

# microbial biotechnology

Open Access

## Influence of copper on expression of *nirS*, *norB* and *nosZ* and the transcription and activity of NIR, NOR and N<sub>2</sub>OR in the denitrifying soil bacteria *Pseudomonas stutzeri*

Amanda Black,<sup>1,\*</sup> Pei-Chun L. Hsu,<sup>1</sup> Kelly E.Hamonts,<sup>1,2</sup> Tim J. Clough<sup>3</sup> and Leo M. Condon<sup>3</sup><sup>1</sup>Bio Protection Research Centre, Lincoln University, PO Box 85084, Lincoln, Christchurch 7647, New Zealand.<sup>2</sup>Hawkesbury Institute for the Environment, University of Western Sydney, Locked Bag 1797, Penrith, NSW 2751, Australia.<sup>3</sup>Faculty of Agriculture and Life Sciences, Lincoln University, PO Box 85084, Lincoln, Christchurch 7647, New Zealand.

### Summary

Reduction of the potent greenhouse gas nitrous oxide (N<sub>2</sub>O) occurs in soil environments by the action of denitrifying bacteria possessing nitrous oxide reductase (N<sub>2</sub>OR), a dimeric copper (Cu)-dependent enzyme producing environmentally benign dinitrogen (N<sub>2</sub>). We examined the effects of increasing Cu concentrations on the transcription and activity of nitrite reductase (NIR), nitric oxide reductase (NOR) and N<sub>2</sub>OR in *Pseudomonas stutzeri* grown anaerobically in solution over a 10-day period. Gas samples were taken on a daily basis and after 6 days, bacterial RNA was recovered to determine the expression of *nirS*, *norB* and *nosZ* encoding NIR, NOR and N<sub>2</sub>OR respectively. Results revealed that 0.05 mM Cu caused maximum conversion of N<sub>2</sub>O to N<sub>2</sub> via bacterial reduction of N<sub>2</sub>O. As soluble Cu generally makes up less than 0.001% of total soil Cu, extrapolation of 0.05 mg l<sup>-1</sup> soluble Cu would require soils to have a total concentration of Cu in the range of, 150–200 µg g<sup>-1</sup> to maximize the proportion of N<sub>2</sub>O reduced to N<sub>2</sub>. Given that many intensively farmed agricultural soils are deficient in Cu in terms

of plant nutrition, providing a sufficient concentration of biologically accessible Cu could provide a potentially useful microbial-based strategy of reducing agricultural N<sub>2</sub>O emissions.

### Introduction

Since the industrial revolution, global agricultural intensification and the use of artificial fertilizers has increased the amount of reactive nitrogen (N) in the natural environment by an order of magnitude (Galloway *et al.*, 2003; Richardson *et al.*, 2009; Taylor and Townsend, 2010). This has resulted in a reorganization of the global N cycle causing a number of environmental problems including increased nitrous oxide (N<sub>2</sub>O) emissions (Taylor and Townsend, 2010; Magalhaes *et al.*, 2011).

Nitrous oxide accounts for ~10% of the total greenhouse gas emissions and is produced as a by-product of bacterial and fungal respiration pathways in soil. Both denitrification and nitrification respiratory pathways emit N<sub>2</sub>O with rates increasing with the addition of N fertilizer (Taylor and Townsend, 2010; Magalhaes *et al.*, 2011). Denitrification occurs when oxygen (O<sub>2</sub>) is in limited supply and bacteria with denitrifying capability can switch to respiring nitrate (NO<sub>3</sub><sup>-</sup>), converting NO<sub>3</sub><sup>-</sup> to nitrite (NO<sub>2</sub><sup>-</sup>) and the gases nitric oxide (NO) and N<sub>2</sub>O and finally dinitrogen (N<sub>2</sub>) (Fig. 1). This process requires four enzymes to sequentially reduce NO<sub>3</sub><sup>-</sup> to N<sub>2</sub> with each of these enzymes requiring a redox metal cofactor (Fig. 1). Denitrifying soil bacteria such as *Pseudomonas stutzeri* generate N<sub>2</sub>O via the reduction of NO, an endogenous cytotoxin, by reducing N<sub>2</sub>O via the enzyme nitric oxide reductase (NOR), hence bacteria deficient in NOR cannot grow through denitrification (Zumft, 2005a,b). Because so much N<sub>2</sub>O is produced from soils carrying out bacterial denitrification, it implies that the bacterial enzyme nitrous oxide reductase (N<sub>2</sub>OR), or the bacterial population as a whole, do not always carry out the reduction of N<sub>2</sub>O to N<sub>2</sub> efficiently or in synchrony with pathways upstream (i.e. nitrifier-denitrification) (Richardson *et al.*, 2009). Thus, managing N<sub>2</sub>O emissions requires consideration of the factors affecting the production of N<sub>2</sub>O at both the molecular and soil microbial ecology levels.

Nitric oxide reductase is a key enzyme in the production of N<sub>2</sub>O and is responsible for catalysing the

Received 27 August, 2014; accepted 24 January, 2016. \*For correspondence. E-mail [amanda.black@lincoln.ac.nz](mailto:amanda.black@lincoln.ac.nz); Tel. +64 3 423 0906; Fax +64 3 32503864.

*Microbial Biotechnology* (2016) 9(3), 381–388

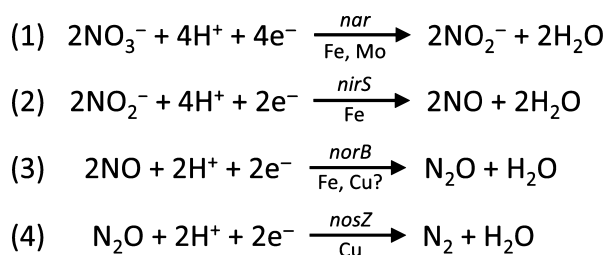
doi:10.1111/1751-7915.12352

### Funding information

This research was funded by the New Zealand Ministry of Innovation, Business and Employment (Ministry of Science and Innovation contract number LINX0904).

© 2016 The Authors. *Microbial Biotechnology* published by John Wiley & Sons Ltd and Society for Applied Microbiology.

This is an open access article under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.



**Fig. 1.** Nitrate is reduced to nitrogen gas under anaerobic condition via the denitrification process of *Pseudomonas stutzeri*. Arrows indicate an operon or gene required for each reaction to occur with metal cofactor requisite for the enzyme complex.

reduction of NO to N<sub>2</sub>O (Fig. 1, equation 3). Under intensive pasture management, N<sub>2</sub>O is the predominant end-product during the denitrification process, which is a main contributor to global N<sub>2</sub>O emissions through the agricultural nitrogen cycle (Mosier *et al.*, 1998). The structure of NOR has been well studied and is known to be a member of the haem-copper oxidase superfamily (Zumft *et al.*, 1994). The homology analogue of this protein taken from *Paracoccus denitrificans*, *Pseudomonas aeruginosa*, *Bacillus azotoformans* and *Ralstonia eutropha* were also comprehensively characterized (DeBoer *et al.*, 1996; Pohlmann *et al.*, 2000; Suharti *et al.*, 2001) *P. aeruginosa*. NOR is expressed by *P. stutzeri* under anaerobic conditions (Korner, 1993) and is found exhibiting as a complex consisting of several components including high-spin cytochrome b, low-spin Fe(III) haem centre and a cytochrome c subunit or short-chain NOR acting as the electron donor at the active centre (Zumft, 2005a,b). Although the majorities of NORs reported consist of iron-binding site, remarkable structural similarities was found between *cbb3* cytochrom oxidase and NOR which contains a high-spin haem-copper (CuB) active site (Vanderost *et al.*, 1994). Furthermore, there is emerging evidence that NOR from *Bacillus azotoformans* to be a hybrid between copper-containing cytochrome oxidases and NOR found in Gram-negative bacteria (Suharti *et al.*, 2001; Al-Attar and de Vries, 2015). Due to the similarities between *cbb3* cytochrom oxidase and NOR, and lack of crystallography structure evidence, it can be questionable whether NOR from *P. stutzeri* is also regulated by copper metal cofactor. Moreover, Cu-dependency of NOR from *P. stutzeri* has not been investigated before.

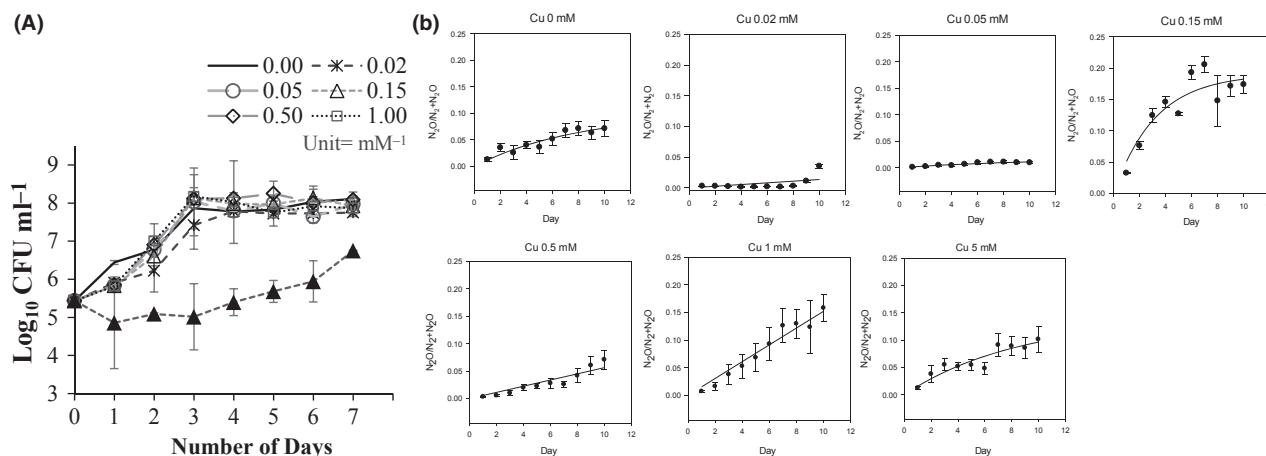
The reduction of N<sub>2</sub>O to N<sub>2</sub> is strongly exergonic (Fig. 1, equation 4) ( $\Delta G^\circ = -339.5 \text{ kJ mol}^{-1}$ ), and electron delocalization stabilizes the molecule and leads to an activation energy barrier of 250 kJ mol<sup>-1</sup> (Tolman, 2010). However, N<sub>2</sub>OR, first identified in 1982 (Zumft and Matsubara, 1982), is the only known enzyme capable of reducing N<sub>2</sub>O to N<sub>2</sub> during the denitrification process (Zumft and Kroneck, 2007). The N<sub>2</sub>OR enzyme

is a Cu-dependent enzyme with a three-dimensional structure revealing a multi-Cu sulfide centre Cu<sub>z</sub> [4: Cu:2S] where the two-electron reduction of N<sub>2</sub>O takes place (Richardson *et al.*, 2009). The Cu requirement for the active dimeric form of N<sub>2</sub>OR requires the bacterium to have an adequate supply of Cu. An absence of Cu in some culture studies has resulted in a rise in N<sub>2</sub>O emissions (Granger and Ward, 2003). While a Cu-deficient denitrifying bacterial community can still remain viable, it is likely that they will release much higher levels of N<sub>2</sub>O. Several studies have documented the central role of Cu in enzyme structure and function (Matsubara *et al.*, 1982; Farrar *et al.*, 1998; Zumft, 2005a,b; Pomowski *et al.*, 2010); however, the effects of Cu on the expression of these genes *nirS*, *norB* and *nosZ* to increase Cu bioavailability have yet to be described. We hypothesize that denitrifying bacteria have the ability to sense Cu availability and adjust the synthesis of N<sub>2</sub>OR in response to environmental conditions. In this study, we examine the effects of increasing Cu concentrations on the expression and activity of nitrite reductase (NIR), NOR and N<sub>2</sub>OR in *P. stutzeri* and the potential implications for molecular and stoichiometric influences on N<sub>2</sub>O emissions from agricultural soils.

## Results and discussion

### *The effect of increasing copper concentrations on nitrous oxide accumulation and conversion into dinitrogen in anaerobic cultures of Pseudomonas stutzeri*

Growth of *P. stutzeri* from anaerobic cultures over 7-day period showed no significant difference throughout all Cu concentration, except 5.00 and 20.00 mM of Cu (Fig. 1A), and the growth reached stationary phase at day 3 with  $\leq 1.00$  mM of Cu. Colony counts of *P. stutzeri* cultures containing 5.00 mM Cu were significantly reduced compared with those in Cu concentrations  $< 5.00$  mM with a delay in growth after day 3 (Fig. 1A). This could indicate the onset of toxicological effects, although denitrification was occurring at relatively high rate (Fig. 2B). As expected, given *P. stutzeri* the highest concentration of Cu (20.00 mM) inhibited the growth completely (result not shown). Daily measurements revealed production of N<sub>2</sub>O and subsequent reduction to N<sub>2</sub> varied significantly with changes in Cu concentrations (Fig. 2B). Interestingly, the Cu-deficient broth (control, 0.00 mM) did not produce the highest concentrations of N<sub>2</sub>O compared with the Cu-containing treatments as has been suggested in a previous study to be the most likely outcome (Granger and Ward, 2003). Furthermore, a comparison between the total amount of N<sub>2</sub> produced per Cu treatment revealed no significant differences. In fact, the highest amount of N<sub>2</sub>O produced was observed in the culture containing 0.15 mM, which corresponded



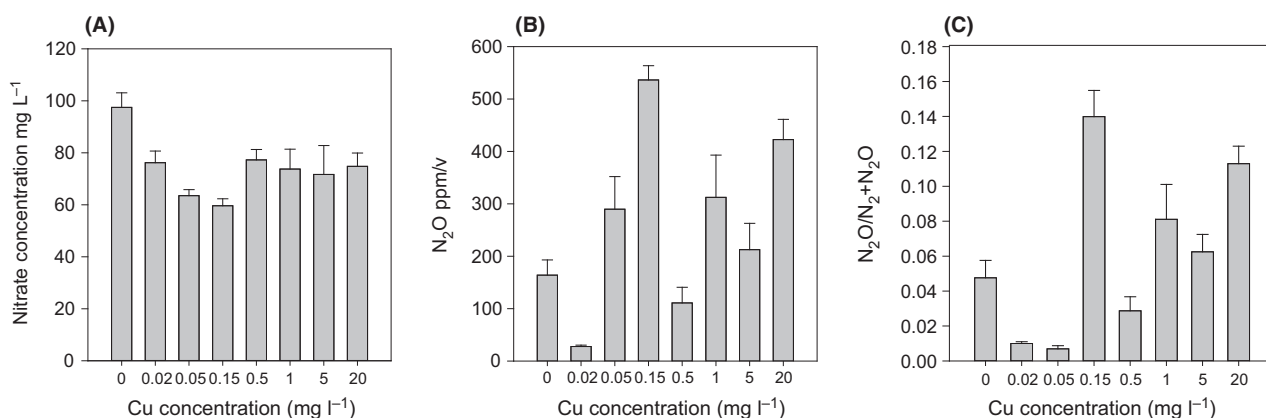
**Fig. 2.** Growth curve of *Pseudomonas stutzeri* over the course of 7 days (A). Mean  $\text{N}_2\text{O}:(\text{N}_2+\text{N}_2\text{O})$  ratio of each copper treatment 0, 0.02, 0.05, 0.15, 0.5, 1, 5 and 20 mM over 10-day period (B). Standard error of the mean (SEM) are represented as error bars.

to the amount of  $\text{NO}_3^-$  consumed (Fig. 3A and B). The proportion of  $\text{N}_2\text{O}$  to  $\text{N}_2\text{O} + \text{N}_2$  in the headspace differed significantly ( $P < 0.001$ ) between Cu treatments, with the lowest proportion of  $\text{N}_2\text{O}$  to total N measured in the treatments containing 0.02 and 0.05 mM of Cu (Fig. 3C). Over the 10-day sampling period (apart from 0.50 mM Cu treatment), treatments  $\geq 0.15$  mM Cu resulted in a significantly higher ( $P < 0.001$ ) total yield of  $\text{N}_2\text{O}$  than  $\text{N}_2$ . Studies that have investigated the effect of Cu addition on the conversion rate of  $\text{N}_2\text{O}$  to  $\text{N}_2$  concluded that the absence of Cu resulted in an accumulation of  $\text{N}_2\text{O}$  (Granger and Ward, 2003; Manconi *et al.*, 2006; Felgate *et al.*, 2012). However in these studies, the Cu concentration at which  $\text{N}_2\text{OR}$  maintained an optimum activity and where the lowest proportion of  $\text{N}_2\text{O}$  to  $\text{N}_2$  levels were generated, were not evaluated in further detail. Additionally, the presence of sulfide, especially  $\text{H}_2\text{S}$  in a Cu-deficient environment has been known to affect the reduction of  $\text{N}_2\text{O}$  to  $\text{N}_2$  (Manconi *et al.*, 2006; Pan *et al.*, 2013). Given that in our study,  $\text{S}_2^{2-}$  and  $\text{H}_2\text{S}$  could have been generated during anaerobic incubation of the broth from  $\text{CuSO}_4$  (source of the Cu) and cysteine, this may have also contributed to the accumulation of  $\text{N}_2\text{O}$ .

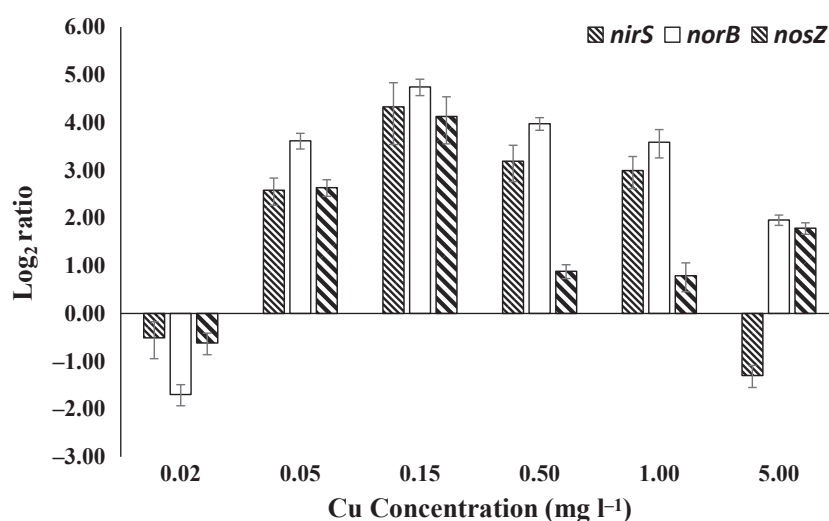
#### The effect of copper availability on the expression of *nirS*, *norB* and *nosZ*

From the Cu treatments it emerges that a Cu concentration of 0.15 mM is the level at which *nirS*, *norB* and *nosZ* expression are at their highest (Fig. 4), although  $\text{N}_2\text{O}$  level and the mean  $\text{N}_2\text{O}:(\text{N}_2+\text{N}_2\text{O})$  ratio was at the highest of all Cu concentration given. Our results show inhibition of *nosZ* transcription from a Cu concentration of 0.50 mM onwards. Interestingly, even though the *nirS* enzyme contains a haem-iron cofactor, it appears the transcription of *nirS* is responsive to changes in Cu con-

centrations (Fig. 4). Furthermore, *norB* expression was also declined with increased Cu concentration. Similarly, in a previous study conducted by Magalhães *et al.* (2011), a pronounced inhibition by Cu on the transcription of *nosZ* and *nirS* was detected. In the treatment containing 0.15 mM of Cu, the  $\text{N}_2\text{O}:(\text{N}_2+\text{N}_2\text{O})$  ratio was elevated as well as the observation of comparatively high levels of denitrifying genes expressed. This suggests that there could exist an alternative pathway for *P. stutzeri* to synthesize  $\text{N}_2\text{O}$  at higher levels of Cu; thus, the level of  $\text{N}_2\text{O}$  produced due to NOR activity could be overestimated in this study. X-ray crystallographic structures of the periplasmic membrane protein *cbb3* cytochrome oxidase of *P. stutzeri* revealed a high-spin haem-copper (CuB) binuclear centre of which the catalytic active site was also found reducing NO to  $\text{N}_2\text{O}$  during denitrification process via the proton pathway through K-channel (Forte *et al.*, 2001; Buschmann *et al.*, 2010). Increased Cu concentration might also contribute in elevating the  $\text{N}_2\text{O}$  level via NO reduction by *cbb3* cytochrome oxidase in this study. Therefore, expression of genes involved in *cbb3* cytochrome oxidase (*ccoQ-NOS*) should further be investigated. This may provide a more detailed insight of  $\text{N}_2\text{O}$  formation regulated by Cu from an alternative pathway. The enzyme  $\text{N}_2\text{OR}$  is considered as the only protein catalysed by Cu due to the multi-Cu-sulfide redox centres (Pomowski *et al.*, 2010). Surprisingly, expression of *nosZ* did not respond to the incensement of Cu concentration in this study, instead, the transcriptional level of *nosZ* decreased tremendously at 0.50 mM of Cu onward (Fig. 4). This suggested high level of Cu could be displaying inhibitory effect. This result differs with a study conducted by Felgate *et al.* (2012) where transcription of *nosZ* of *P. denitrificans* was found upregulated at high Cu concentration ( $13 \mu\text{mol l}^{-1}$  which is equivalent to 2 mM).



**Fig. 3.** Mean nitrate (A) and  $N_2O$  (B) concentrations after 10 days and mean  $N_2O:(N_2+N_2O)$  ratio (C). Standard error of the mean (SEM) are represented as error bars. Groups with different letters differ significantly at the 5% level.



**Fig. 4.** Expression ratios of the *Pseudomonas stutzeri* *nirS*, *norB* and *nosZ* at 5 days post-inoculation. Expression ratios were calculated and normalized against reference genes *fdxA*, *ropD* and *gyrB*. Expression ratios are the difference in gene expression of *Pseudomonas stutzeri* cultured in basal salt solution with different copper concentration relative to the RNA expression in culture incubated without the presence of copper. Error bars are the SEM for all sample replicates.

Felgate *et al.* (2012) tested both pure (*P. denitrificans*) and mixed denitrifying cultures using 13.00  $\mu\text{M}$  and 0.50  $\mu\text{M}$  Cu in excess and limiting  $\text{NO}_3^-$  and C availability to investigate the production of  $\text{N}_2\text{O}$  and the accumulation of intermediate products, namely  $\text{NO}_2^-$ . Although the Cu concentrations used were an order of magnitude lower than the lowest concentrations used in this study and atypical of bioavailable Cu concentrations found in agricultural soils (Black *et al.*, 2011), results demonstrated that if sufficient  $\text{NO}_3^-$  was present when Cu was depleted, *P. denitrificans* would maintain biomass, but release  $\text{N}_2\text{O}$  at a rate > 1000 times the rate of the corresponding Cu replete cultures. It was also noted that the  $\text{N}_2\text{O}$  electron acceptor lost under Cu-depleted conditions was compensated for by increased ( $\approx 20\%$ ) consumption

of  $\text{NO}_3^-$  compared with cultures replete in Cu. This observation of  $\text{N}_2\text{O}$  being produced more quickly but consumed more slowly is mirrored when comparing the control and 0.02 mM Cu treatment concentration (Fig. 2A and B), although nitrate consumption did not increase. The  $\text{N}_2\text{O}:(\text{N}_2\text{O}+\text{N}_2)$  trend observed in this study reflected observations made in a denitrifying community sourced from river sediments which were enriched in Cu due to agriculture run-off (Magalhaes *et al.*, 2011). The suggested reason for the observed pattern was the different sensitivities of each of the enzymes that catalyse the first three steps of denitrification ( $\text{NO}_3^- \rightarrow \text{NO}_2^-$ ,  $\text{NO}_2^- \rightarrow \text{NO}^-$  and  $\text{NO}^- \rightarrow \text{N}_2\text{O}$ ) (Magalhaes *et al.*, 2011). Similarly, the authors found that denitrification rates were highly affected by Cu



concentrations, moreover they also observed a decrease in the diversity of *nirK*, *nirS* and *nosZ* and the corresponding transcribed enzymes with increasing Cu.

Results from this study suggest that Cu bioavailability can influence the expression and activity of N<sub>2</sub>OR and NIR, as well as the growth rates of *P. stutzeri* and imply that it may be possible to use stoichiometry to manage N<sub>2</sub>O emissions from agricultural soils. This approach has been previously suggested as a possible strategy to mitigate N<sub>2</sub>O emissions by providing an adequate source of essential micronutrients particularly Cu, Mo and Fe for redox reactions (Richardson *et al.*, 2009). Although the reduction of N<sub>2</sub>O to N<sub>2</sub> is not energetically favoured under optimal conditions, this reduction does occur at no loss of energy requirements to the bacteria. Moreover, our results confirm that there indeed exists an optimal Cu concentration threshold for *P. stutzeri* with respect to maximizing N<sub>2</sub>O consumption.

## Conclusion

In summary, our findings do support a key role for Cu in the regulation of N<sub>2</sub>O emissions, by demonstrating a Cu concentration gradient effect in the production and consumption of N<sub>2</sub>O. While total soil Cu is still the most common soil measure of bioavailability, bioavailable Cu is typically < 0.001% of total soil Cu (Bolan *et al.*, 2003; McLaren *et al.*, 2010; Black *et al.*, 2011). Thus, values of 0.02–0.15 Cu mM used in this study are more realistic representations of biologically available Cu in agricultural soil environments to consider for potential management strategies of N<sub>2</sub>O emissions (Richardson *et al.*, 2009). Extrapolating the Cu concentrations used in this study into an agricultural setting imply in some situations, current soil levels of Cu maybe deficient to allow this enzymatic pathway to operate at an optimal level. Calculating back from total soil Cu concentrations to biologically available Cu (0.05–0.10 mM Cu in solution) that is required to attain maximum conversion of N<sub>2</sub>O to N<sub>2</sub>, equates to a total amount of soil Cu in the range 150–200 µg g<sup>-1</sup> (Black *et al.*, 2011). Soils containing higher concentrations of Cu may result in inhibition of denitrification via a shift in denitrifier community composition (Manconi *et al.*, 2006; Taylor and Townsend, 2010; Felgate *et al.*, 2012) and correspondingly, Cu-deficient soils may result in N<sub>2</sub>O production exceeding N<sub>2</sub>O conversion to N<sub>2</sub>.

Using one species of denitrifying bacteria in a simple basal salt solution has provided some insight into the effects of increasing Cu bioavailability on the transcription and activity of N<sub>2</sub>OR and NIR to reduce N<sub>2</sub>O to N<sub>2</sub>. We observed that adequate bioavailable Cu concentrations (0.15 mM) resulted in the greatest transcription of the *nirS*, *norB* and *nosZ*, which is not the optimal

consumption of N<sub>2</sub>O to produce N<sub>2</sub>. However, the level of N<sub>2</sub>O may be overestimated due to another possible alternative pathway of NO reduction by *cbb3* cytochrome oxidase. Based on our experience with measuring amounts of bioavailable soil Cu, extrapolation of this soluble Cu concentration to an agricultural soil environment equates to total soil Cu concentrations in the range of 150–200 µg g<sup>-1</sup>. Furthermore, a majority of soils globally under intensive agriculture, are now considered too deficient in Cu to complete the last stages of the denitrification process to N<sub>2</sub> (Skrbic and Durisic-Mladenovic, 2010). Therefore, in addition to other mitigation methods, supplying adequate Cu would contribute to an overall effective management of N<sub>2</sub>O emissions in increasing N intensification systems. Future studies will need to investigate if similar Cu concentrations may apply to other soil denitrifying bacteria, including investigating the possibility of adopting a Cu strategy for whole soil microbial communities. However, any strategy employed would have to consider any potential toxicity issues for grazing animals.

## Experimental procedures

### *Cultivation conditions of P. stutzeri (ATCC 17588)*

The denitrifying bacteria *P. stutzeri* (ATCC 17588) was chosen due to the presence of *nirS* (Fe-cofactor) instead of *nirK* (Cu-cofactor). Bacteria were first grown in cultures under aerobic conditions. The basal salt solution of the aerobic medium comprised the following (mM): 1.5 KH<sub>2</sub>PO<sub>4</sub>, 5.6 NH<sub>4</sub>Cl, 0.5 MgCl<sub>2</sub>·6H<sub>2</sub>O, 0.7 CaCl<sub>2</sub>·2H<sub>2</sub>O, 0.4 MgSO<sub>4</sub>·7H<sub>2</sub>O and 30 KNO<sub>3</sub>. The solution was autoclaved and after cooling 99% D-Na lactate (Sigma Aldrich, St Louis, Missouri, United States) was added to a final concentration of 31 mM in 100 ml volume (Johnsson *et al.*, 2006). The aerobic culture was grown in 100 ml solution contained per 250 ml Erlenmeyer flasks for 4 days before 1 ml of culture-containing solution, containing approximately 10<sup>6</sup> bacterial cells, was transferred into the anaerobic Cu medium experiment.

The basal salt solution of the anaerobic medium comprised (mM) 0.06 KH<sub>2</sub>PO<sub>4</sub>, 5.6 NH<sub>4</sub>Cl, 0.5 MgCl<sub>2</sub>·6H<sub>2</sub>O, 0.8 0.7 CaCl<sub>2</sub>·2H<sub>2</sub>O, 0.4 MgSO<sub>4</sub>·7H<sub>2</sub>O and 11 mM of <sup>15</sup>N-enriched KNO<sub>3</sub> (10 atom%). The pH was adjusted to 8 using 1 M NaOH, with sufficient KH<sub>2</sub>PO<sub>4</sub> in solution to provide buffering capacity (Johnsson *et al.*, 2006). Cu sulfate (CuSO<sub>4</sub>) was added to each gas-tight medical boston bottle at concentrations equivalent to 0.00, 0.02, 0.05, 0.15, 0.50, 1.00, 5.00, 20.00 mM of Cu. The basal solution (100 ml per bottle) with Cu treatments was autoclaved and 99% D-Na lactate and cysteine-HCl (Sigma Aldrich) were added to produce final concentrations of 4.40 and 4.50 mM respectively. Copper treatments were performed in triplicate and each treatment

replicate was inoculated with 1 ml of  $10^6$  bacterial cells before being fitted with gas-tight Teflon<sup>®</sup> septa and purged using 99.99% Argon (Ar) (remaining 0.01% comprising  $\text{CO}_2 < 5 \text{ ppm}$ ,  $\text{N}_2 < 25 \mu\text{l l}^{-1}$ ,  $\text{O}_2 < 10 \mu\text{l l}^{-1}$ ,  $\text{H}_2\text{O} < 10 \mu\text{l l}^{-1}$ ,  $\text{CO}$ ,  $\text{C}_2\text{H}_6$ ,  $\text{CH}_4 < 1 \mu\text{l l}^{-1}$ ) to create an anaerobic environment with minimum  $\text{N}_2$ . Non-inoculated controls were run simultaneously. The total headspace volume of each bottle was 150 ml. Cultures were grown in a pressurized Ar atmosphere at room temperature (23°C) and were agitated on an orbital shaker for 10 days. Colony-forming units were used to estimate the number of viable cells for each replicated treatment each day using 10-fold dilution (up to  $10^{-9}$ ) with 100  $\mu\text{l}$  of diluted cells plated out onto solidified Luria–Bertani agar (Oxoid Thermo Fisher Scientific Inc. Waltham, MA, United States) and incubated for 3 days at room temperature (24°C) for 3 days.

#### Gas sampling and analyses

Gas samples were taken on a daily basis for 10 days (2 days after inoculation) where 1 ml of headspace was extracted and added to 11 ml of helium (99.99% pure) contained in 12 ml glass Exetainers<sup>®</sup>. Sample volume removed was replaced with 1 ml of Ar. Samples were analysed for Ar,  $\text{O}_2$ ,  $\text{N}_2$ ,  $\text{N}_2\text{O}$  and  $^{15}\text{N}$  using a Continuous Flow Isotope Ratio Mass Spectrometer (PDZ Europa TGII/20-20). The concentration of  $^{15}\text{N-N}_2$  was below the limit of quantification and thus not reported. Accuracies of Cu concentrations per treatment were verified at  $\pm 10\%$  at the end of the experiment using inductively coupled plasma optical emissions spectrophotometer (ICP-OES).

#### Anion and cation analyses

After 10 days, 10 ml of the growth medium was filtered using a 0.22  $\mu\text{m}$  cellulose filter (DISMIC, Advantec, Tokyo, Japan) and analysed for  $\text{NO}_2^-$  and  $\text{NO}_3^-$  concentrations using a Dionex DX-120 Suppressed Ion

Exchange Chromatograph (Dionex Corporation, Sunnyvale, CA, USA). Copper concentrations were confirmed using an ICP-OES Varian 720-ES fitted with a SPS-3 auto-sampler and ultrasonic nebulizer.

#### Expression of *nirS*, *norB* and *nosZ*

Bacterial RNA was extracted from the centrifuged bacterial pellet (20 000 r.p.m. for 10 min at 4°C) at day 5 using Roche High Pure RNA Isolation Kit (Cat. No. 11 828 665 001; Basel, Switzerland) according to the manufacturer's specifications. The RNA was treated twice with RNase-free DNase I recombinant (Roche) to remove any genomic DNA contamination. The RNA was then stored in RNA*later* (Invitrogen, Boston, MA, USA) at  $-80^\circ\text{C}$  before cDNA synthesis using Superscript III Supermix First Strand cDNA Synthesis Kit (Invitrogen) according to the manufacturer's instructions. The quality of the extracted RNA and cDNA was confirmed by gel electrophoresis, and yields of RNA and cDNA were measured with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

Transcripts of *nirS*, *norB* and *nosZ* encoding the cytochrome *cd<sub>1</sub>*, haem NIR, NOR and  $\text{N}_2\text{OR}$ , respectively, were quantified by qRT-PCR. Specific primers for three reference genes *fdxA*, *ropD* and *gyrB*, and one denitrification gene, *norB*, were designed from *Pseudomonas stutzeri* ATCC 17588 genome sequence (accession: PRJNA68131) using the web-based program Primer-BLAST (Ye *et al.*, 2012) to generate ~200 bp DNA sequences (Table 1). The cDNA products were amplified with an ABI Prism 7000 (Applied Biosystems, Mulgrave Australia) using SensiFAST<sup>™</sup> SYBR<sup>®</sup> Hi-ROX Kit (Bio-line, London, UK) according to supplier's instructions. Quantitative RT-PCR was performed with an initial denaturation at 95°C for 2 min, followed by 40 cycles of 95°C for 5 s, annealing at 60°C for 10 s and extension at 72°C for 20 s, with exception to the *nirS* which was amplified with an extension step for 30 s at 72°C.

**Table 1.** A list of oligonucleotide primers used in this study.

Gene	Primer name	Sequences (5'–3')	Fragment	Reference
<i>fdxA</i>	<i>fdxA</i> (F)	CCGGTGGACTGCTTCTACGA	208 bp	Present study
	<i>fdxA</i> (R)	CGGCCAGTGCATCCTTCTTC		
<i>gyrB</i>	<i>gyrB</i> (F)	GAATACCTCACCCAGTCGGC	198 bp	Present study
	<i>gyrB</i> (R)	CGTGACCCGAGGCAGATAGA		
<i>ropD</i>	<i>ropD</i> (F)	AGTACGATGCCCTGGTCGAG	198 bp	Present study
	<i>ropD</i> (R)	CGATGGCTTCGGCGTACTTG		
<i>nirS</i>	<i>nirS</i> Cd3aF	AACGYSAAAGGARACSSG	406 bp	Kandeler <i>et al.</i> (2006)
	<i>nirS</i> R3Cd	GASTTCGGRTGSGTCTTSAYGAA		
<i>norB</i>	<i>norB</i> (F)	CCATGCTCAAGGGTCGCAAG	197 bp	Present study
	<i>norB</i> (R)	CAGGACGAAGGCCAACATCG		
<i>nosZ</i>	<i>nosZ</i> 1840F	CGCRACGGCAASAAGGTSMSSTG	185 bp	Henry <i>et al.</i> (2006)
	<i>nosZ</i> 2090R	CAKRTGCAKSGCRTGGCAGAA		

Raw data were analysed based on Pfaffl *et al.* (2004) mathematical model to determine the relative quantitation of the target genes (*nirS*, *norB* and *nosZ*) that are normalized by three non-regulated reference genes (*fdxA*, *ropD* and *gyrB*). High resolution melting analysis was performed to determine PCR integrity and primer dimers at the end of each run. Each qRT-PCR efficiency is within the value between 1.60 and 2.10 and a  $R^2$  value of > 0.980. The qRT-PCR was performed in triplicate, and the means and standard errors were calculated.

#### Statistical analysis

Statistical analysis on gas results, ion chromatography for broth chemistry and colony-forming units were performed on log-transformed data using one-way ANOVA in GenStat (GenStat14; VSN International, Hemptstead UK). Non-linear regression to evaluate the relationships between ratios of  $N_2O:N_2+N_2O$  per treatment over the 10-day period was performed in GenStat (GenStat 16.1; VSN International).

#### Acknowledgements

This research was funded by the New Zealand Ministry of Innovation, Business and Employment (Ministry of Science and Innovation contract number LINX0904). We thank Roger Creswell and Neil Smith for their technical assistance and Tom W. Speir and Steve A. Wakelin for their critical input.

#### References

- Al-Attar, S., and de Vries, S. (2015) An electrogenic nitric oxide reductase. *FEBS Lett*, **589**: 2050–2057.
- Black, A., McLaren, R.G., Reichman, S.M., Speir, T.W., and Condon, L.M. (2011) Evaluation of soil metal bioavailability estimates using two plant species (*L. perenne* and *T. aestivum*) grown in a range of agricultural soils treated with biosolids and metal salts. *Environ Pollut*, **159**: 1523–1535.
- Bolan, N., Adriano, D., Mani, S., and Khan, A. (2003) Adsorption, complexation, and phytoavailability of copper as influenced by organic manure. *Environ Toxicol Chem*, **22**: 450–456.
- Buschmann, S., Warkentin, E., Xie, H., Langer, J.D., Ermler, U., and Michel, H. (2010) The structure of *cbb(3)* cytochrome oxidase provides insights into proton pumping. *Science*, **329**: 327–330.
- DeBoer, A.P.N., VanderOost, J., Reijnders, W.N.M., Westerhoff, H.V., Stouthamer, A.H., and VanSpanning, R.J.M. (1996) Mutational analysis of the *nor* gene cluster which encodes nitric-oxide reductase from *Paracoccus denitrificans*. *Eur J Biochem*, **242**: 592–600.
- Farrar, J.A., Zumft, W.G., and Thomson, A.J. (1998) Cu-A and Cu-Z are variants of the electron transfer center in nitrous oxide reductase. *Proc Natl Acad Sci USA*, **95**: 9891–9896.
- Felgate, H., Giannopoulos, G., Sullivan, M.J., Gates, A.J., Clarke, T.A., Baggs, E., *et al.* (2012) The impact of copper, nitrate and carbon status on the emission of nitrous oxide by two species of bacteria with biochemically distinct denitrification pathways. *Environ Microbiol*, **14**: 1788–1800.
- Forte, E., Urbani, A., Saraste, M., Sarti, P., Brunori, M., and Giuffrè, A. (2001) The cytochrome *cbb(3)* from *Pseudomonas stutzeri* displays nitric oxide reductase activity. *Eur J Biochem*, **268**: 6486–6490.
- Galloway, J.N., Aber, J.D., Erisman, J.W., Seitzinger, S.P., Howarth, R.W., Cowling, E.B., *et al.* (2003) The nitrogen cascade. *Bioscience*, **53**: 341–356.
- Granger, J., and Ward, B.B. (2003) Accumulation of nitrogen oxides in copper-limited cultures of denitrifying bacteria. *Limnol Oceanogr*, **48**: 313–318.
- Henry, S., Bru, D., Stres, B., Hallet, S., and Philippot, L. (2006) Quantitative detection of the *nosZ* gene, encoding nitrous oxide reductase, and comparison of the abundances of 16S rRNA, *narG*, *nirK*, and *nosZ* genes in soils. *Appl Environ Microbiol*, **72**: 5181–5189.
- Johnsson, A., Arlinger, J., Pedersen, K., Odegaard-Jensen, A., and Albinsson, Y. (2006) Solid-aqueous phase partitioning of radionuclides by complexing compounds excreted by subsurface bacteria. *Geomicrobiol J*, **23**: 621–630.
- Kandeler, E., Deiglmayr, K., Tschirko, D., Bru, D., and Philippot, L. (2006) Abundance of *narG*, *nirS*, *nirK*, and *nosZ* genes of denitrifying bacteria during primary successions of a glacier foreland. *Appl Environ Microbiol*, **72**: 5957–5962.
- Korner, H. (1993) Anaerobic expression of nitric-oxide reductase from denitrifying *Pseudomonas-stutzeri*. *Arch Microbiol*, **159**: 410–416.
- Magalhaes, C.M., Machado, A., Matos, P., and Bordalo, A.A. (2011) Impact of copper on the diversity, abundance and transcription of nitrite and nitrous oxide reductase genes in an urban European estuary. *FEMS Microbiol Ecol*, **77**: 274–284.
- Manconi, I., van der Maas, P., and Lens, P. (2006) Effect of copper dosing on sulfide inhibited reduction of nitric and nitrous oxide. *Nitric Oxide*, **15**: 400–407.
- Matsubara, T., Frunzke, K., and Zumft, W.G. (1982) Modulation by copper of the products of nitrite respiration in *Pseudomonas-perfectomarinus*. *J Bacteriol*, **149**: 816–823.
- McLaren, R.G., Black, A., and Clucas, L.M. (2010) Changes in Cu, Ni, and Zn availability following simulated conversion of biosolids-amended forest soils back to agricultural use. *Aust J Soil Res*, **48**: 286–293.
- Mosier, A., Kroeze, C., Nevison, C., Oenema, O., Seitzinger, S., and van Cleemput, O. (1998) Closing the global  $N(2)O$  budget: nitrous oxide emissions through the agricultural nitrogen cycle - OECD/IPCC/IEA phase II development of IPCC guidelines for national greenhouse gas inventory methodology. *Nutr Cycl Agroecosyst*, **52**: 225–248.
- Pan, Y., Ye, L., and Yuan, Z. (2013) Effect of  $H_2S$  on  $N_2O$  reduction and accumulation during denitrification by

- methanol utilizing denitrifiers. *Environ Sci Technol*, **47**: 8408–8415.
- Pfaffl, M.W., Tichopad, A., Prgomet, C., and Neuvians, T.P. (2004) Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper - Excel-based tool using pair-wise correlations. *Biotechnol Lett*, **26**: 509–515.
- Pohlmann, A., Cramm, R., Schmelz, K., and Friedrich, B. (2000) A novel NO-responding regulator controls the reduction of nitric oxide in *Ralstonia eutropha*. *Mol Microbiol*, **38**: 626–638.
- Pomowski, A., Zumft, W.G., Kroneck, P.M.H., and Einsle, O. (2010) Crystallization of purple nitrous oxide reductase from *Pseudomonas stutzeri*. *Acta Crystallogr Sect F Struct Biol Cryst Commun*, **66**: 1541–1543.
- Richardson, D., Felgate, H., Watmough, N., Thomson, A., and Baggs, E. (2009) Mitigating release of the potent greenhouse gas N(2)O from the nitrogen cycle - could enzymic regulation hold the key? *Trends Biotechnol*, **27**: 388–397.
- Skrbic, B., and Durisic-Mladenovic, N. (2010) Chemometric interpretation of heavy metal patterns in soils worldwide. *Chemosphere*, **80**: 1360–1369.
- Suharti, Strampraad, M.J.F., Schroder, I., and de Vries, S. (2001) A novel copper A containing menaquinol NO reductase from *Bacillus azotoformans*. *Biochemistry*, **40**: 2632–2639.
- Taylor, P.G., and Townsend, A.R. (2010) Stoichiometric control of organic carbon-nitrate relationships from soils to the sea. *Nature*, **464**: 1178–1181.
- Tolman, W.B. (2010) Binding and activation of N<sub>2</sub>O at transition-metal centers: recent mechanistic insights. *Angew Chem Int Ed Engl*, **49**: 1018–1024.
- Vanderoost, J., Deboer, A.P.N., Degier, J.W.L., Zumft, W.G., Stouthamer, A.H., and Vanspanning, R.J.M. (1994) The heme-copper oxidase family consists of 3 distinct types of terminal oxidases and is related to nitric-oxide reductase. *FEMS Microbiol Lett*, **121**: 1–9.
- Ye, J., Coulouris, G., Zaretskaya, I., Cutcutache, I., Rozen, S., and Madden, T.L. (2012) Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. *BMC Bioinformatics*, **13**: 1–11.
- Zumft, W.G. (2005a) Nitric oxide reductases of prokaryotes with emphasis on the respiratory, heme-copper oxidase type. *J Inorg Biochem*, **99**: 194–215.
- Zumft, W.G. (2005b) Biogenesis of the bacterial respiratory Cu-A, Cu-S enzyme nitrous oxide reductase. *J Mol Microbiol Biotechnol*, **10**: 154–166.
- Zumft, W.G. and Kroneck, P.M.H. (2007) Respiratory transformation of nitrous oxide (N(2)O) to dinitrogen by Bacteria and Archaea. In: *Advances in Microbial Physiology*. Poole, R.K. (ed). Academic Press Ltd-Elsevier Science Ltd, 24-28 Oval Road, London Nw1 7DX, England, pp. 107.
- Zumft, W.G., and Matsubara, T. (1982) A novel kind of multi-copper protein as terminal oxidoreductase of nitrous-oxide respiration in *pseudomonas-perfectomarinus*. *FEBS Lett*, **148**: 107–112.
- Zumft, W.G., Braun, C., and Cuypers, H. (1994) Nitric-oxide reductase from *Pseudomonas-stutzeri* - primary structure and gene organization of a novel bacterial cytochrome bc complex. *Eur J Biochem*, **219**: 481–490.