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1 Identification of the Temporal Control on Nitrate Removal Rate

2 Variability in a Denitrifying Woodchip Bioreactor

3
4 Albin Nordström^{a*}, Roger B. Herbert^a

5
6 *^aUppsala University, Department of Earth Sciences, Villavägen 16, SE-752 36, Uppsala,*
7 *Sweden*

8 9 Contact information (Albin Nordström)

10 *Corresponding author

11 E-mail address: albin.nordstrom@geo.uu.se

12 Telephone number: +46 18 471 22 72

13 ORCID: 0000-0002-0311-8368

14 Contact information (Roger B. Herbert)

15 E-mail address: roger.herbert@geo.uu.se

16 Telephone number: +46 18 471 22 66

17 ORCID: 0000-0002-7561-757X

18
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Identification of the Temporal Control on Nitrate Removal Rate Variability in a Denitrifying Woodchip Bioreactor

Nitrate (NO_3^-) removal rates in a denitrifying woodchip bioreactor (DWB) removing NO_3^- from mine water in a subarctic climate was modeled with the purpose of determining the processes controlling variability in NO_3^- removal rates over time. The Eyring equation was used to define the temperature dependency, while a rate law was used to describe the NO_3^- concentration dependency of the NO_3^- removal rates. The results show that the temperature and NO_3^- concentration dependency of the NO_3^- removal rates changes over time in the DWB due to the preferential selection of conceptualized NO_3^- - reducing bacteria favoring low temperatures. It is suggested that the selection of the low temperature NO_3^- reducers in the DWB represented an increased dependence on cross-feeding between a fermentative community, producing the reactive organic carbon substrate, and a denitrifying community, consuming the organic carbon substrate, with the temporal variability in NO_3^- removal rates being controlled by the stabilization of the microbial community structure. It is also suggested that the life expectancy of DWBs is more related to the stability of the cross-feeding between the fermenting microbial community and the denitrifying microbial community, than to the total carbon content.

Keywords: woodchip bioreactor, modeling, macromolecular rate theory, selection, temperature, longevity

1. Introduction

Denitrifying fixed-bed bioreactors are techniques for the removal of NO_3^- from water (Christianson and Schipper, 2016; Schipper et al. 2010). NO_3^- rich water is passed through a carbon-rich porous matrix which supplies the electron donors required for (heterotrophic) denitrification, defined as the sequential reduction of NO_3^- to nitrogen gas (N_2) (net denitrification shown in reaction 1).

There has been a growing interest in the denitrifying fixed-bed technique in recent years due to its relatively low construction and operational costs (Schipper et al. 2010), which arise from the use of comparatively inexpensive lignocellulosic material (wood-byproducts) as the carbon source. Woodchips as a carbon source have been increasingly preferred due to the relatively high permeabilities that may be attained with the material and for providing sustained NO_3^- removal rates up to decades following installation (Robertson, 2010). However, the processes and underlying parameters that control the absolute NO_3^- removal rates attained in these systems and how these change over time, which control the dimensioning (Moorman et al. 2015) and longevity of the DWB, are still elusive and require additional exploration.

It is generally recognized that temperature controls a substantial part of the variability in NO_3^- removal rates in DWBs (Addy et al. 2016; Schmidt and Clark, 2013), with other significant factors being NO_3^- concentrations, organic carbon availability, and DWB age (Addy et al. 2016). Indeed, NO_3^- removal rates have been observed to be proportional to labile organic carbon concentrations (typically indicated as dissolved organic carbon, DOC) (cf. Hassanpour et al. 2017) and to be significantly higher during a period directly after the installation of the DWB when the level of labile organic carbon is greatest (Addy et al. 2016;

Robertson, 2010; Schipper et al. 2010). During this initial period, DWB operation is associated with a high export of organic carbon from the system (Hassanpour et al. 2017; Hoover et al. 2016; Schipper et al. 2010), and NO_3^- removal rates may be relatively independent of temperature (Hassanpour et al. 2017).

While DWBs consist primarily of a cellulose and lignin-rich substrate, and lignin is a relatively recalcitrant organic compound, hydrolysis and fermentation processes in DWBs will produce low molecular weight (labile) compounds such as sugars and volatile fatty acids. As such, the organic substrate in a DWB will consist of a continuum of organic compounds with different reactivities (cf. Boudreau and Ruddick, 1991). We hypothesize therefore that the general reduction in NO_3^- removal rates with time in DWBs is due to a greater amount of labile organic carbon (easily mineralized, ‘high quality’) during the initial stages of DWB operation, with the organic carbon becoming increasingly recalcitrant (less available, ‘low quality’) over time. In accordance with Bosatta and Ågren (1999), we define carbon ‘quality’ as ‘the number of enzymatic steps required to release as carbon dioxide a carbon atom from an organic compound’ where low quality carbon is characterized by a larger number of steps required for the oxidation of organic carbon to inorganic CO_2 (cf. Bosatta and Ågren, 1999). Using this definition, a decrease in the carbon quality is synonymous with an increased biogeochemical cycling of organic carbon prior to its final oxidation to inorganic carbon (H_2CO_3^* , HCO_3^-) by terminal electron accepting processes (TEAPs), such as denitrification (reaction 1). This definition emphasizes the processes consuming, rather than the chemical properties of, the organic carbon, wherefore any changes in the carbon quality over time is reflected in the structure and functioning of the microbial processes in the DWB. Indeed, the type of organic carbon and the microbial structure in DWBs are related (Porter et al. 2015).

The objective of this study was to explore the relationship between NO_3^- removal rates and carbon quality in a DWB. However, carbon quality is a rather ambiguous term. Instead of

quantifying this parameter, this study seeks to infer the role of carbon quality from changes in the temperature dependence of NO_3^- removal rates through the perspective of an adjustment in the microbial community structure to environmental conditions; i.e. we believe that temporal changes in the NO_3^- removal rates are the result of changes in the composition of the NO_3^- reducing microbial community. If there was a decrease in the carbon ‘quality’ over time in the DWB, this would impact enzymatic processes feeding on varying ‘qualities’ of organic carbon (Sierra, 2012), which in turn would manifest itself in temporal variations in the temperature-dependent NO_3^- removal rates. The results of this study will add to our understanding of the processes that control both short-term and long-term NO_3^- removal in DWBs.

2. Materials and methods

2.1 Study site and DWB system

The data used in this study was collected from a previously described DWB (cf. Nordström and Herbert, 2018) installed for the removal of NO_3^- in mine drainage from the Kiruna iron ore mine, northern Sweden. This section briefly summarizes relevant aspects of the DWB studied; the reader is referred to the original publication by Nordström and Herbert (2018) for additional detail.

The Kiruna iron ore mine is operated by the mining company Luossavaara-Kiirunavaara Aktiebolag (LKAB) and located in Kiruna, northern Sweden (67°51’N 20°13’E) where the mean annual air temperature was -3°C for the period 1961-1990 (SMHI, 2017). Mine and process water with elevated NO_3^- concentrations, originating from the use of ammonium nitrate-based explosives, is discharged through a tailings impoundment-clarification pond system and eventually released into the Rakkuri river system (LKAB, 2016).

The DWB was constructed as a subsurface system close to the outlet of the clarification

pond at the mine site during May - June 2015, and was filled with a mixture of decorticated pine woodchips and digested sewage sludge (Figure S.1 in supporting information (SI) summarizes details on the design). The woodchips (porosity 0.54, laboratory estimate) were bought from a local supplier while the digested sewage sludge was shipped from Luleå, northern Sweden. Water from the clarification pond, with an average NO_3^- concentration of 1.57 mmol L^{-1} , was delivered to the DWB using a submersible pump, and the hydraulic residence time (HRT) of water in the DWB was controlled by changing the pump discharge.

The DWB was operated for two consecutive field seasons, referred to as the first and second operational years. The first operational year extended from 22 June to 20 November 2015, and the second operational year from the 9 May 2016 to 21 October 2016. During the operational years, the daily mean DWB temperature varied between 0.8 and 17°C and was calculated from 16 spatially distributed temperature probes (T107, Campbell Scientific®) embedded within the woodchip - sewage sludge mixture. The HRT in the DWB was in the range 0.8-2.1 days (SI, Table S.1). There was no flow through the DWB between the two operational years. The calculated HRTs were corrected for the dual-porosity nature of the woodchip-sewage sludge media (cf. Nordström and Herbert, 2017a; SI, section S.1).

2.2 Sampling and analysis

The DWB featured 20 sampling points including inlet, outlet, and 18 polyvinyl chloride pipes for pore water sampling. The sampling points were spatially distributed at various depths in the DWB and at regular intervals between the inlet and outlet. Pore water profiles obtained on days 22, 57, 85, 113, 330, 365, 400, 428, 456, and 477 of DWB operation were used in the study. Samples were analyzed by LKAB's accredited laboratory services following standardized procedures.

2.3 Data analysis

2.3.1 Determination of nitrate removal rates

NO_3^- concentrations from the influent, effluent, and five pore water sampling points located along the bottom of the DWB (SI, Figure S.1a) were used to determine NO_3^- removal rates. These sampling points were assumed to be located along a theoretical flow line along the bottom of the DWB, such that each consecutive observation between the inlet and outlet represented the change in NO_3^- concentration with time. NO_3^- removal rates were calculated as the difference in NO_3^- concentration between each consecutive point along the theoretical flow line, divided by the time elapsed for transport between each observation. Since the cross-sectional area of DWB was not constant along the flow path, advection velocity varied in the direction of flow. This in turn affected the time elapsed between different sampling points used for calculating the NO_3^- removal rates (SI, section S.2). If there was no change in NO_3^- concentration between two consecutive sampling points, or if there was an observed increase in NO_3^- concentration, the NO_3^- removal rate was not considered for this interval.

2.3.2 Modeling procedure

This study uses two related kinetic expressions for investigating denitrification occurring in the DWB, an n^{th} -order rate law and the Monod equation. As a first step, the n^{th} -order rate law is fitted to rate and concentration data to obtain general reaction orders and Gibbs free energies of activation ($\Delta G^\ddagger$, the minimum energy required for reaction, see below). The Monod equation is subsequently fit to rate and concentration data to obtain values of the half-saturation index, which is used as an indicator of substrate (NO_3^-) limitation. $\Delta G^\ddagger$ is then independently calculated for each obtained NO_3^- concentration profile and ‘macromolecular rate theory’ (MMRT) is used to investigate the relation between reaction rate and temperature for enzymatic processes. Finally, the relative temperature sensitivity of the reaction rate is

calculated and used as a proxy for the ‘quality’ of the organic carbon substrate.

The n^{th} -order rate law is expressed as follows:

$$v = k[NO_3^-]^n \quad (2)$$

where v is the field-based NO_3^- removal rate, $[NO_3^-]$ is nitrate concentration, and n is the rate order, commonly equal to 0 or 1 for NO_3^- removal rates in DWBs (Halaburka et al. 2017; Jaynes et al. 2016; Robertson, 2010). k is the temperature dependent rate constant of the reaction rate, here expressed accordingly to the Eyring equation (Garcia-Viloca et al. 2004):

$$k = \gamma(T)c_0^{1-n} \frac{k_b T}{h} e^{\frac{-\Delta G^\ddagger}{RT}} \quad (3)$$

where k_b , h , R , and c_0 are Boltzmann’s constant, Planck’s constant, the universal gas constant, and the standard state concentration (1 mol L⁻¹), respectively. $\gamma(t)$ is the generalized transmission coefficient and is generally close to unity (Feller, 2013). In this study, it was assumed that $\gamma(T)$ was equal to unity.

In addition to equation (2), above, NO_3^- removal rates in DWBs are likely obeying Michaelis-Menten kinetics (Ghane et al. 2015; Nordström and Herbert, 2017a; Schipper et al. 2010) as described by the Monod equation (equation 4).

$$v = k \frac{[NO_3^-]}{[NO_3^-] + K_s} \quad (4)$$

K_s , the half-saturation index, is defined as the NO_3^- concentration when v is half of the maximum NO_3^- removal rate (Arnaldos et al. 2015), which in this study is defined as the temperature dependent rate constant (k) from equation (3). According to the Monod equation, the NO_3^- removal rate will move from an approximately first order control if $K_s \gg [NO_3^-]$, to an approximately zeroth order control if $K_s \ll [NO_3^-]$ (Arnaldos et al. 2015).

2.3.3 Parameter estimation

Equation (2), with k expressed according to the Eyring equation, was fitted to the determined NO_3^- removal rates (v) as a function of temperature and NO_3^- concentration through a non-linear least squares (NLS) regression, in R (R Core Team, 2017), for the determination of $\Delta G^\ddagger$ and the reaction order of NO_3^- removal. Fitting was performed in two scenarios referred to as E_0 and E_1 where zeroth ($n=0$) and first ($n=1$) order NO_3^- removal rates were assumed, respectively. The Monod equation was then fitted to the determined NO_3^- removal rates as a function of NO_3^- concentration using NLS regression and a 1000 iteration non-parametric bootstrap procedure to investigate if the NO_3^- removal rates were limited by NO_3^- availability (SI, section S3). Since it has been previously determined that NO_3^- removal rates in DWBs observed within the initial 13 months of operation are significantly higher than NO_3^- removal rates in DWBs ‘older’ than 13 months (Addy et al. 2016), we fitted the scenarios to data from the first and second operational years separately.

Traditional models for the temperature dependency of reaction rates (e.g. the Arrhenius equation and the Eyring equation) are unable to explain temperature optimums of enzymatic reaction rates (such as NO_3^- reduction studied here) where the reaction rate is maximized with respect to temperature; below and above the temperature optimum, the reaction rate declines. However, recent work on enzymatic reaction rates (see Hobbs et al. 2013; Schipper et al. 2014) have established a relationship between the temperature dependency of $\Delta G^\ddagger$ (equation 5) and the temperature optimum of an enzymatic reaction rate (equation 6). According to MMRT, $\Delta G^\ddagger$ for enzymatic reaction rates are non-linearly dependent on temperature due to the high heat capacities (C_p) of enzymes (Arcus et al. 2016; Hobbs et al. 2013; Schipper et al. 2014).

$$\Delta G^\ddagger = [\Delta H_{T_0}^\ddagger + \Delta C_p^\ddagger(T - T_0)] - T[\Delta S_{T_0}^\ddagger + \Delta C_p^\ddagger \ln(T/T_0)] \quad (5)$$

T and T_0 is the temperature and a reference temperature. $\Delta H_{T_0}^\ddagger$ and $\Delta S_{T_0}^\ddagger$ are the differences in enthalpy and entropy of activation, respectively, between the ground state enzyme substrate

complex and the transition state enzyme substrate complex at T_0 (Hobbs et al. 2013). $\Delta C_p^\ddagger$ is the difference in heat capacity between the ground state and the transition state and is typically negative, resulting in a temperature optimum (T_{opt}) of the enzymatic reaction rate (equation 6, Hobbs et al. 2013).

$$T_{opt} = \frac{\Delta H_{T_0}^\ddagger - \Delta C_p^\ddagger T_0}{-\Delta C_p^\ddagger - R} \quad (6)$$

To determine $\Delta C_p^\ddagger$ and T_{opt} for NO_3^- removal in the DWB, we first determined the temperature dependency of $\Delta G^\ddagger$ by selecting the ‘optimal’ $\Delta G^\ddagger$ for NO_3^- removal for each of the sampled NO_3^- concentrations separately according to a Monte Carlo procedure (see section S.4 in SI). Equation (5) was then fitted to the variability in the ‘optimal’ $\Delta G^\ddagger$ with temperature through a non-linear least squares (NLS) regression in combination with a 1000 iteration non-parametric bootstrap procedure using the R package ‘nlstools’ (Baty et al. 2015; SI, section S.4). In accordance with the discussion by Schipper et al. (2014), $\Delta H_{T_0}^\ddagger$, $\Delta S_{T_0}^\ddagger$, $\Delta C_p^\ddagger$, and T_{opt} should here be regarded as a community average, with the NO_3^- reducing consortium in the DWB being represented by several individual microbial species that are adapted to exploit marginally different temperature ranges. In addition, since the determined NO_3^- removal rates were obtained over a period of two years and at different spatial coordinates in the DWB, the determined $\Delta H_{T_0}^\ddagger$, $\Delta S_{T_0}^\ddagger$, $\Delta C_p^\ddagger$, and T_{opt} are also spatiotemporal averages. We therefore regarded each of the bootstrapped sets of $\Delta H_{T_0}^\ddagger$, $\Delta S_{T_0}^\ddagger$, $\Delta C_p^\ddagger$, and T_{opt} as representing an individual microbial species that were relatively prolific in the DWB at different times and temperatures in the DWB.

Finally, the relative temperature sensitivity of the NO_3^- removal rate was defined using Q_{10} , the relative change in reaction rate by a 10°C change in temperature, which is related to $\Delta C_p^\ddagger$ according to equation (7) (Schipper et al. 2014).

$$Q_{10} = e^{\left(\frac{10(\Delta H^\ddagger - 5\Delta C_p^\ddagger)}{RT^2}\right)} \quad (7)$$

where $\Delta H^\ddagger$, the enthalpy of activation, is linearly dependent on temperature [i.e. $\Delta H^\ddagger = \Delta H_{T0} + \Delta C_p^\ddagger(T-T_0)$] (Schipper et al. 2014). Q_{10} was then used as an indicator of the carbon quality.

The goodness of fit of all regression exercises was evaluated using the Nash-Sutcliffe model Efficiency coefficient (NSE). All calculations were performed with concentrations expressed in moles per liter.

2.4 Modeling

Based on the acquired rate – concentration – temperature relationships (above), the observed NO_3^- concentration profiles and DWB effluent NO_3^- concentrations were simulated using equation (8)

$$[NO_3^-]_{l_i} = \frac{k_b T}{h} e^{\frac{-\Delta G^\ddagger}{RT}} \cdot [NO_3^-]_{l_{i-1}}^n \cdot \Delta t_i(l, HRT) \quad (8)$$

where the subscript l refers to the horizontal distance of the pore water sampling point from the inlet of the DWB, and Δt is the time elapsed between each sampling point as a function of the spatial coordinate of the sampling point and the HRT in the DWB at the time of profile sampling, according to our conceptual flow model (SI, section S.2). $\Delta G^\ddagger$ is obtained from equation (5), above. Since there were a total of seven sampling points, including inlet and outlet, the DWB was discretized into six cells with the position of the inlet, five pore water sampling points, and outlet demarcating the nodes of the model where equation (8) was evaluated.

3. Results and Discussion

3.1 Determined NO_3^- removal rates and apparent NO_3^- removal kinetics

NO_3^- removal rates were obtained for daily average DWB temperatures from 2.6 to 16.8°C, and ranged between 0.76 $\mu\text{mol N L}^{-1} \text{ h}^{-1}$ to 206.30 $\mu\text{mol N L}^{-1} \text{ h}^{-1}$ (0.14 $\text{g N m}^{-3} \text{ d}^{-1}$ to 37.45 $\text{g N m}^{-3} \text{ d}^{-1}$) with an average removal rate of $39.28 \pm 6.37 \mu\text{mol N L}^{-1} \text{ h}^{-1}$ ($7.13 \pm 1.16 \text{ g N m}^{-3} \text{ d}^{-1}$) where L^{-1} and m^{-3} pertain to the pore volume and the DWB volume, respectively. The determined NO_3^- removal rates approximately cover the range of NO_3^- removal rates previously observed in DWBs (cf. Addy et al. 2016; Schipper et al. 2010), with the NO_3^- removal rates for the first operational year being higher and more variable ($9.7 \pm 10.7 \text{ g N m}^{-3} \text{ d}^{-1}$) than for the second operational year ($5.5 \pm 5.2 \text{ g N m}^{-3} \text{ d}^{-1}$). The first operational year was characterized by a relatively high export of the total organic carbon (TOC) from the DWB studied here (Nordström and Herbert, 2018), with 50% of the total estimated TOC emission occurring before day 47, and 75.2% during the first operational year (Nordström and Herbert, 2017b), suggesting that NO_3^- removal rates decreased in association with a decrease in the availability of organic carbon. NO_3^- removal rates determined during the second operational year are therefore regarded as ‘stable’ and operationally relevant.

3.1.1 *Nth-order model fit and identification of general temporal trends*

By fitting the NO_3^- removal rates to concentration data from the first and second operational years according to scenario E_0 and E_1 , we find that our conceptual model (equation 2) generally provides a poor fit ($\text{NSE} < 0.6$) for the determined NO_3^- removal rates (Figures 1a-b; SI Table S.3). This implies that our conceptual model of the NO_3^- removal rate was inadequate for the representation of the NO_3^- removal rate studied here, which we here interpret as the improper assignment of a deterministic $\Delta G^\ddagger$ and/or rate order for each operational year and that either or both varied on an intra-annual basis. However, we find that

the fit for the first order rate scenario (E_1 , $NSE = 0.546$) was superior to the zeroth order ($E_0 = 0.119$) rate scenario for the first operational year, while E_0 ($NSE = 0.019$) was marginally superior to E_1 ($NSE = 0.017$) for the second operational year. This corresponds to an accompanying increase in $\Delta G^\ddagger$ between the two operational years (SI, Table S.3), which suggests that the temperature dependency of the NO_3^- removal rates was different when comparing the first and second operational years. Indeed, the respective operational years were separated by the winter intermission (SI, Table S.1) during which there was no influx of NO_3^- to the DWB and mean daily DWB temperatures were comparatively low and stable (see below). This likely resulted in the starvation of the NO_3^- reducing community and the endogenous respiration of the bacterial community with a resultant decrease in active biomass. Such conditions can trigger an adaption (selection) of the microbial community to the environmental conditions (Hao et al. 2010; Oviedo et al. 2013), which supports a separate treatment of the NO_3^- removal kinetics during the first and second operational years. In addition, long-term NO_3^- removal rates are generally regarded as being operationally zeroth order with respect to NO_3^- concentration in DWBs (Halaburka et al. 2017; Robertson, 2010; Schipper et al. 2010); which supports the interpretation of the NO_3^- removal rate as zeroth order during the second operational year of this study. Accordingly, the respective temporal decrease and change in NO_3^- removal rates and kinetics observed in this study (see above) were the result of a selection of NO_3^- reducers. However, the basis of this selection remains elusive, i.e. whether the apparent selection was characterized by a reduction in rate order or an increase in $\Delta G^\ddagger$, and requires further exploration. Indeed, decreasing the rate order has mathematically the same effect as increasing $\Delta G^\ddagger$ on the conceptualized NO_3^- removal rate (cf. equations 2 and 3).

3.2 Selection as an explanation for changes in NO_3^- removal kinetics over time

Since the NO_3^- concentrations in the DWB were similar between the two operational years (cf. Figure 1c), the apparent inter-annual decrease in rate order (see above) suggests a general decrease in K_s (see equation 4), which could indicate a selection of NO_3^- reducers that were more successful at NO_3^- - limiting concentrations (e.g. Papaspyrou et al. 2014). Indeed, it is generally accepted that an organism with a relatively low K_s will perform better under substrate (NO_3^-) limiting conditions (Arnaldos et al. 2015). The fit of the Monod equation (equation 4) to rate and concentration data (Figure 1c), however, had poor predictive power (i.e. low NSE values) for either operational year. Although unreliable, the results indeed point to a decrease in K_s from $1939.8 \mu\text{mol NO}_3^- \text{ L}^{-1}$ (NSE = 0.019) to $539.86 \mu\text{mol NO}_3^- \text{ L}^{-1}$ (NSE = -0.273) between the two operational years (Figure 1c), and in addition, a reduction in the rate constant k (SI, section S.3).

K_s determined at the scale of the DWB studied here does not necessarily reflect a biological trait of the NO_3^- reducers. Indeed, as discussed by Arnaldos et al. (2015), on a macroscopic scale K_s represents the combined effects of the enzymatic ‘affinity’ toward the substrate (NO_3^-) and limitations in mass transport (in this study, to the active sites of NO_3^- reduction within the woodchip media). In fact, given diffusional limitation on the mass transport of NO_3^- , K_s scales with the maximum NO_3^- removal rate and the size of the NO_3^- reducing community resulting in a variable K_s (Shaw et al. 2013, 2015). This could explain our inability to determine a reliable K_s for neither of the operational years in this study (SI, section S.3). However, from a general point of view, the size of the NO_3^- reducing community at a given time is the net sum of the production and decay of the NO_3^- reducing biomass (cf. Rittman and McCarty, 2001). The rate of biomass production increases with the rate of substrate (NO_3^-) removal (cf. Rittman and McCarty, 2001), wherefore changes in the size of the NO_3^- reducing community (with accompanying changes in K_s , see above) are an expected

outcome from changes in the NO_3^- removal rate. Hence, the general apparent temporal patterns in rate order were the result of the general decrease in k (increase in $\Delta G^\ddagger$) between the two operational years, rather than a decrease in K_s . Accordingly, the increase in $\Delta G^\ddagger$ between the two operational years characterized the selection of NO_3^- reducers between the two operational years. The reason for the apparent division between the first and second operational years in rate orders was hence due to significant differences in $\Delta G^\ddagger$ between the two operational years.

3.2.1 Temperature and community selection

According to our conceptual model for the NO_3^- removal rate (cf. equations 2 and 3), the magnitude of $\Delta G^\ddagger$ reflects the temperature dependency of the NO_3^- removal rate and the variability in $\Delta G^\ddagger$ represent changes in the temperature dependency of the NO_3^- reducing community.

The temperature in the DWB during the winter intermission was low and stable in comparison to an operational year, on average being 1.6°C and varying between 1.1 and 1.9°C ; while the average temperature during an operational year was $\sim 8.9^\circ\text{C}$ and with the temperature varying between 0.8 and 17.3°C . We therefore propose that the apparent variability in $\Delta G^\ddagger$ between the two operational years was the result of a selection of NO_3^- reducers favoring low temperature conditions, i.e. a selection of NO_3^- reducers with increasingly lower T_{opt} . Indeed, temperature as a selective pressure on the functioning and composition of microbial communities has been demonstrated in previous studies (Hanke et al. 2016; Ogilvie et al. 1997; Porter et al. 2015). As described in section 2, MMRT was used to calculate the temperature dependence of $\Delta G^\ddagger$, where after we investigated if there was a selection of NO_3^- reducers favoring low temperature conditions between the two operational years. If this was the case, $\Delta C_p^\ddagger$ (and T_{opt}) for NO_3^- reduction should have decreased as $\Delta C_p^\ddagger$ becomes increasingly negative for low temperature-adapted enzymes (Arcus et al. 2016), with

a resulting decrease in T_{opt} of the enzymatic process (Arcus et al. 2016; Robinson et al. 2017).

The ‘optimal’ $\Delta G^\ddagger$ (SI, section S.4) for NO_3^- removal varied between 93.9 to 96.5 kJ mol⁻¹ and 112.7 to 116.5 kJ mol⁻¹ for the first and second operational year, respectively (Figure 2); while the mean T_{opt} for NO_3^- reduction decreased from 24.2°C to 16.0°C between the two operational years, indicating a general tendency for the selection of NO_3^- reducers favoring low temperatures with time (Table 1). This is in accordance with our previous proposal (see above). [Table 1 near here] From the range of determined T_{opt} (Table 1), NO_3^- reducers with comparatively high T_{opt} ($\gtrsim 30^\circ\text{C}$) were selectively removed from the NO_3^- reducing consortium between the two operational years in favor of increasingly low temperature specializations of NO_3^- reducers (Table 1). In addition, the lowest T_{opt} between the two operational years decreased from 16.4°C to 13.3°C (Table 1), which, in addition to a selection, suggests an adaption of the NO_3^- reducing community to lower temperatures. Indeed, environments where the temperature is comparatively low and invariant are required for the adaption of microorganisms favoring low temperatures, as the catalytic efficiency of low temperature adaptations of microorganisms becomes increasingly sensitive to small temperature changes (Arcus et al. 2016).

3.2.2 Intra-annual seasonality

The mean MMRT parameters (Table 1) successfully described the variation in the ‘optimal’ $\Delta G^\ddagger$ with temperature (NSE = 0.82) for the second operational year, albeit they were less successful (NSE = 0.54) for the first operational year (Figure 2, Table 1). This indicates that the mean MMRT parameters for the second operational year (Table 1) were generally representative for the entire duration of the second operational year, hence representing a comparatively homogeneous temperature dependence of the NO_3^- reducing consortium. The lower NSE for the mean MMRT parameters for the first operational year indicates that the temperature dependency of the NO_3^- reducing consortium was more heterogeneous during the

first operational year. This is also evident when comparing the ranges of T_{opt} for the first and second operational years (Table 1), with the ranges being larger for the first operational year than for the second operational year. Accordingly, in addition to the general selection (and adaption) of NO_3^- reducers between the two operational years, there was also a tendency for the homogenization of the NO_3^- reducing consortium with respect to temperature dependency. We believe this apparent homogenization to be related to a decreased spatial variability in the community of NO_3^- reducers over time. Indeed, Nordström and Herbert (2018) observed that dissimilatory nitrate reduction to ammonium (DNRA), a secondary pathway for NO_3^- reduction in the DWB studied here, was more prolific during the first operational year, and was active when NO_3^- concentrations were low (generally close to the outlet of the DWB), and/or when temperatures decreased to below 5°C .

Using all sets of MMRT parameters from the bootstrap procedure (see above), we simulated the effluent NO_3^- concentrations in the DWB. This was done to study the selection of NO_3^- reducers over time based on temperature, and with assumed first and zeroth order reactions rates for the first and second operational year, respectively. From Figure 3 it is seen that the mean MMRT parameters for each operational year satisfactorily ($\text{NSE} > 0.6$) represented the variation in observed effluent NO_3^- concentrations between the observed and simulated effluent NO_3^- concentrations. In addition, for the simulation of the observed NO_3^- concentration profiles, the mean MMRT parameters generally resulted in high fits ($\text{NSE} > 0.6$) between the simulated and observed NO_3^- concentration profiles (SI, Figure S.4). [Figure 3 near here] This shows that the MMRT framework successfully described the internal dynamics of NO_3^- removal in the DWB, and shows that for long-term, accurate, prediction of NO_3^- removal it is necessary to consider adjustments in the NO_3^- reducing community, which in the DWB studied here are represented by a temporal decrease in T_{opt} .

Figure 3 indicates however that there were occasions during both the first and second

operational years when the mean MMRT parameters were not representative for the NO_3^- reducing consortium. For example, as the DWB temperature decreased around day ~70 during the first operational year (Figure 3), the mean MMRT parameters from the first operational year consistently predict higher effluent NO_3^- concentrations than were observed (Figure 3), with a NO_3^- reducing consortium with a higher T_{opt} being visually superior (Figure 3). This suggests that NO_3^- reducers favoring lower temperatures were generally unsuccessful during the first operational year and were outcompeted by NO_3^- reducers with comparatively high T_{opt} . Indeed, the digested sludge used to inoculate the DWB (see above) was previously operating in an anaerobic digester at 37°C wherefore the success of NO_3^- reducers with comparatively high T_{opt} during the first operational year could be due to a remnant microbial consortium. In addition, following the winter intermission at the start of the second operational year, the mean MMRT parameters for the second year predicts higher effluent NO_3^- concentration than what was observed, and a NO_3^- reducing consortium favoring even lower temperatures than 16°C (Table 1) was visually superior (days ~330-370, Figure 3). Accordingly, it is likely that the low temperature in the DWB during the winter intermission allowed for a NO_3^- reducing consortium with T_{opt} less than 16°C. However, NO_3^- reducers favoring increasingly lower temperatures were subsequently outcompeted following the initialization of the second operational year due to their increased relative sensitivity to small changes in temperature (see above). Hence, the temporal dynamics of NO_3^- reduction in the DWB studied here was more complex than a general division between the two operational years. Indeed, the structure of microbial communities in DWBs have been previously shown to exhibit a high degree of seasonality on intra-annual cycles driven by variations in temperature, degree of saturation of the woodchip media, and distance to the DWB surface (Porter et al. 2015).

3.2.3 Temporal control on NO_3^- removal rates in the DWB

Returning to our initial hypothesis that the temporal control on NO_3^- removal rates in DWBs was related to a decrease in the carbon quality, the variation in Q_{10} (equation 7) representing the relative temperature sensitivity of the reaction rate (section 2) was used as a proxy to study changes in the carbon quality over time. As explained in the study by Sierra (2012), processes feeding on low quality organic carbon exhibit a higher relative temperature sensitivity (i.e. higher values of Q_{10}).

Using the mean $\Delta H^\ddagger_{T0}$ and $\Delta C^\ddagger_p$ (Table 1), Q_{10} is predicted to vary between ~4.6-84.9 and ~4.2-6257 for the first and second operational years, respectively, for the temperature range generally experienced by the DWB studied here (1-17°C), with higher Q_{10} for lower temperatures (SI, Figure S.5). It is here worth noting that the calculated upper ranges of Q_{10} values are much higher than Q_{10} previously determined for NO_3^- removal in DWBs that generally range ~2-5 (e.g. Elgood et al. 2010; Schmidt and Clark, 2013; Warneke et al. 2011). However, Q_{10} defined according to equation (7) curves strongly with temperature, in comparison to more traditional definitions of Q_{10} , and is expected to predict higher values at lower temperatures as further explained by Schipper et al. (2014).

The increased relative temperature sensitivity of the NO_3^- removal rate over time, particularly at lower temperatures, in the DWB is here interpreted as an indication that there was a temporal decrease in carbon quality available in the DWB, particularly at lower temperatures. In accordance with our previous definition of carbon ‘quality’ (see above), we interpret the apparent decrease in carbon quality over time and at lower temperatures as an increased cycling of organic carbon in the DWB prior its final utilization by TEAPs. Since NO_3^- reduction through denitrification in DWBs using lignocellulosic material as the electron source is dependent on upstream fermenting processes for the supply of carbon (Schipper and Vojvodić-Vuković, 2001), the temporal decrease in carbon quality suggests that there was an

457 increased activity of a fermenting microbial community producing organic carbon substrates
458 that later were consumed by the denitrifying community for NO_x reduction in the DWB (i.e.
459 increased cross-feeding). Accordingly, the denitrifying community and NO_3^- removal rates in
460 the DWB became increasingly dependent on the cross-feeding between the fermentative and
461 denitrifying microbial community. This interpretation is supported by a recent study by Hanke
462 et al. (2016) who showed that competition for a shared substrate (carbon) and cross-feeding
463 between fermenters and denitrifiers controlled denitrification rates in tidal marine sediments,
464 with the nature of the interactions and competition being related to changes in temperature. In
465 addition, cross-feeding between fermenters and TEAPs is thermodynamically favorable in
466 systems where there is competition for a shared substrate, and a proposed selective pressure
467 for the microbial interactions in such systems is the rate of energy harvest (González-
468 Cabaleiro et al. 2015). Interestingly, the diversity in biogeochemical processes in the DWB
469 studied here decreased between the two operational years with less energetically efficient (in
470 comparison to denitrification) biogeochemical processes such as DNRA and sulfate reduction
471 being generally outcompeted by denitrification during the second operational year (Nordström
472 and Herbert, 2018). The decrease in the diversity of the biogeochemical processes was
473 suggested to be due to a change in the (primary) organic carbon species produced by
474 fermentation and made available for TEAPs over time (Nordström and Herbert, 2018), which
475 indicates a change in the fermentative microbial community. It is likely that the selection of
476 NO_3^- reducers (see above) occurred in response to a change in the organic carbon substrate
477 produced by the fermenting microbial community as different species of denitrifiers are
478 observed to be prolific on different species of organic carbon substrates, see for example the
479 study by Li et al. (2015). Accordingly, the changes in the NO_3^- removal kinetics over time
480 observed in this study are suggested to have been the result of the development/selection of a
481 microbial structure with increasingly (energetically) efficient exchange with the fermentative

microbial community at the environmental conditions imposed by temperature, with the kinetics of the NO_3^- removal rate becoming increasingly controlled by the temperature dependence of the cross-feeding between the fermentative and denitrifying microbial community. In light of these results, any long-term stabilization of NO_3^- removal rates in DWBs are due to the stabilization of the cross-feeding between fermenters and denitrifiers; future studies should emphasize the biogeochemical cycling of carbon in order to better understand the processes controlling operationally relevant NO_3^- removal rates in DWBs.

3.3 Implications for DWBs

The results of this study point to an increased dependency of the denitrifying community reducing the NO_3^- on the supply of organic carbon from fermentation with time from DWB start-up. Hence, the long term (> 2 years) NO_3^- removal efficiency in DWBs is determined by the interplay between fermentation and denitrification; the longevity of DWBs is not directly related to the total carbon contained in the woodchips, a common indicator used to predict the longevity of NO_3^- removal in DWBs (e.g. Long et al. 2011; Moorman et al. 2010). Indeed, provided that the cross-feeding between the fermentative and denitrifying communities becomes unfavorable, it is likely that the denitrifying community becomes deprived of carbon with a resultant decrease in, or termination of, denitrification prior the depletion of the woodchip carbon storage. In addition, from the observation that the interdependency between the fermentative and denitrifying community increases at lower temperatures (see above), it is also likely that DWBs operating in low temperature environments may be at an increased risk of a ‘premature’ decline in NO_3^- removal efficiencies providing unfavorable conditions for cross-feeding. For example, Hanke et al. (2016), studying the interaction between denitrifiers and fermenters in tidal marine sediments, observed that at 25°C denitrifiers generally outcompeted or engaged in a successful cross-feeding with fermenters, whereas at 10°C denitrification became increasingly unfavorable despite the availability of a carbon substrate.

Accordingly, the longevity of DWBs may be temperature dependent, with a suggested shorter life expectancy for DWBs operating in lower temperature environments.

4. Conclusions

Changes in NO_3^- removal rates in a DWB operated for the duration of two-years was studied with the objective of identifying the parameters controlling the generally observed decline in NO_3^- removal rates with time from DWB start-up. It was hypothesized that this decline was due to a decrease in the ‘carbon quality’ over time, with carbon becoming increasingly recalcitrant with time. By defining ‘carbon quality’ as a property of the microbial community structure, the results of this study suggested that NO_3^- removal in the DWB studied here became increasingly dependent on the cross-feeding between the fermentative (carbon supplying) and the denitrifying (NO_3^- removing) microbial communities. The increased dependence on the interplay between the fermentative and denitrifying microbial communities was associated with an increased recycling of carbon within the fermentative community and a homogenization of the NO_3^- reducing community over time. These changes in the microbial structure over time were interpreted to reflect the development/selection of a more energy efficient microbial community that was better suited for the conditions imposed by the operational environment. Hence, the sustainability of long-term, ‘stable’, NO_3^- removal rates would be controlled by the stability of the cross-feeding between the fermentative and denitrifying community, rather than the total carbon contained in the woodchips.

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Tables

Table 1. Range of fitted macromolecular rate theory parameters.

Year	Parameter	Range of fitted parameters			NSE
		Minimum	Mean	Maximum	
1	$\Delta H_{T_0}^\ddagger$ ^a (kJ mol ⁻¹)	-156.3	-33.5	67.8	0.54
	$\Delta S_{T_0}^\ddagger$ ^b (J mol ⁻¹ K ⁻¹)	-862.9	-437.1	-86.5	
	$\Delta C_p^\ddagger$ ^c (kJ mol ⁻¹ K ⁻¹)	-17.9	-10.7	-4.9	
	T _{opt} ^d (°C)	16.4	24.2	38.2	
2	$\Delta H_{T_0}^\ddagger$ ^a (kJ mol ⁻¹)	-584.2	-262.7	34.5	0.82
	$\Delta S_{T_0}^\ddagger$ ^b (J mol ⁻¹ K ⁻¹)	-2392.8	-1290.4	-270.0	
	$\Delta C_p^\ddagger$ ^c (kJ mol ⁻¹ K ⁻¹)	-49.6	-27.9	-8.0	
	T _{opt} ^d (°C)	13.3	16.0	29.6	

^aEnthalpy of activation at the reference temperature T_0 (298.15 K). ^bEntropy of activation at the reference temperature T_0 (298.15 K). ^cThe difference in heat capacity between the enzyme-substrate complex (ES) and the enzyme-substrate transition species ($ES^\ddagger$). ^dThe optimum temperature of the NO_3^- removal rate. ^eNash-Sutcliffe model efficiency coefficient between the variation in 'optimal' $\Delta G^\ddagger$ with temperature and the mean MMRT parameters.

679
680 *Figure 1. The temperature dependence of the rate constant for NO_3^- removal (k), assuming zeroth ($k = v$, Figure*
681 *1a) and first ($k = v/[\text{NO}_3^-]$, Figure 1b) order reaction rates; the variation in the determined NO_3^- removal rates*
682 *with NO_3^- concentration (Figure 1c), for the first ($\circ$, days 0-151) and second ($\bullet$, days 322-490) operational*
683 *years. E_0 and E_1 are zeroth and first order model scenarios, respectively, and Monod refers to the Monod*
684 *equation. [1 COLUMN FITTING]*

Figure 2. Temperature dependency of 'optimal' Gibb's free energy of activation ($\Delta G^\ddagger$) determined for each profile sampling occasion for the first ($\circ$, days 0-151) and second ($\bullet$, days 322-490) operational years. Numbers on data points indicate day of sampling. Color-graded line shows all bootstrap estimates, while black line shows mean bootstrap estimates. [1 COLUMN FITTING]

Figure 3. Simulated NO_3^- effluent concentrations using MMRT. Mean MMRT indicates line simulated using the mean MMRT parameters determined in the bootstrap procedure. NSE = Nash-Sutcliffe Model efficiency Coefficient. [1.5 COLUMN FITTING]