Influence of copper on expression of nirS, norB and nosZ and the transcription and activity of NIR, NOR and N₂OR in the denitrifying soil bacteria Pseudomonas stutzeri

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Summary
Reduction of the potent greenhouse gas nitrous oxide (N₂O) occurs in soil environments by the action of denitrifying bacteria possessing nitrous oxide reductase (N₂OR), a dimeric copper (Cu)-dependent enzyme producing environmentally benign dinitrogen (N₂). We examined the effects of increasing Cu concentrations on the transcription and activity of nitrite reductase (NIR), nitric oxide reductase (NOR) and N₂OR 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 N₂OR respectively. Results revealed that 0.05 mM Cu caused maximum conversion of N₂O to N₂ via bacterial reduction of N₂O. As soluble Cu generally makes up less than 0.001% of total soil Cu, extrapolation of 0.05 mg l⁻¹ Cu would require an additional 0.05 mg l⁻¹ Cu to maximise the proportion of N₂O reduced to N₂. 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 N₂O 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 (N₂O) 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 N₂O with rates increasing with the addition of N fertilizer (Taylor and Townsend, 2010; Magalhaes et al., 2011). Denitrification occurs when oxygen (O₂) is in limited supply and bacteria with denitrifying capability can switch to respiring nitrate (NO₃⁻), converting NO₃⁻ to nitrite (NO₂⁻) and the gases nitric oxide (NO) and N₂O and finally dinitrogen (N₂) (Fig. 1). This process requires four enzymes to sequentially reduce NO₃⁻ to N₂ with each of these enzymes requiring a redox metal cofactor (Fig. 1). Denitrifying soil bacteria such as Pseudomonas stutzeri generate N₂O via the reduction of NO, an endogenous cytotoxin, by reducing N₂O via the enzyme nitric oxide reductase (NOR), hence bacteria deficient in NOR cannot grow through denitrification (Zumft, 2005a,b). Because so much N₂O is produced from soils carrying out bacterial denitrification, it implies that the bacterial enzyme nitrous oxide reductase (N₂OR), or the bacterial population as a whole, do not always carry out the reduction of N₂O to N₂ efficiently or in synchrony with pathways upstream (i.e. nitrifier-denitrification) (Richardson et al., 2009). Thus, managing N₂O emissions requires consideration of the factors affecting the production of N₂O at both the molecular and soil microbial ecology levels.

Nitric oxide reductase is a key enzyme in the production of N₂O and is responsible for catalysing the
reduction of NO to N2O (Fig. 1, equation 3). Under intensive pasture management, N2O is the predominant end-product during the denitrification process, which is a main contributor to global N2O 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 (Komer, 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 (Vanderoost 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 cytochrome 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 N2O to N2 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\(^{-1}\) (Tolman, 2010). However, N2OR, first identified in 1982 (Zumft and Matsubara, 1982), is the only known enzyme capable of reducing N2O to N2 during the denitrification process (Zumft and Kroneck, 2007). The N2OR enzyme is a Cu-dependent enzyme with a three-dimensional structure revealing a multi-Cu sulfide centre Cu\(_4\) [4: Cu2S] where the two-electron reduction of N2O takes place (Richardson et al., 2009). The Cu requirement for the active dimeric form of N2O reductase requires the bacterium to have an adequate supply of Cu. An absence of Cu in some culture studies has resulted in a rise in N2O 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 N2O. 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 N2OR 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 N2OR in P. stutzeri and the potential implications for molecular and stoichiometric influences on N2O 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 stationery 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 N2O and subsequent reduction to N2 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 N2O 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 N2 produced per Cu treatment revealed no significant differences. In fact, the highest amount of N2O produced was observed in the culture containing 0.15 mM, which corresponded...
Studies that have investigated the effect of Cu addition to microbial systems have been performed (Manconi et al., 2012). Given that in our study, S2O32− treatment resulted in a significantly higher ($P < 0.001$) total yield of N2O than N2. Studies that have investigated the effect of Cu addition to the system have concluded that the absence of Cu resulted in an accumulation of N2O (Granger and Ward, 2003; Manconi et al., 2006; Felgate et al., 2012). However, in these studies, the Cu concentration at which N2OR maintained an optimum activity and where the lowest proportion of N2O to N2 levels were generated, were not evaluated in further detail. Additionally, the presence of sulfide, especially H2S, in a Cu-deficient environment has been known to affect the reduction of N2O to N2 (Manconi et al., 2006; Pan et al., 2013). Given that in our study, S2O32− and H2S could have been generated during an aerobic incubation of the broth from CuSO4 (source of the Cu) and cysteine, this may have also contributed to the accumulation of N2O.

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 N2O level and the mean N2O:(N2 + N2O) 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 concentrations (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 N2O:(N2 + N2O) 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 N2O at higher levels of Cu; thus, the level of N2O 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 N2O 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 N2O level via NO reduction by cbb3 cytochrome oxidase in this study. Therefore, expression of genes involved in cbb3 cytochrome oxidase (ccO-NOS) should further be investigated. This may provide a more detailed insight of N2O formation regulated by Cu from an alternative pathway. The enzyme N2OR 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 μmol l$^{-1}$ which is equivalent to 2 mM).

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Felgate et al. (2012) tested both pure \( \textit{P. denitrificans} \) and mixed denitrifying cultures using 13.00 mM and 0.50 mM Cu in excess and limiting NO\(_3^-\) and C availability to investigate the production of N\(_2\)O and the accumulation of intermediate products, namely NO\(_2^-\)/C\(_0\) and N\(_2\)/C\(_0\). 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 NO\(_3^-\) was present when Cu was depleted, \textit{P. denitrificans} would maintain biomass, but release N\(_2\)O at a rate > 1000 times the rate of the corresponding Cu replete cultures. It was also noted that the N\(_2\)O electron acceptor lost under Cu-depleted conditions was compensated for by increased (~20%) consumption of NO\(_3^-\) compared with cultures replete in Cu. This observation of N\(_2\)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 N\(_2\)O:(N\(_2\)O+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 N\(_2\)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 N2OR and NIR, as well as the growth rates of P. stutzeri and imply that it may be possible to use stoichiometry to manage N2O emissions from agricultural soils. This approach has been previously suggested as a possible strategy to mitigate N2O 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 N2O to N2 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 N2O consumption.

Conclusion

In summary, our findings do support a key role for Cu in the regulation of N2O emissions, by demonstrating a Cu concentration gradient effect in the production and consumption of N2O. 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 N2O 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 may be 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 N2O to N2, equates to a total amount of soil Cu in the range 150–200 μg g−1 (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 N2O production exceeding N2O conversion to N2.

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 N2OR and NIR to reduce N2O to N2. 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 N2O to produce N2. However, the level of N2O 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−1. 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 N2 (Skrbic and Durisic-Mladenovic, 2010). Therefore, in addition to other mitigation methods, supplying adequate Cu would contribute to an overall effective management of N2O 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 KH2PO4, 5.6 NH4Cl, 0.5 MgCl2·6H2O, 0.7 CaCl2·2H2O, 0.4 MgSO4·7H2O and 30 KNO3. 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 106 bacterial cells, was transferred into the anaerobic Cu medium experiment.

The basal salt solution of the anaerobic medium comprised (mM) 0.06 KH2PO4, 5.6 NH4Cl, 0.5 MgCl2·6H2O, 0.8 0.7 CaCl2·2H2O, 0.4 MgSO4·7H2O and 11 mM of 15N-enriched KNO3 (10 atom%). The pH was adjusted to 8 using 1 M NaOH, with sufficient KH2PO4 in solution to provide buffering capacity (Johnsson et al., 2006). Cu sulfate (CuSO4) 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

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replicate was inoculated with 1 ml of $10^6$ bacterial cells before being fitted with gas-tight Teflon® septa and purged using 99.99% Argon (Ar) (remaining 0.01% comprising CO₂< 5 ppm, N₂< 25 μl l⁻¹, O₂< 10 μl l⁻¹, H₂O< 10 μl l⁻¹, CO, C₂H₆, CH₄< 1 μl l⁻¹) to create an anaerobic environment with minimum N₂. 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 10⁶) to create a dilution that included viable cells (23°C) at day 10 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 RNAlater (Invitrogen, Boston, MA, USA) at −80°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 cd₇₅, haem NIR, NOR and N₂OR, 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 SYBR® 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.

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 RNAlater (Invitrogen, Boston, MA, USA) at −80°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 cd₇₅, haem NIR, NOR and N₂OR, 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 SYBR® 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 (bp) | Reference |
|------|-------------|------------------|---------------|-----------|
| fdxA | fdxA(F)     | CCGTGGACTGCTTCTTACGA | 208 | Present study |
| fdxA | fdxA(R)     | CGGCCACGTGACCTTTTCTTC | 198 | Present study |
| gyrB | gyrB(F)     | GAATACCTCACCCAGTCGGC | 198 | Present study |
| ropD | ropD(F)     | AGTACGATGCCCTGGTCGAG | 198 | Present study |
| ropD | ropD(R)     | CGGAGGTCTCCGGGTACCTTG | 406 | Kandeler et al. (2006) |
| nirS | nirSrCd3aF  | AAGCYSAGAAGARACSGG | 197 | Present study |
| nirS | nirSrCd3b  | GASTTCCGRTGSSGTGCTTSAAYGAA | | |
| norB | norB(F)     | CCATGCTCAAGGGTCGCAAG | 185 | Henry et al. (2006) |
| norB | norB(R)     | CAGGAGCAAGGGCGAACATCG | | |
| nosZ | nosZ1840F   | CGCRCAGGCAASAGGTSMSSGT | | |
| nosZ | nosZ2009R   | CAGKTRGCAKSGCRTGGCAGAA | | |
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, Hemptead UK). Non-linear regression to evaluate the relationships between ratios of $N_2O:N_2$ was performed in GenStat (GenStat 16.1; VSN International).

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**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 Condron, 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., Ermier, 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., Bagg, 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 Giuffre, 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., Tscherko, 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.

Moslion, A., Kroese, 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$_2$S on N$_2$O reduction and accumulation during denitrification by
methanol utilizing denitrifiers. *Environ Sci Technol*, **47**: 8408–8415.

Pfafl, 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(2)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.