

EFFICACY OF SUBSURFACE DENITRIFICATION TO ATTENUATE NITRATE IN SHALLOW GROUNDWATERS

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Abstract

Leaching of nitrate (NO_3^-) from grazed pastoral systems and other intensive land uses is of a key concern in the deterioration of surface and groundwater quality in New Zealand's agricultural catchments. However, the extent of NO_3^- leaching from agricultural soils to receiving waters depends on its flow pathways and potential attenuation (removal) in the subsurface environment.

Denitrification, a multistep sequence of N reduction reactions, is one of the key mechanism that under favorable conditions can attenuate NO_3^- in a subsurface environment including shallow groundwaters (first saturated zone < 10 meters below ground surface). Environmental benefit of subsurface denitrification, however, may be limited if subsurface denitrification is incomplete and the terminal product of NO_3^- reduction is nitrous oxide (N_2O , a harmful greenhouse gas) rather than dinitrogen (N_2 , an inert and harmless gas making up 78% of the atmosphere).

We are undertaking a field study assessing whether NO_3^- reduction in shallow groundwaters is incomplete and environmentally harmful (i.e. N_2O release) or complete and environmentally benign (i.e. N_2 release) in New Zealand agricultural catchments. From August 2017 to January 2018 we have collected, monthly shallow groundwater samples from 6 pastoral farms located in various hydrogeological settings in the Manawatu and Rangitikei River catchments. The collected groundwater samples were analysed for their levels of dissolved oxygen (DO), nitrate (NO_3^-), sulfate (SO_4^{2-}), iron (Fe^{2+}), manganese (Mn^{2+}), dissolved gases (N_2O and N_2) and the abundance of denitrifier genes *nosZ*, *nirS*, and *nirK*. The groundwater redox conditions of the study sites range from moderately/highly oxidized ($\text{DO} > 1 \text{ mg L}^{-1}$) to moderately/highly reduced ($\text{DO} < 1 \text{ mg L}^{-1}$).

Our preliminary results indicate occurrence of benign denitrification at the reducing shallow groundwater sites with lower DO contents ($\text{DO} < 1 \text{ mg L}^{-1}$), as compared with the oxidizing shallow groundwater sites (less-reducing) with higher DO contents ($\text{DO} > 1 \text{ mg L}^{-1}$). This study is continued with further monitoring and experiments for examination of processes and regulatory factors driving subsurface denitrification in shallow groundwaters at the study sites.

Key Words: Agriculture, Pastoral farming, Water quality, Nitrate leaching, Groundwater redox, New Zealand.

Introduction

Globally, pastoral livestock grazing and other intensive agricultural land uses with increased fertilizer nitrogen (N) use exhibit risks of nitrate (NO_3^-) leaching to groundwater and surface waters across agricultural landscapes. Land use intensification and excessive use of N fertilizers can result in the release of NO_3^- in the environment, which ends up in the water bodies (Zhang *et al.*, 2015; Rivett *et al.*, 2008; Wong *et al.*, 2015). Nitrate pollution in waters is linked to public health concerns, toxicity to aquatic species, eutrophication of water bodies, emission of greenhouse gases to the atmosphere (Galloway *et al.*, 2003; Galloway *et al.*, 2008; Reay *et al.*, 2012; Steffen *et al.*, 2015), and represents an economic loss to farming sector. Hence, management of NO_3^- losses to waters requires development of effective approaches to minimize NO_3^- leaching from agricultural soils and a better understanding of its flow pathways and attenuation from land to receiving waters.

Among the various transformation pathways of NO_3^- in its flow from land to waters, subsurface denitrification is identified as a key process that transforms NO_3^- to benign dinitrogen (N_2) (Seitzinger *et al.*, 2006). Denitrification is a multistep biochemical process during which NO_3^- is converted to gaseous N_2 . However, an incomplete denitrification can lead to release of harmful gas nitrous oxide (N_2O) from soil and groundwater (Butterbach-Bahl *et al.*, 2013). Also, there is spatial and temporal variation in subsurface denitrification capacity across agricultural landscapes (Jahangir *et al.*, 2013). This variation in subsurface denitrification capacity generally arises from changes in hydrogeological and geochemical characteristics of the subsurface environment (Jahangir *et al.*, 2013, Rivas *et al.*, 2017; Collins *et al.*, 2017).

Pastoral grazed systems are inherently leaky with respect to NO_3^- , a key water contaminant implicated in the deterioration of surface and groundwater quality in New Zealand agricultural catchments. Due to intensive farming and year-round grazing, pastoral farms in New Zealand exhibit a high potential for NO_3^- leaching to subsurface and indirect N_2O emissions to the atmospheric environment (Saggar *et al.*, 2013). Hence, a sound understanding and knowledge of hydrogeological and geochemical characteristics affecting transport and transformation of NO_3^- in the subsurface environment are essential for developing effective and targeted strategies for reducing NO_3^- leaching to waters and net N_2O emissions to the atmosphere. Variability in the extent of subsurface denitrification has been identified in the previous studies in New Zealand and globally (Anderson *et al.*, 2014; Clague *et al.*, 2013; Jahangir *et al.*, 2013; Peterson *et al.*, 2013; Rissmann, 2011; Stenger *et al.*, 2008; Elwan *et al.*, 2015; Rivas *et al.*, 2017; Collins *et al.*, 2017). However, there is very limited information available on the fate of NO_3^- transformed as benign N_2 and/or harmful N_2O in the subsurface environment of New Zealand agricultural catchments.

This study aims to investigate processes and regulatory factors driving subsurface denitrification in shallow groundwaters in New Zealand catchments. We hypothesize that (i) subsurface denitrification in shallow groundwaters is dominantly complete denitrification (i.e. greater dissolved N_2 than N_2O emission) at the reduced groundwater conditions than the oxidizing groundwaters, and (ii) the reducing groundwaters have higher abundance of denitrifier genes especially *nosZ* (N_2O reductase) gene than the oxidizing groundwaters.

Materials and Methods

We have selected 6 study sites under pastoral farms located in various hydrogeological settings in the Manawatu and Rangitikei River catchments, located in lower parts of North Island, New Zealand. Each site has been installed with 3 PVC piezometers (28 mm inner diameter) at three different depths, except the Dannevirke (CAM) site that has only two piezometers (Table 1). The depth of piezometers range from 3.3 to 9.0 m below ground level (bgl). The piezometers are screened in the last 50 or 60 cm, allowing access to shallow groundwaters at different depths (Table 1).

All the study sites are under dairy farming, except the Woodville (ARM) site under sheep and beef farming (Table 1). Soil types at the study sites range from heavy soil (Clay loam) at the Woodville (ARM) site to stony silt loam at the Pahiatua (BUR and CAM sites), to lighter soil (Foxton Brown Sand/ Pukepuke Black Sand/ Himitangi Sand) at the Santoft (TAY) site. The Pahiatua (BUR and SR) and Dannevirke (CAM) sites are located in loess over gravel setting, while the Palmerston North (DFI) and Woodville (ARM) sites are located in alluvial setting. The Santoft (TAY) site is located in a sand flat geological unit. The recharge rate of groundwater in the piezometers at the CAM site is contrasting and relatively much slower, as compared to the other sites. This suggests that the piezometers at the CAM site are installed into relatively low hydraulic conductivity setting. Refer to Rivas *et al.* (2017) and Collins *et al.* (2017) for further details of hydrogeological settings and groundwater redox variations in the study areas.

Collection of shallow groundwater samples

We have been collecting three replicated shallow groundwater samples from each of the piezometers every month from August 2017. This monthly groundwater sampling and analysis is ongoing and here we analyze and present the measurements for the first 5 months from August 2017 to January 2018. The groundwater samples are collected as per the National Protocol for SOE Groundwater Sampling in NZ (Daughney, 2006). A peristaltic pump (Solinst Canada Ltd. Model 410) fitted with Teflon outting tubing was used to collect groundwater samples at a low flow rate ranging from 0.5 to 1 L min⁻¹ from the study piezometers. The groundwater samples from each piezometer were collected after purging at least three well volumes and until the field parameters (DO, temperature, pH, electrical conductivity and oxidation-reduction potential) measured by a YSI multi-parameter probe (In Situ Inc. USA) were stabilized.

After purging, we collected the groundwater samples for hydrochemistry, dissolved gases, and molecular measurements. The groundwater samples for cation and anion analysis were collected in 50 ml plastic containers after filtered through a syringe fitted with a 0.45 µm syringe-tip (polypropylene Terumo®) filter (Microscience Polyethersulphone). The samples collected for metal ions analysis were acid preserved by adding nitric acid into the filtered samples. For the measurement of dissolved N₂ and N₂O gases, the groundwater samples were collected in 125 ml Wheaton serum bottles (Sigma-Aldrich®). The groundwater samples were collected in 5l Nasco whirl-pak™ sterile plastic bags for molecular analysis. Due to very slow

groundwater recharge rate in the CAM1 piezometer it was not practically possible to collect groundwater samples for dissolved gases and microbial analysis.

Immediately after collection the groundwater samples were stored in chilly bins with frozen ice packs until transported to the fridge or freezer in the lab. The collected groundwater samples were analysed within 2-4 weeks of the sampling. Ion Chromatograph (IC) and Microwave Plasma Atomic Emission Spectroscopy (MP-AES) instruments, available in the Soil Water Laboratory at Massey University, were used for chemical (cations and anions) analysis of the groundwater samples. The amounts of dissolved gases, N₂O and N₂ were analysed using gas chromatograph in the Environmental Chemistry Laboratory, Manaaki Whenua Landcare Research. The qPCR of denitrifier genes was performed using the lightcycler at the Institute of Fundamental Sciences, Massey University.

Chemical analysis

We used a Dionex™ Aquion™ IC (ThermoFisher Scientific) for the analysis of anions such as nitrate (NO₃⁻) and sulfate (SO₄²⁻) in the groundwater samples. We also used a 4200 MP-AES (Agilent Technologies, Santa Clara, CA USA) for the analysis of dissolved metal ions such as iron (III) and manganese (IV) in the groundwater samples. These instruments detection limit for each ion was as follows Fe²⁺: 0.01 mg L⁻¹, Mn: 0.05 mg L⁻¹, NO₃⁻-N: 0.003 mg L⁻¹, and SO₄²⁻: 0.01 mg L⁻¹.

Dissolved gases analysis

We measured dissolved N₂O and N₂ gases in the collected groundwater samples using a robotized auto sampler (Gilson GX271 Liquid handler) and Shimadzu 2010-Plus gas chromatograph system (Shimadzu Corp, Kyoto, Japan) with an electron capture detector (ECD), flame-ionization detector (FID) and thermal conductivity detector (TCD) (McMillan *et al.*, 2014). First, a 50 ml headspace was created in the collected groundwater filled serum bottles by simultaneous groundwater extraction and the addition of Ultra-High Purity (99.999%) helium (Gas Code 220, BOC, Auckland, New Zealand) (Clagnan *et al.*, 2018). Then, the serum bottles were kept on a rotary shaker for 1 hour to equilibrate the headspace with dissolved gases in the groundwater samples. The headspace gas was then analyzed for dissolved N₂O and N₂ gases using gas chromatography. The instrument detection limit for N₂O and N₂ is 0.01 and 85 μ mole L⁻¹, respectively.

Molecular analysis

The collected groundwater samples in sterile plastic bags were first filtered (500 ml) through 0.22 μm S-Pak® membrane filters (Millipore France) (Jahangir *et al.*, 2013). The filters were then subject to DNA extraction using a DNA isolation kit Genomic DNA kit (Plant) (dnature, New Zealand). The filters were stored frozen until the DNA extraction was performed using the protocols of the kit. The extracted DNA was quantified and quality assessed using the DS-11 spectrophotometer (DeNovix Inc. Wilmington, DE USA). The extracted DNA was then used for the Polymerase chain reaction (PCR) analysis of *nosZ*, *nirS*, and *nirK* genes. PCR

reactions were set up and conducted through Roche 480 lightcycler using the procedure and reaction setup described in Jha *et al.* (2017) and Morales *et al.* (2015).

Quantification of bacterial *nirS*, *nirK*, and *nosZ* genes was accomplished using quantitative PCR (qPCR), as described in Jha *et al.*, (2017). The primers used were *nirS* Cd3aF, R3cd (Enwall *et al.*, 2010), *nirK* Copper 583F, 909R (Dandie *et al.*, 2011), and *nosZ* 2F, 2R (Henry *et al.*, 2006). Amplification efficiencies were within the expected range of values ($E = 90 - 110\%$). The reactions were linear over seven orders of magnitude and sensitive down to 10^2 copies. Refer to Morales *et al.* (2015) for details of the reaction conditions used for this analysis.

Data Analysis

Groundwater chemistry, dissolved gases and molecular data generated through various analyses as described above were first checked using Shapiro Wilk test (Shapiro & Wilk, 1965) for normality and appropriately transformed if the normality was not met. The parameters such as NO_3^- -N, DO, N_2O , N_2 , *nir*(S+K), and *nosZ* gene abundances were log transformed and other parameters such as Mn^{2+} , SO_4^{2-} , and Fe^{2+} were power transformed (1/3). The NO_3^- -N and Mn^{2+} values below the detection limits were assigned a value of $1/\sqrt{2}$ of the detection limit (Finkelstein and Verma, 2001).

The effects of site location, piezometer depths, and groundwater redox status on the measured groundwater chemical parameters, dissolved gases, and denitrifier gene abundances were assessed by a multiple-level analysis of variance (ANOVA) using a general linear model procedure in statistical software Minitab. Tukey's Studentized Range Test at $\alpha = 0.05$ significance level was used *post hoc* to reveal significant differences among the means of variables analyzed.

Table 1: Location of the study sites for collection of shallow groundwater samples in the Manawatu and Rangitikei catchments.

Site code	Location of sampling site	No. of Piezometers at each site	Piezometer Depth (m, bgl)	Screen depth (m, bgl)	Land Use	Soil Type* (<i>from NZ FSL layers</i>)	Rock Type* (<i>from NZ QMAP layer</i>)
DFI	Palmerston North	P1	5.9	0.5	Dairy	Manawatu fine sandy loam	Alluvium
		P2	7.8	0.5			
		P3	9.0	0.5			
TAY	Santoft	P1	3.3	0.5	Dairy	Foxton brown sand, Pukepuke black sand, and Himitangi sand	Sand flat
		P2	3.4	0.5			
		P3	5.2	0.5			
ARM	Woodville	P1	5.3	0.5	Beef/Sheep	Kairange silt loam and clay loam	Alluvium
		P2	5.9	0.5			
		P3	7.8	0.5			
SR	Pahiatua	P1	4.7	0.5	Dairy	Kopua stony silt loam	Loess over gravel
		P2	5.7	0.5			

		P3	6.7	0.5			
BUR	Pahiatua	P1	3.6	0.5	Dairy	Kopua stony silt loam	Loess over gravel
		P2	4.3	0.6			
		P3	6.1	0.6			
CAM	Dannevirke	P1	4.8	0.5	Dairy	Takapau silt loam	Loess over gravel
		P2	7.7	0.5			

*Rivas (2018), Collins (2015) and New Zealand Fundamental Soil Layer and Rock type from the New Zealand Land Resource Inventory based on the location of the study sites (<https://soils.landcareresearch.co.nz/soil-data/the-lris-portal/>).

Results and Discussion

Groundwater redox status and chemistry

Using McMahon and Chapelle (2008) redox assessment criteria, the measured groundwater chemistry parameters were used to assess the redox status of the groundwater samples collected from the study sites (Table 2). Here, we used DO as indicator of groundwater redox status (<1 mg L⁻¹ of DO as anoxic and >1 mg L⁻¹ of DO as oxic) and presence of NO₃⁻-N (≥ 0.5 mg L⁻¹), Mn²⁺ (≥ 0.05 mg L⁻¹), and Fe²⁺ (≥ 0.1 mg L⁻¹) suggested the reduction process occurring. This analysis suggested that the shallow groundwaters at the DFI, ARM, and TAY sites are reducing groundwaters (DO < 1 mg L⁻¹), while the shallow groundwaters at the BUR and SR are oxic groundwaters (DO > 1 mg L⁻¹). This analysis further characterized the DFI and ARM sites as the NO₃⁻-N, Mn (IV) and Fe (III) reducing conditions, and the TAY site as NO₃⁻-N and Mn (IV) reducing conditions. The two piezometers at the CAM site were contrasting with the CAM1 being characterized as oxic condition, while the CAM2 being as anoxic, and NO₃⁻-N and Mn (IV) reducing condition. Rivas (2018) and Collins (2015) reported a similar characterization of shallow groundwaters at the study sites.

Interestingly, there is a significant variation in the groundwater redox and chemical parameters collected from different sites (Table 2), also reported earlier (Rivas *et al.*, 2017, Collins *et al.*, 2017). However, there is no significant variation in these parameters in the groundwater samples collected from three piezometer depths at the same site, except CAM site (Table 2). The two piezometers at the CAM site had contrasting dissolved oxygen (DO) with the significantly lower DO (mean DO = 0.22 mg L⁻¹) in the deeper piezometer, CAM P2 (Table 2). The shallow piezometer (P1) at the CAM site, CAM P1 had DO levels > 4 mg L⁻¹. DO was observed significantly ($P < 0.05$) highest (mean DO ranging from 7.28 - 7.43 mg L⁻¹) at the SR site, followed by the BUR site (mean DO ranging from 2.65 – 4.07 mg L⁻¹). Apart from the ARM site, in all the sites DO was observed slightly decreasing with the piezometer depth. This contrasting nature of reducing and oxidizing (less-reducing) conditions of shallow groundwaters have been previously documented and attributed to variability in hydrogeological settings in terms of soil and rock types found at the study sites (Rivas *et al.*, 2017; Collins *et al.*, 2017).

The NO₃⁻-N content in the groundwater samples was significantly lower (mean NO₃⁻-N ranging from 0.01 – 1.33 mg L⁻¹) in the reducing sites (DO < 1 mg L⁻¹) as compared to the non-reducing sites (mean NO₃⁻-N ranging from 3.39 – 7.73 mg L⁻¹; DO > 1 mg L⁻¹). This positive correlation between the DO and NO₃⁻-N contents corresponds with the occurrence of subsurface denitrification at low DO levels (Rivett *et al.*, 2008; Clague *et al.*, 2013; Jahangir *et al.*, 2013; Rivas *et al.*, 2017; Collins *et al.*, 2017).

Interestingly, the reducing groundwater sites (DFI, ARM, and TAY) had significantly higher iron (Fe²⁺) content (mean Fe²⁺ ranging from 0.15 to 4.21 mg L⁻¹) as compared to the non-reducing sites (SR, CAM, and BUR) (mean Fe²⁺ ranging from 0.01 to 0.08 mg L⁻¹) (Table 2). The higher Fe²⁺ content under anoxic condition suggests a coupled microbial NO₃⁻-N reduction

with Fe (III) oxidation under the anaerobic condition where NO_3^- -N serves as an electron acceptor (Straub *et al.*, 2001).

Variation of dissolved gases (N_2O and N_2) in shallow groundwaters

We observed a significant variability in both dissolved N_2O and N_2 contents measured across the oxidized and reducing groundwaters at the study sites (Figures 1 and 2). However, there was no significant effect of the piezometer depth on the measured dissolved N_2O and N_2 contents at the study sites (Figures 1 and 2). The dissolved N_2O content was measured significantly ($P < 0.05$) higher in the BUR site (measuring a mean value of $0.17 \mu\text{moles N}_2\text{O L}^{-1}$ in the three piezometers) and SR site (measuring a mean value of $0.12 \mu\text{moles N}_2\text{O L}^{-1}$ in the three piezometers). The BUR and SR sites are both assessed as oxic groundwaters (mean DO ranging from 2.65 to 7.43 mg L^{-1} , Table 2).

At the reducing groundwater sites (DFI, TAY, ARM, and CAM2), dissolved N_2O and N_2 contents were similar with the piezometers, depths (mean values ranging only from 0.004 to $0.039 \mu\text{moles L}^{-1}$ of N_2O and from 594 to $1066 \mu\text{moles L}^{-1}$ of N_2) (Figures 1 and 2). When compared across the groundwater redox conditions, the sites categorized as oxic groundwaters (BUR and SR) had significantly higher mean dissolved N_2O (mean values ranging from 0.115 to $0.343 \mu\text{moles L}^{-1}$) than the anoxic sites DFI, TAY, ARM and CAM2 (mean values ranging from 0.004 to $0.039 \mu\text{moles L}^{-1}$). In contrast to the dissolved N_2O levels, the oxic groundwater sites (BUR and SR) had lower dissolved N_2 levels (mean values ranging from 308 to 572 $\mu\text{moles L}^{-1}$) than the anoxic (DFI, TAY, ARM and CAM2) sites, which had almost double the amount of dissolved N_2 (mean values ranging from 594 to $1066 \mu\text{moles L}^{-1}$).

The measured amounts of dissolved N_2O and N_2 contents could be affected by hydrogeological (e.g. depth to water table, groundwater recharge rate, hydraulic conductivity) as well as geochemical (e.g. redox status, presence of electron donors and acceptors) conditions of the subsurface environment. We plan further observations and analysis of excess N_2 as a measure of subsurface denitrification in shallow groundwaters at the study sites. However, the observations presented in this study support our first hypothesis and show the potential of complete denitrification (lack of N_2O emission) occurring at the reducing shallow groundwater sites (Figures 1 and 2). The low levels of dissolved N_2O and high levels of N_2 at the DFI, TAY and ARM sites suggest a predominant complete 'benign' denitrification of NO_3^- to harmless N_2 in shallow groundwater at the reducing sites. However, the oxic groundwater sites (BUR and SR), with DO higher than the threshold level of 1 mg L^{-1} (Rivett *et al.*, 2008; Rivas *et al.*, 2017), appears to be participating more in partial incomplete denitrification, leading to relatively higher dissolved N_2O than N_2 levels in the shallow groundwater at the study sites.

Table 2: Groundwater chemistry, identification of redox status and process in shallow groundwaters collected at 6 pastoral farm sites (Table 1) in the Manawatu and Rangitikei catchments, from August 2017 to January 2018. The data presented are mean \pm standard error ($n = 3$). The subscript letter values denote one-way analysis of variance (ANOVA) test. Values sharing the same letter in each column are not significantly different. Columns shaded in blue are anoxic ($\text{DO} < 1 \text{ mg L}^{-1}$) and in yellow are oxic ($\text{DO} > 1 \text{ mg L}^{-1}$) sites.

Site	Piezometer	Dissolved Oxygen (O_2) (mg L^{-1})	Nitrate (NO_3^- -N) (mg L^{-1})	Iron (Fe^{2+}) (mg L^{-1})	Manganese (Mn^{2+}) (mg L^{-1})	Sulfate (SO_4^{2-}) (mg L^{-1})	Redox Status and Process
DFI	P1	0.99 ± 0.00^G	0.20 ± 0.00^H	1.34 ± 0.31^C	0.07 ± 0.02^D	3.55 ± 0.00^I	Anoxic, NO_3^- -N, Mn (IV), and Fe (III) reduction
	P2	0.19 ± 0.01^J	0.02 ± 0.00^I	3.49 ± 0.26^{AB}	0.16 ± 0.01^B	2.97 ± 0.00^J	
	P3	0.14 ± 0.01^J	0.08 ± 0.01^I	3.09 ± 0.32^{AB}	0.15 ± 0.00^{BC}	4.16 ± 0.00^H	
TAY	P1	0.50 ± 0.01^H	0.92 ± 0.00^G	0.28 ± 0.02^C	0.51 ± 0.04^A	9.41 ± 0.01^C	Anoxic, NO_3^- -N, and Mn (IV)
	P2	0.42 ± 0.03^{HI}	1.33 ± 0.00^F	4.21 ± 0.77^A	0.66 ± 0.01^A	5.18 ± 0.01^F	
	P3	0.09 ± 0.01^J	0.01 ± 0.00^I	3.22 ± 0.28^{AB}	0.19 ± 0.00^B	21.55 ± 0.09^A	
ARM	P1	0.17 ± 0.01^J	0.02 ± 0.00^I	4.02 ± 0.14^A	0.10 ± 0.00^D	4.64 ± 0.01^G	Anoxic, NO_3^- -N, Mn (IV), and Fe (III) reduction
	P2	0.25 ± 0.04^J	0.03 ± 0.01^I	2.70 ± 0.33^B	0.08 ± 0.00^D	1.41 ± 0.00^L	
	P3	0.12 ± 0.01^J	0.02 ± 0.00^I	0.60 ± 0.03^C	0.20 ± 0.01^B	0.70 ± 0.34^M	
CAM	P2	0.22 ± 0.02^J	0.03 ± 0.00^I	0.15 ± 0.01^C	0.57 ± 0.01^A	2.44 ± 0.00^K	Anoxic, NO_3^- -N, and Mn (IV)
SR	P1	7.43 ± 0.02^A	3.39 ± 0.02^E	0.01 ± 0.00^C	0.01 ± 0.00^E	5.06 ± 0.00^{FG}	Oxic, O_2 reduction
	P2	7.28 ± 0.05^{AB}	3.45 ± 0.01^{DE}	0.08 ± 0.03^C	0.02 ± 0.00^E	5.04 ± 0.00^{FG}	
	P3	7.40 ± 0.02^B	3.44 ± 0.01^{DE}	0.01 ± 0.02^C	0.02 ± 0.00^E	4.98 ± 0.00^{FG}	
BUR	P1	2.69 ± 0.06^E	3.50 ± 0.04^D	0.01 ± 0.00^C	0.01 ± 0.00^E	5.85 ± 0.01^H	Oxic, O_2 reduction
	P2	4.07 ± 0.04^D	4.60 ± 0.00^C	0.03 ± 0.00^C	0.02 ± 0.00^E	6.33 ± 0.01^E	
	P3	2.65 ± 0.06^F	7.52 ± 0.01^B	0.01 ± 0.00^C	0.02 ± 0.01^E	4.64 ± 0.01^D	
CAM	P1	4.64 ± 0.13^C	7.73 ± 0.04^A	0.04 ± 0.01^C	0.11 ± 0.01^{CD}	12.13 ± 0.09^B	Oxic, O_2 reduction

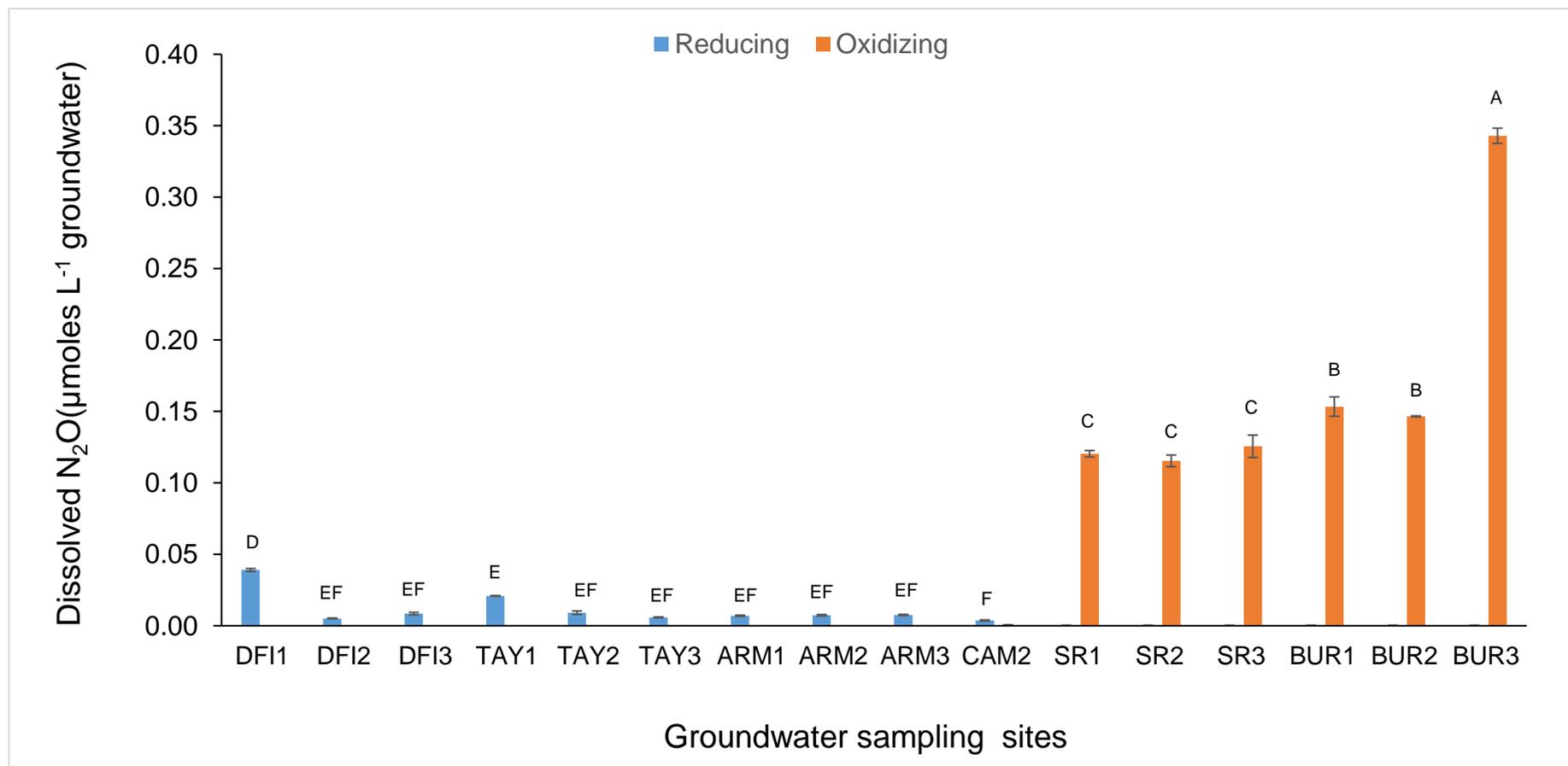


Figure 1. Variation in dissolved nitrous oxide (N₂O) concentration observed in shallow groundwater samples collected at 6 pastoral farm sites (Table 1) in the Manawatu and Rangitikei catchments, from August 2017 to January 2018. Data points are the mean ± standard error ($n = 3$). The blue bars are anoxic sites ($DO < 1 \text{ mg L}^{-1}$) and the orange bars are the oxic sites ($DO > 1 \text{ mg L}^{-1}$). Letter values on the bars denote one-way ANOVA test. Values sharing the same letter are not significantly different. Sample names denote site and piezometer for the collection of groundwater sample (Table 1). Due to very slow recharge of groundwater, CAM1 was not sampled and analysed for dissolved gases analysis.

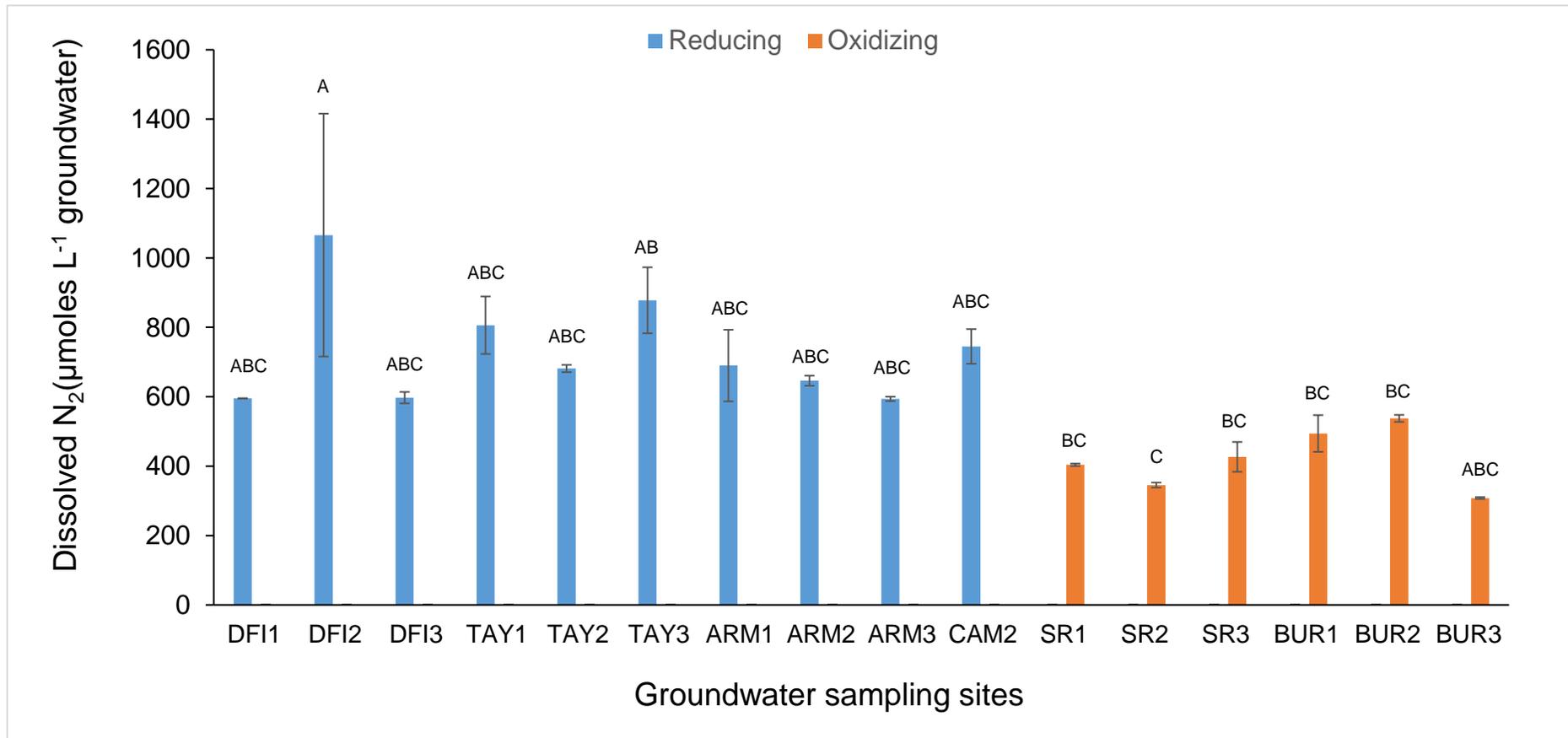


Figure 2. Variation in dissolved dinitrogen (N₂) concentration observed in shallow groundwater samples collected at 6 pastoral farm sites (Table 1) in the Manawatu and Rangitikei catchments, from August 2017 to January 2018. Data points are the mean ± standard error (*n* = 3). The blue bars are anoxic sites (DO < 1 mg L⁻¹) and the orange bars are the oxic sites (DO > 1 mg L⁻¹). Letter values on the bars denote one-way ANOVA test. Values sharing the same letter are not significantly different. Sample names denote site and piezometer for the collection of groundwater sample (Table 1). Due to very slow recharge of groundwater, CAM1 was not sampled and analysed for dissolved gases analysis.

Variation in total DNA and denitrifier genes abundances in shallow groundwaters

The shallow groundwater samples used in this study varied in their total DNA content. The amount of DNA extracted (measured in ng from 1 L of groundwater) was lowest at the DFI site (a mean value of 8.6 ng L⁻¹) and highest at the TAY site (a mean value of 37.0 ng L⁻¹). The anoxic groundwater (DFI, TAY, ARM and CAM2) sites had higher DNA content (a mean value of 20.13 ng L⁻¹) than the oxic groundwater (BUR and SR) sites (a mean value of 11.83 ng L⁻¹).

We found in general a higher abundance of the nitrite (NO₂⁻) reductase gene (*nirS+K*) than the N₂O reductase gene (*nosZ*) in the shallow groundwater samples analyzed (Figures 3 and 4). Other studies have also observed similar results. For example, Barrett *et al.*, 2013 also observed that *nirS* and *nirK* gene copy numbers were significantly higher than the *nosZ* gene copy numbers in groundwater samples collected from 36 different multilevel piezometers in grazed grassland and barley cultivated land in Ireland. The abundance of *nirS+K* genes was measured relatively similar among the study sites (Figure 3).

Interesting, the abundance of the *nosZ* gene varied among the study sites (Figure 4). The mean *nosZ* gene copies were significantly highest in the TAY (22896 copies L⁻¹) and ARM sites (18138 copies L⁻¹) and least in the SR site (5673 copies L⁻¹) (Figure 4). In general, the abundance of *nosZ* gene copies was significantly higher (overall mean ~ 15107 copies L⁻¹) at the reducing groundwater sites (DFI, TAY, CAM, and ARM), as compared to the oxidized groundwater sites (SR and BUR; overall mean ~ 8261 copies L⁻¹). We were not able to look into the variation in denitrifier gene abundances between the two contrasting piezometers at the CAM site due to slow recharge of groundwater for sampling at the CAM1 piezometer.

Relationships between groundwater redox species, dissolved gases and denitrifier genes abundances in shallow groundwaters

A further analysis suggests that the dissolved N₂ contents measured in the groundwater samples is correlated positively with Fe²⁺ content ($r = 0.421, P < 0.05$), DNA content ($r = 0.292, P < 0.05$), *nosZ* gene copies ($r = 0.221, P < 0.05$) and negatively with DO ($r = -0.595, P < 0.05$) and NO₃⁻-N contents ($r = -0.381, P < 0.05$). On the other hand, the dissolved N₂O was positively correlated with NO₃⁻-N content ($r = 0.695, P < 0.05$) and DO contents ($r = 0.499, P < 0.05$) and negatively correlated with Fe²⁺ ($r = -0.520, P < 0.05$). McAleer *et al.* (2017) have reported similar correlation of N₂O emission and excess N₂ with NO₃⁻, Fe²⁺ and DO content in an agricultural sandstone catchment in Ireland.

Our correlation analysis between groundwater redox, chemical, and dissolved gases parameters indicates that the studied shallow groundwaters exhibit a complete 'benign' subsurface denitrification of NO₃⁻-N under low DO levels (<1 mg L⁻¹), producing relatively very low levels of N₂O but high N₂. However, a weak correlation of dissolved N₂ and *nosZ* gene copies indicated that there are some outliers such as the DFI site. This site interestingly shows a very high dissolved N₂ (Figure 2) and very low DO and NO₃⁻-N contents (Table 2), but overall much less DNA content and little abundance of denitrifier *nosZ* gene (Figure 4). However, the DFI

site shows high levels of Fe^{2+} content, which might suggest that the subsurface denitrification occurring at this site, could be mainly autotrophic or abiotic denitrification and not mediated by microbial population (Rivett *et al.*, 2008). This will need further investigations.

Overall, the observations presented so far also support our second hypothesis, that the groundwater sites (apart from the DFI site) that are under reducing conditions (low DO levels) also shows an abundance of denitrifier *nosZ* genes. This abundance of *nosZ* genes supports the occurrence of complete ‘benign’ subsurface denitrification, reducing dissolved NO_3^- -N all the way to harmless dinitrogen in reducing shallow groundwaters. However, this will be further investigated with quantification of excess N_2 at the study sites.

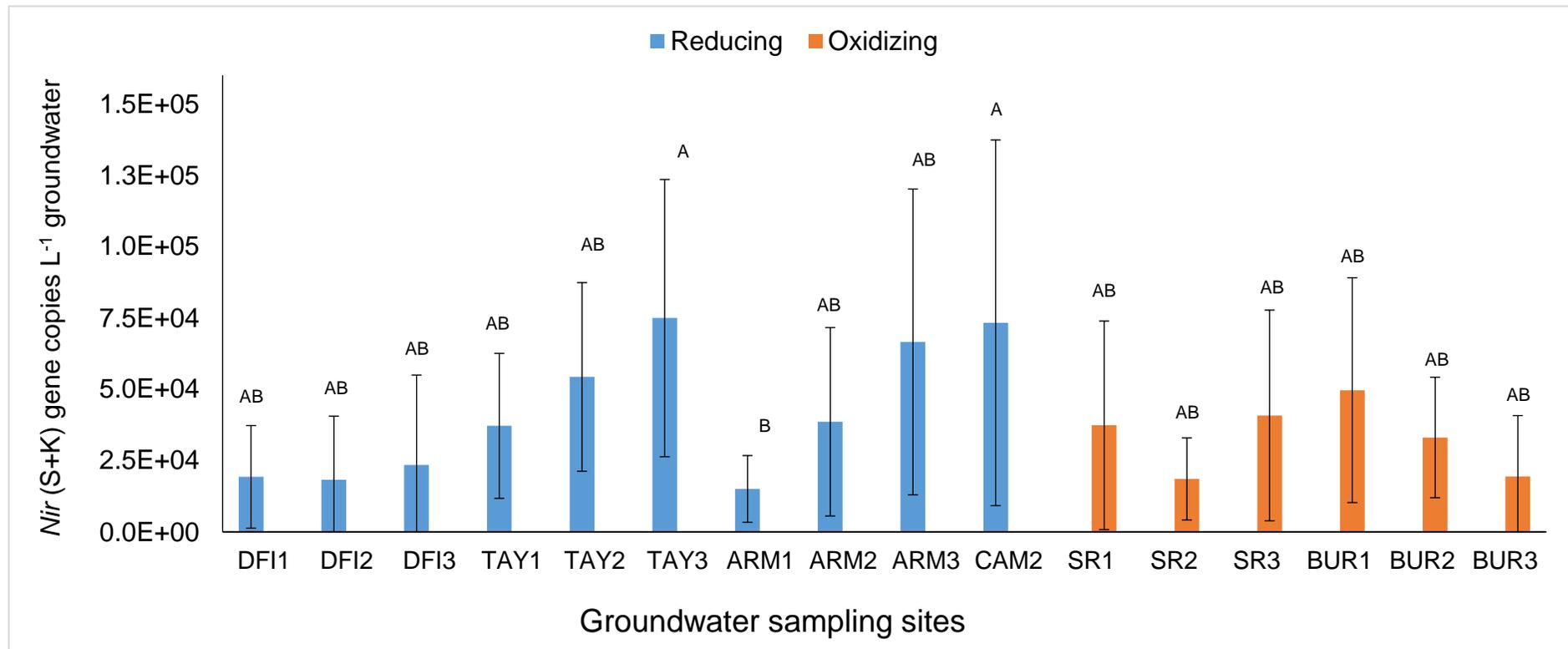


Figure 3. Variation in denitrifier gene abundance (*nir S+K*) observed in shallow groundwater samples collected at 6 pastoral farm sites (Table 1) in the Manawatu and Rangitikei catchments, from August 2017 to January 2018. Data points are the mean \pm standard error ($n = 3$). The blue bars are anoxic sites ($\text{DO} < 1 \text{ mg L}^{-1}$) and the orange bars are the oxic sites ($\text{DO} > 1 \text{ mg L}^{-1}$). Letter values on the bars denote one-way ANOVA test. Values sharing the same letter are not significantly different. Sample names denote site and piezometer for the collection of groundwater sample (Table 1). Due to very slow recharge of groundwater, CAM1 was not sampled and analysed for microbial analysis.

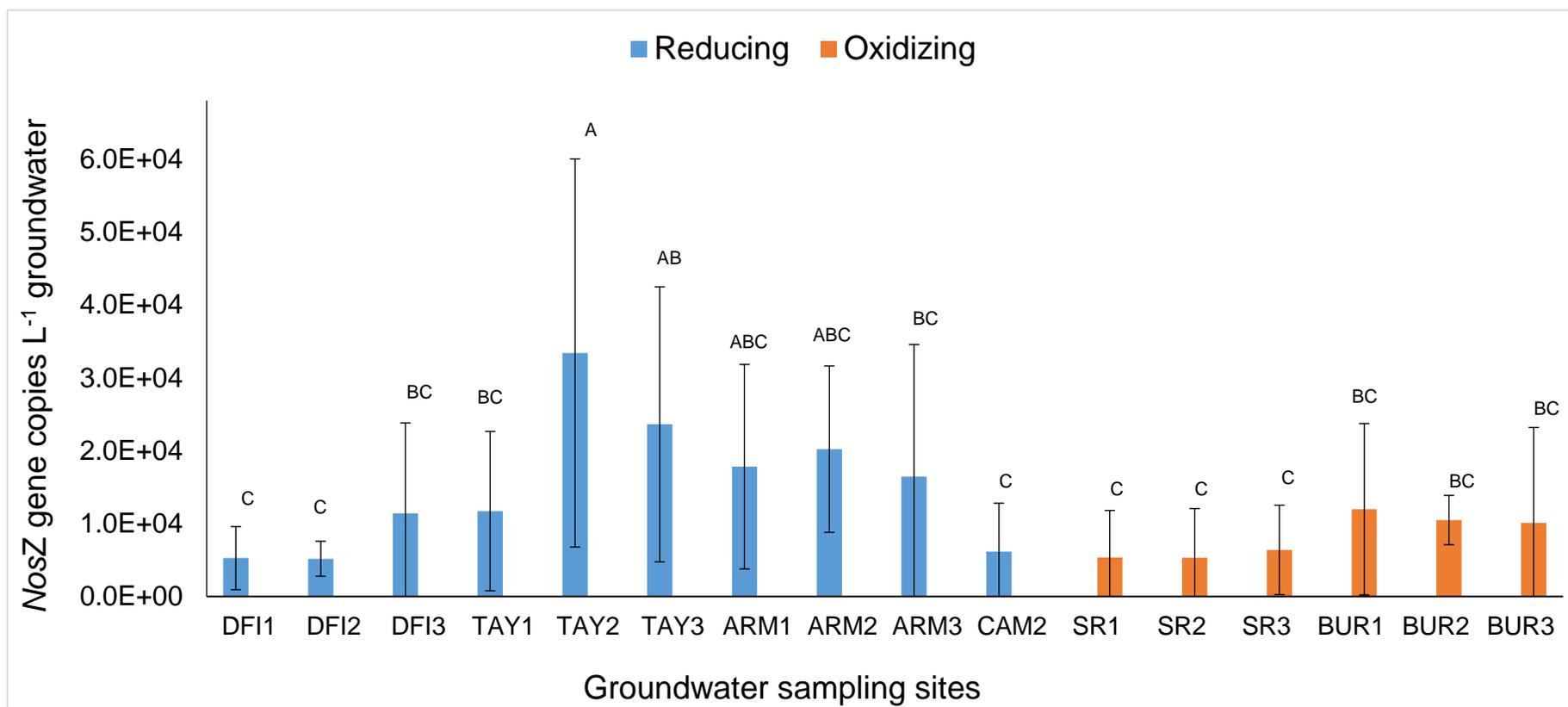


Figure 4. Variation in denitrifier gene abundance (*nosZ*) observed in shallow groundwater samples collected at 6 pastoral farm sites (Table 1) in the Manawatu and Rangitikei catchments, from August 2017 to January 2018. Data points are the mean \pm standard error ($n = 3$). The blue bars are anoxic sites ($DO < 1 \text{ mg L}^{-1}$) and the orange bars are the oxic sites ($DO > 1 \text{ mg L}^{-1}$). Letter values on the bars denote one-way ANOVA test. Values sharing the same letter are not significantly different. Sample names denote site and piezometer for the collection of groundwater sample (Table 1). Due to very slow recharge of groundwater, CAM1 was not sampled and analysed for microbial analysis.

Conclusions

Effective and targeted strategies for reducing NO_3^- leaching from agricultural lands to waters and net N_2O emissions to the atmosphere require a sound understanding and quantification of NO_3^- flows and its transformations in surface soils and subsurface environment (beyond the root zone). This study highlights the variability in the redox conditions, chemical characteristics, dissolved gases (N_2O and N_2) contents and abundance of denitrifier genes (*nosZ*) in shallow groundwater samples collected from 6 contrasting sites across the Manawatu and Rangitikei catchments. The preliminary results presented here show that the oxidizing shallow groundwater ($\text{DO} > 1 \text{ mg L}^{-1}$) had high dissolved NO_3^- -N, lower *nosZ* gene abundance and higher dissolved N_2O levels as compared to the reducing shallow groundwaters ($\text{DO} < 1 \text{ mg L}^{-1}$). Our observations so far suggest the occurrence of predominantly a complete 'benign' denitrification that results in very low N_2O production under the reducing groundwater conditions. We suggest further observations and analysis of this efficacy of subsurface denitrification to attenuate NO_3^- -N in shallow groundwaters across contrasting New Zealand catchments. This new observations on efficacy and variability of subsurface denitrification will offer a transformative opportunity for targeted and effective nitrogen management practices and policies for improved water quality outcomes in our sensitive agricultural catchments.

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