only with the largest addition step (10 mmol), the reaction rate at room temperature is higher (at 10°C even lower) than with 5 mmol.

Comparing all four C sources, reaction rates with a doubled amount of electron donor do not increase proportionally, but depending on the C source. Only ascorbic acid at 10°C and ethanol at room temperature increase proportionally with injection quantity. For almost, all C compounds and addition steps, reaction rate increases with input amount. Only when adding 10 mmol ethanol, the reaction rate is lower than in the previous addition step.

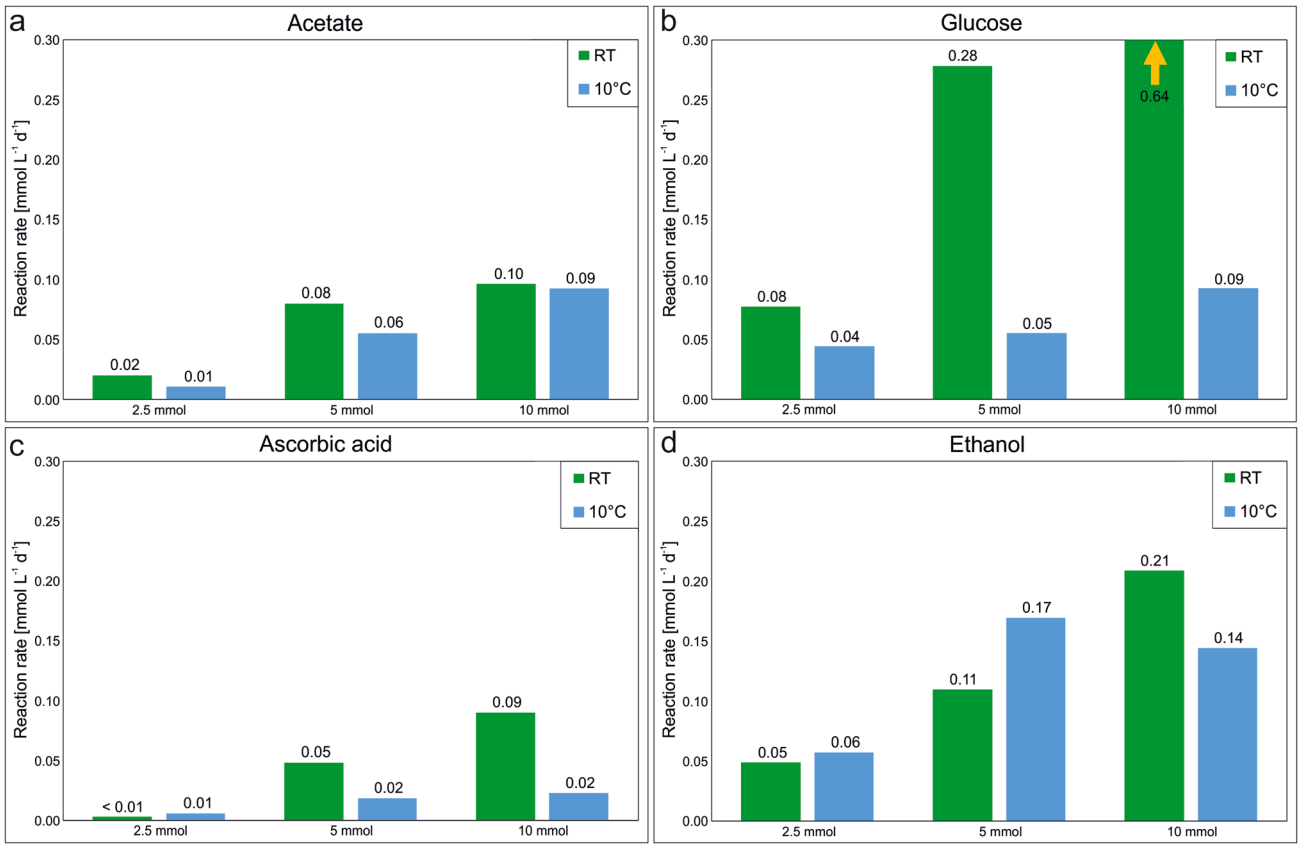


Figure 5. Reaction rate of initiated denitrification by (a) Acetate, (b) Glucose, (c) Ascorbic acid, and (d) Ethanol, injection of electron donor in 3 steps (2.5 mmol, 5, and 10 mmol), room temperature (RT): green, 10°C: blue.

3.3. Stoichiometrically Degraded Percentages

Since reaction rates do not increase proportionally to the added amount, Figure 6 shows how much NO_3^- is stoichiometrically degraded and how much could potentially be degraded. The following simplified reaction for heterotrophic denitrification considers the electron donor with one C atom (Equation 1) with an oxidation state of zero. Consequently, the number of C atoms of the reducing substances is included in the calculation:

$$5\ CH_2O + 4\ NO_3^- \rightarrow 2\ N_2 + 4\ HCO_3^- + CO_2 + 3\ H_2O \quad (1)$$

For most reactions, it becomes obvious that the stoichiometric reduction potential is not fully used. In the following, the utilization of the stoichiometric reduction potential is described as efficiency. At room temperature with 2.5 and 5 mmol acetate (Figure 6a), only about 60% of the stoichiometrically possible NO_3^- is degraded, decreasing to 45% for 10 mmol. A different observation is made at 10°C: while 2.5 mmol acetate only degraded 31% NO_3^-, this value increases to 38% (5 mmol) and 46% (10 mmol).

When adding glucose (Figure 6b), the stoichiometrically degraded NO_3^- concentration decreases from 40 to approx. 20% at room- and groundwater temperature. Degradation at 10°C with 2.5 and 5 mmol is below that at room temperature. Thus, glucose causes a less efficient NO_3^- degradation as compared to acetate.

The by far lowest efficiency is achieved with ascorbic acid (Figure 6c). At room temperature, the stoichiometric degradation percentage is between 3% and 19%, increasing with higher addition. At 10°C, NO_3^- degradation in all addition steps is below 10% of the stoichiometrically possible total degradation. In the last addition step, a slight increase of the degraded NO_3^- portion is observed.

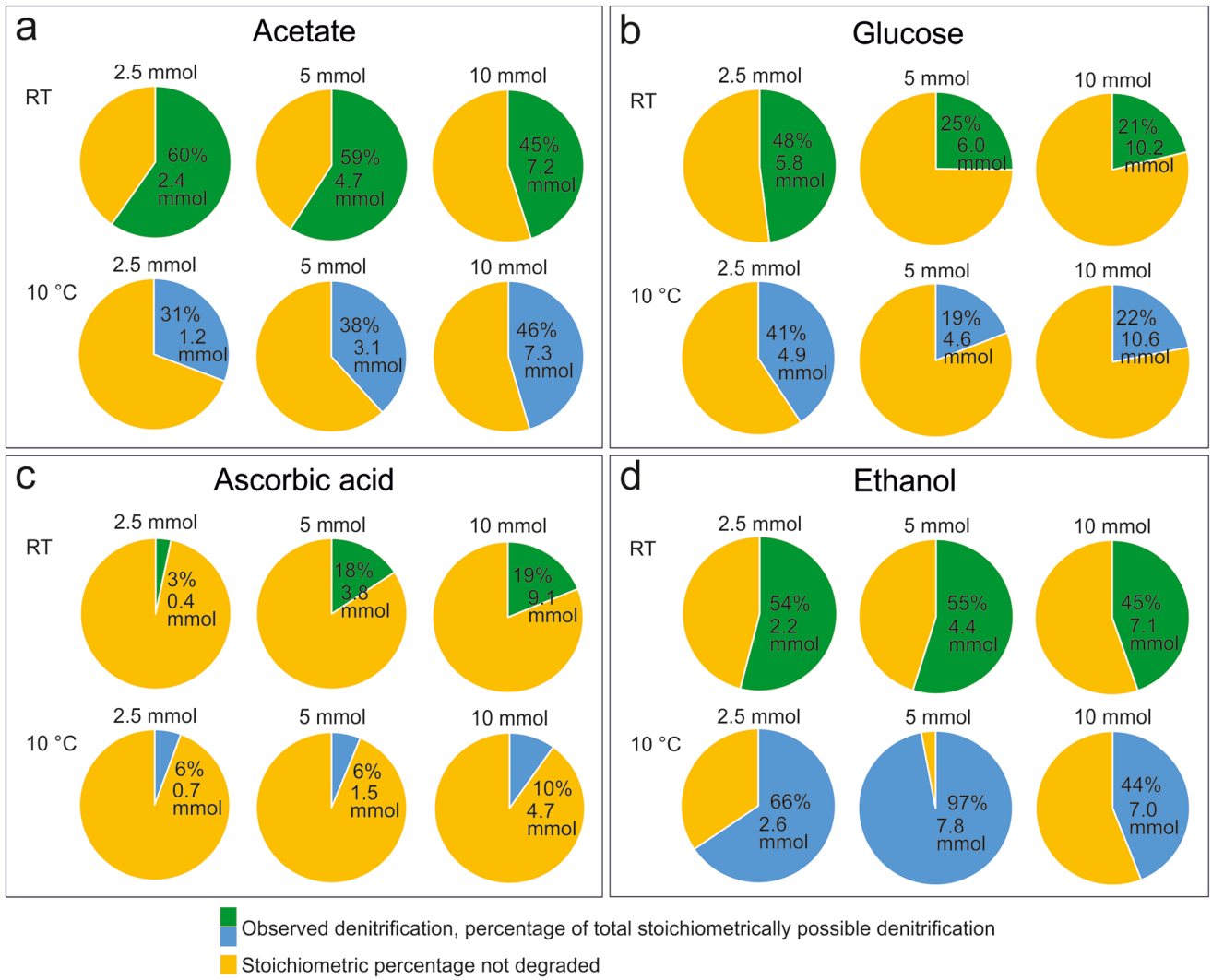


Figure 6. Stoichiometric degradation of initiated denitrification by (a) Acetate, (b) Glucose, (c) Ascorbic acid, and (d) Ethanol, injection of electron donor in 3 steps (2.5 mmol, 5, and 10 mmol), observed denitrification of total possible stoichiometric degradation at room temperature (RT): green, at 10°C: blue.

Ethanol, on the other hand, induces the most efficient NO_3^- degradation (Figure 6d). While at room temperature, the stoichiometrically degraded NO_3^- fraction is just 55% (2.5 and 5 mmol) and 45% (10 mmol), a larger fraction is actually degraded at 10°C: 66% (2.5 mmol) and 97% with 5 mmol. However, when 10 mmol is added, efficiency decreases again to 44%.

The stoichiometrically degraded NO_3^- contents differ between all reducing substances, and for the three concentrations of added electron donor. Concentration also has an important role in reaction efficiency. Furthermore, stoichiometric proportions differ considerably between room- and groundwater temperature. Ascorbic acid causes inefficient NO_3^- degradation, while with ethanol, especially at 10°C, an efficient NO_3^- degradation is induced.

3.4. Microbiology

The 16S amplicon sequencing yielded 587,377 quality-filtered reads, which were further clustered into 37 bacterial OTUs. Taxonomic assignment revealed eight bacterial phyla and one unidentified phylum present in the experiments (Figure 7). Water samples without addition of organic C (control) were dominated by Actinobacteriota and Proteobacteria in both temperature approaches. However, the microbial community structure was strongly influenced by temperature and organic C. For instance, samples supplemented with

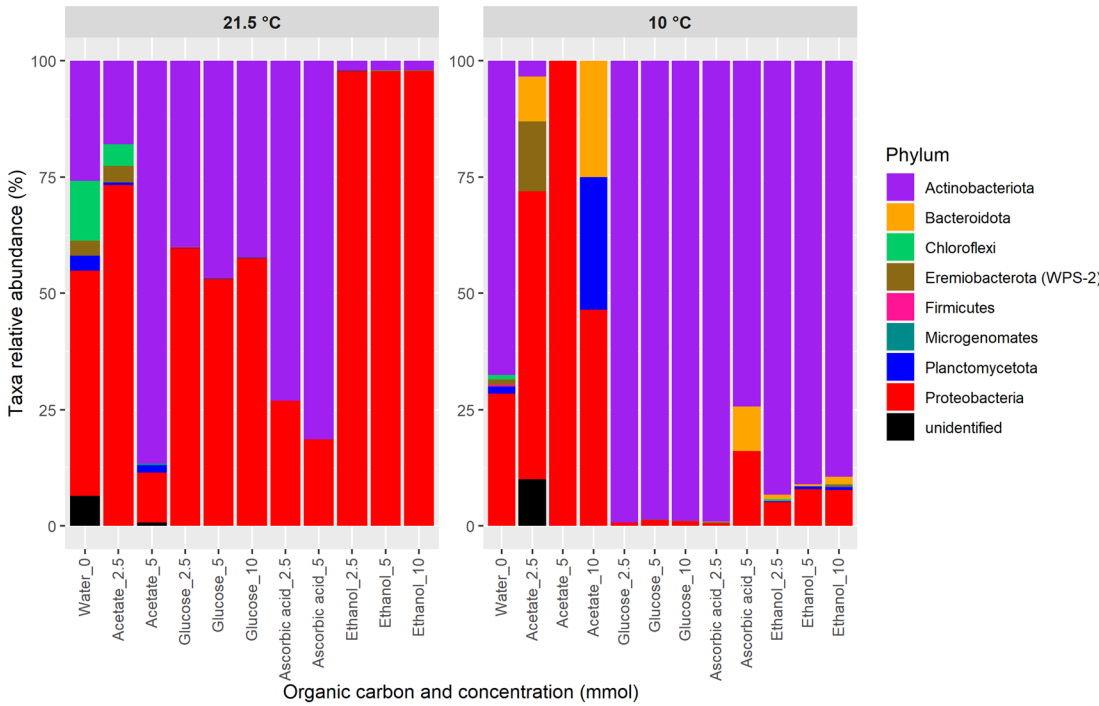


Figure 7. Relative abundances of bacterial phyla in samples containing different organic C additions (concentration steps 2.5, 5, and 10 mmol), incubated at room temperature (21.5°C) or at 10°C, and a control sample (water) without C_{org} addition.

glucose and incubated at room temperature (21.5°C) were also dominated by Proteobacteria (53%–60% relative abundance) and Actinobacteria (40%–47%). However, when incubated at 10°C, Actinobacteria dominated (99%) the communities. A similar pattern was detected for samples containing ethanol, where the communities were dominated by Proteobacteria at room temperature (98%), but by Actinobacteriota at 10°C (89%–93%). The opposite pattern was observed with acetate addition, where Actinobacteriota dominated at room temperature (87%), and Proteobacteria at 10°C (100%). Furthermore, less abundant phyla such as Chloroflexi were mainly detected at room temperature, while Bacteroidota, Planctomycetota and Eremiobacteriota were mainly detected at 10°C.

At the same temperature, the addition of organic C appears to be the decisive parameter influencing the community structure, while the effects of concentration were minor for all C, except for acetate. At room temperature, Proteobacteria dominated samples containing glucose (53%–60%) and ethanol (98%), while Actinobacteriota were dominant in ascorbic acid (73%–81%). At groundwater temperature, Actinobacteriota dominated overall in glucose, ascorbic acid and ethanol (89%–99%). Concentration effects were negligible for these organic C, however, acetate concentration revealed to be responsible for major community shifts. At room temperature, bacteria communities are dominated by Proteobacteria (73%) on 2.5 mmol acetate and by Actinobacteriota (87%) on 5 mmol acetate. At 10°C, Proteobacteria dominate at 2.5 (62%), 5 (100%), and 10 (46%) mmol acetate.

4. Discussion

For the remediation of groundwater polluted with NO_3^-, all four electron donors used in this study (acetate, glucose, ascorbic acid, and ethanol) may generally be used, since NO_3^- degradation is induced with all tested substances. Results of isotope analysis after acetate addition underline NO_3^- reduction at room- and groundwater temperature. Less enriched signatures of $\delta^{15}N\text{-}NO_3^-$ and $\delta^{18}O\text{-}NO_3^-$ at 10°C, emphasize the differences in denitrification for both temperatures and illustrate that less denitrification occurs with acetate at 10°C. Furthermore, $\varepsilon^{15}N/\varepsilon^{18}O$ values from 1.06 to 1.92 are in agreement with published data for induced biodenitrification, where in laboratory experiments values close to 1 (Carrey et al., 2013; Margalef-Marti et al., 2019) and on the field-scale values close to 2 are obtained (Critchley et al., 2014; Otero

et al., 2009). Only the reaction with 2.5 mmol acetate at 10°C produced $\varepsilon^{15}N/\varepsilon^{18}O$ <1, since only a small amount of NO_3^- was degraded.

Despite successfully initiated denitrification, results show different NO_3^- degradation efficiencies of the C compounds. At first glance, glucose seems to be the best choice for enhanced NO_3^- degradation. With its addition, by far the highest reaction rates observed in this study are achieved at room temperature. Other investigations also describe successful NO_3^- degradation by glucose at room temperatures (Akunna et al., 1993; Carrey et al., 2014; Ge et al., 2012). Nevertheless, this study suggests that the consideration of groundwater temperature is an important factor. At 10°C, reaction rates of glucose differ from those at room temperature and are orders of magnitudes lower. Ethanol, on the other hand, not only shows the best reaction rates at 10°C, but is also by far the most effective additive for reducing NO_3^- concentrations. The stoichiometrically degraded NO_3^- proportion with ethanol at 10°C is up to 42.1% higher than at room temperature. Martin et al. (2009) also found that NO_3^- concentrations of up to 200 mg/L were degraded at 6°C by ethanol. Several studies investigated the effect of temperature on biological denitrification; they often observe a degradation rate increase with increasing temperature (e.g., Dawson & Murphy, 1972; Elefsiniotis & Li, 2006; Hoover et al., 2016). However, results of the present study show that denitrification rates can even be higher at lower temperatures, depending on the C source. Consequently, reaction kinetics does not always increase with rising temperature. The study of Comer-Warner et al. (2018) underlines this interpretation, because here also the temperature sensitivity depends on other factors like substrate (grain size), organic matter content, and geological origin. Considering the complexity of potentially involved parameters, a linear relationship between decreasing or increasing temperature with the NO_3^- reduction potential appears unlikely. Rather, we assume different denitrification potentials for different organic substrates and temperatures.

Greskowiak et al. (2017) compare biodegradation rate constants of 82 different organic compounds. They report that in some studies, temperature was not specified and experiments were conducted at room temperature. The present study indicates that biodegradation rate constants of various organic compounds may also change considerably if groundwater temperature is taken into account.

Furthermore, this investigation shows proportional increases in denitrification rates only for ascorbic acid at 10°C and ethanol at room temperature. In most experiments, however, reaction rates are not doubled after double electron donor concentration. Nevertheless, denitrification rates generally increase with increasing addition, except for 10 mmol ethanol.

Stoichiometrically degraded NO_3^- percentages also differ in the experiments. Comparing the three concentration steps of the respective electron donors, it becomes clear that with increasing C addition, the stoichiometrically equal amount of NO_3^- is not quantitatively degraded. Degraded proportions increase or decrease with increasing injection quantity and also differ between room- and groundwater temperature. Thus, the same percentage of NO_3^- is not necessarily degraded with a given organic C in different concentrations. Consequently, electron donor concentration is also important for the efficiency of a NO_3^- degrading substance. However, if NO_3^- concentration decreases below the analytical detection limit, the interpretation of electron donor efficiency becomes imprecise. It is assumed that no more efficient NO_3^- degradation would have occurred, since H_2S formation and sulfate reduction were only observed after addition of 10 mmol glucose at room temperature. Carrey et al. (2014) also observed conditions under which excess glucose led to sulfate reduction. As described above, not only the correct electron donor concentration should be chosen to achieve an optimal NO_3^- degradation, but also excess C should be avoided to minimize sulfate reduction.

Beside denitrification rate, temperature also has an influence on the formation of NO_2^-, which is more toxic (De Beer et al., 1997) than NO_3^-. Nitrite occurs considerably more frequently at 10°C and is present in higher concentrations (cf. Figure 2). Carrey et al. (2014) describe NO_2^- formation at room temperature using glucose but in the present study, no NO_2^- is formed at room temperature with higher glucose concentrations (5 and 10 mmol). At 10°C, the maximum concentration of the entire study is 23 mg/L. When ethanol is added, NO_2^- is formed mainly at 10°C, whereby concentrations are considerably lower. With acetate and ascorbic acid, NO_2^- occurs at room and groundwater temperature. The transport modeling suggests a much lower accumulation of NO_2^- than the results in the laboratory experiments actually show. Consequently, this cannot yet be completely correctly represented in the model by the temperature influence. Nitrite

accumulation can be related to temperature but also to pH, which is in a slightly acidic range (4.1–6.0) in all test series. Carrey et al. (2014) also indicate a possible reason for NO_2^- accumulation in pH conditions. In their study, pH was in a slightly basic range (8.4–8.7). Other studies indicate that the pH optimum for denitrification was between 7.6 and 8.6 (Karanasios et al., 2010; Lee & Rittmann, 2003) and that NO_3^- degradation was considerably inhibited already at pH 6.5–7.0 (Glass & Silverstein, 1998). It can therefore be assumed that NO_3^- reduction at 10°C is also inhibited by the slightly acidic pH value described above. It also cannot be ruled out that NO_2^- accumulation may be favored by pH conditions outside the mentioned optimum.

Besides an undesired accumulation of NO_2^-, experiments with ascorbic acid addition showed a high production of biomass, which repeatedly led to clogging in the experimental setup. This is associated with low reaction rates and very ineffective denitrification. Consequently, ascorbic acid is not a good choice for treatment of groundwater with high NO_3^- concentrations, as clogging in wells or in aquifer pores may occur. Biomass formation resulting in clogging was also observed in the evaluation of denitrification potential of wine production wastes (Carrey et al., 2018) and whey (Margalef-Marti et al., 2019).

Differences in biomass production among the four organic C can be explained, in part, by characterizing microbial communities influenced by the choice of electron donors (cf. Figure 7). Actinobacteriota and Proteobacteria were dominant in the experiments, however, occurring bacteria differ in type and relative abundance between the C compounds. Our results are supported by previous studies (Chen et al., 2018; Qin et al., 2017), which detected similar communities with the addition of organic C compounds for NO_3^- removal. Furthermore, our study highlights temperature-dependent differences in denitrification rates. This is supported by our microbiological characterization approach since incubation temperature has a striking impact on microbial community composition and structure (cf. Figure 7). The more effective NO_3^- degradation with ethanol at 10°C may be explained by shifts in the microbial community. While our data are only correlative, the causal link remains to be shown. Either members of Actinobacteriota are more tolerant against ethanol than Proteobacteria or their overall efficiency in denitrification is higher at low temperature.

All results combined, ethanol appears to be the clearly most suitable electron donor among the organic substances investigated in this study for induced biodenitrification in groundwater with a temperature of 10°C. Besides the highest reaction rate, ethanol shows the highest denitrification efficiency. Furthermore, clogging due to excessive production of biomass does not occur. Nevertheless, the transferability of the results to the field scale should be discussed. The circulation column experiments do not represent an infinitely extended aquifer. The determined reduction potential is therefore only valid for the location where the selected organic C was introduced and not, as is often the case, for large parts of an aquifer on average. The column experiment can therefore be compared with a redox boundary in an aquifer, at the location where NO_3^- gets in contact with the geogenic reduction potential of the aquifer. Depending on the extent of the aquifer and NO_3^- contamination, organic C could be injected at several locations in the aquifer.

5. Conclusions

For a better understanding of biological groundwater denitrification enhanced by organic C, aquifer conditions have to be simulated in experiments to enable transfer of results to in situ conditions. In this process, temperature is a crucial factor. The present study demonstrates, depending on the electron donor, different NO_3^- degradation when considering groundwater temperature.

Results show a better NO_3^- degradation by ethanol at groundwater temperatures (10°C) than at room temperature. Comparing the four C compounds tested here, ethanol is by far the most effective electron donor at 10°C. This indicates that reaction kinetics does not necessarily increase with rising temperature, and that the temperature optimum for denitrification by ethanol appears to be rather in the range of 10°C than at room temperature. The added electron donor quantity must also be taken into account for efficiency, since excess C can lead to lower denitrification rates or even to sulfate reduction. Based on this study, it can be assumed that each reducing substance has its own temperature optimum for biodenitrification, whereby some will probably be able to achieve good NO_3^- degradation at room temperatures and others at lower temperatures. This optimum depends on the bacterial community catalyzing biodenitrification whose composition in turn is determined by the choice of electron donor.

Selection of the most suitable electron donor for in situ or ex situ groundwater remediation therefore also depends on an affected area's location and climate which define groundwater temperature. Ethanol seems more suitable than glucose for regions with lower temperature while glucose may be more effective in warmer regions.

Climate change is expected to cause rising temperatures in many regions, which will inevitably lead to rising groundwater temperatures. This development can lead to a dynamic change in the most suitable electron donors for biological denitrification, and should be taken into account in future groundwater remediation efforts.

Data Availability Statement

Data sets for this research are included in this article and data sets for microbiology are available from the European Nucleotide Archive (http://www.ebi.ac.uk/ena/) under study PRJEB42532.

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