The Production of the Greenhouse Gas Nitrous Oxide by Denitrifying Soil Bacteria.

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Submitted July 2011

This thesis is submitted in partial fulfilment of the requirements of the degree of Doctor of Philosophy at the University of East Anglia

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Statement

The work submitted in this thesis is my own work, except where due reference is made to other authors, and has not been submitted to this or any other University for any degree.

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Acknowledgements

I would like to say thank you to my primary supervisor, Prof. David Richardson, for his constant support, encouragement and guidance throughout my PhD, without which, this thesis would not have been completed.

I would also like to say thank you my secondary supervisor Dr Tom Clarke for his endless support and advice inside and outside of work you have been an amazing friend and supervisor.

Andrea and Lucy thank you both for all your support and advice, you have both been incredible friends and work colleagues and I have loved working with you both.

To the members of lab 2.30 past and present, thank you for all the help and advice I have received, you have all been a vital part of my research and social life.

To my friends Becky, Eddie, Doyle and Yann you have all had a great influence on my life and you have all showed so much support over the years, I appreciate everything you have all done for me.

Mum, Dad, Ben and Georgina, all of you have supported my studies from the beginning, thank you all so much for your help, advice and love throughout my life, you are the making of this thesis, as without you I would be completely lost in life.

Charlie, you have had to suffer with me day and night through the good and bad, I cannot express how much I appreciate your endless support, for standing by me and giving me the much needed encouragement over the past two years. You pushed me through to the end and I cannot thank you enough for all your help and love.

I would also like to acknowledge the financial support from the BBSRC, and the Pamaler Salter trust.

Abstract

Over the past few years it has become more apparent that global warming is having a significant effect on our climate. Therefore greenhouse gas emissions are also of great concern. Nitrous oxide (N_2O), commonly known as laughing gas, is a colourless non toxic gas and is a potent greenhouse gas and is involved in the depletion of the Ozone. Nearly two thirds of emissions are from soils, of which agricultural soils are the biggest culprits as they are typically high in nitrates (NO_3^-) which is one of the driving forces for denitrification. *Paracoccus denitrificans* and *Achromobacter xylosoxidans* are model soil denitrifiers capable of reducing NO_3^- to dinitrogen (N_2). Both encode the Cu containing nitrous oxide reductase (NosZ). In order to investigate how Cu depletion could affect the rates of N_2O reduction a series of chemostat cultures were set up with either NO_3^- sufficient carbon limited or NO_3^- limited carbon sufficient medium containing 18 µM, 0.8 µM and <0.5 µM Cu. When *P.denitrificans* was grown in NO_3^- sufficient medium as the Cu concentration decreased an increase in N_2O production was observed. Under NO_3^- limitation little N_2O was produced, and the full 66 kDa NosZ protein was synthesised. In the 0.8 µM and <0.5 µM Cu NO_3^- sufficient cultures *nosZ* transcription was significantly lower than the 18 µM Cu cultures and a truncated form of NosZ was detected. Chemostat cultures of *A.xylosoxidans* displayed a significant increase in nitrite (NO_2^-) production under NO_3^- sufficient conditions, compared to the *P.denitrificans* cultures. When the Cu concentration was reduced there was an increase in NO_2^- and N_2O, due to both enzymes involved in NO_2^- and N_2O reduction containing Cu. The transcription of *nosZ* was not significantly affected by the Cu concentration, but decreased in the NO_3^- limited cultures. Due to the presence of Cu in the denitrification pathway, the concentration of Cu in an environment could play a major role in N_2O emissions, along with the concentration of NO_3^- which is the first step of denitrification. This research forms the basis of how N_2O emitted from agricultural soils could potentially be reduced by monitoring the NO_3^- and Cu concentrations so that the complete reduction of NO_3^- to N_2 by denitrifying bacteria can be achieved.

Chapter 1: Introduction to the accumulation and consumption of nitrous oxide by denitrifying bacteria.

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Introduction

1.1 Nitrogen Cycle

"Water water everywhere, nor a drop to drink" (Samuel Taylor Coleridge 1797-98) the same can be said for dinitrogen (N_2) as approximately 78% of the atmosphere consists of N_2 which no higher organisms can utilise. Nitrogen is vital for life on Earth, it is a major building block in amino acids and so without nitrogen the world would not be as we currently know it. Typically ammonium (NH_4^+) is used in synthesising glutamine, which is the N donor for the synthesis of all other amino acids (Bothe, Ferguson et al. 2007). Over the past 4 billion years prokaryotes have evolved to exploit the inert N_2 in a process called nitrogen fixation where N_2 is converted to NH_4^+. This can then be used by higher organisms, such as plants, as a nitrogen source, or by micro organisms during respiration (Figure 1.1-1). Through the aerobic respiration process of nitrification, NH_4^+ is oxidised to hydroxylamine (NH_2OH), nitrate (NO_3^-) and nitrite (NO_2^-) (Figure 1.1-1). These nitrogenous compounds are in turn reduced to N_2 during denitrification, completing the nitrogen cycle. Anaerobic ammonium oxidation (Anammox) was discovered in 1995 by Mulder *et al.* previous to this the oxidation of NH_4^+ had only been found in aerobic environments (Mulder, van de Graaf et al. 1995). This process is where NH_4^+ and NO_2^- are combined to form N_2 and was originally identified in waste water sludge (Mulder, van de Graaf et al. 1995; Strous, Van Gerven et al. 1997). All of these processes have been identified in various bacteria, archaea and fungi, some of which form a symbiosis with plants, and due to the nature of the nitrogen cycle some intermediates have been found to accumulate. The denitrification path is a great interest to many scientists as it is involved in many aspects of agriculture, waste water and greenhouse gas production, which is discussed in the following section.

NO_3^- (+5)

Denitrification

NO_2^- (+3)

Nitrification

NO (+2)

Heterotrophic nitrification

N_2O (+1)

ANAMMOX

Dissimilatory nitrate reduction

NH_2OH (-1)

N_2 (0)

NH_4^+ (-3)

Nitrogen Fixation

Glutamine
and other amino acids

Figure 1.1-1 The Nitrogen Cycle; showing the reduction of nitrate (NO_3^-) to nitrite (NO_2^-), nitric oxide (NO), nitrous oxide (N_2O), dinitrogen (N_2) and ammonium (NH_4^+), and the oxidation of NH_4^+ to hydroxylamine (NH_2OH) and NO_2^-. Numbers in brackets represent the oxidation state of N.

1.2 Denitrification

Denitrification refers to the dissimilatory microbial reduction of one or both soluble ionic nitrogen oxides, NO_3^- and NO_2^-, to the gaseous oxides, nitric oxide (NO) and nitrous oxide (N_2O), which may be further reduced to N_2. NO is a cytotoxin as well as being involved in depleting the Ozone layer, N_2O is a major greenhouse gas also involved in the breakdown of the Ozone layer. These denitrifying reactions occur in the periplasm and on the cytoplasmic membrane of many bacteria where the different nitrogen oxide species act as terminal electron acceptors. There is a wide range of microorganisms that can undergo denitrification, including *Paracoccus spp., Shewanella spp., Achromobacter spp.(previously Alcaligenes*), *Pseudomonas spp., Burkholderia spp.* and *Rhodobacter spp..* Individual reactions of the denitrification pathway can be found in some types of fungi, archaea, halophilic and hyperthermophilic branches of the prokaryote kingdom, for example the thermophile *Thermus thermophilus* or halophile *Bacillus halodenitrificans*. Denitrification is a very important process in many ways; it is a major problem in farming as it decreases the effectiveness of fertiliser through the reduction of NO_3^- and NO_2^- to N_2O and N_2. However the removal of NO_3^- and NO_2^- are vital in waste water treatment. After treatment waste water is released into the freshwater environment, if the waste is contaminated with NO_3^- and NO_2^- then algal blooms and eutrophication can occur. Denitrification is also a contributor to the greenhouse effect due to the nitrogenous intermediates formed, NO and N_2O, which affect the stratospheric and Ozone chemistry (Knowles 1982; Zumft 1997; Granger and Ward 2003; Bothe, Ferguson et al. 2007; Crutzen, Mosier et al. 2008).

1.3 Enzymes Involved in denitrification

Denitrification involves a variety of metalloproteins that have been well characterised in many bacteria. In a high proportion of Gram negative denitrifiers the enzymes associated with the denitrification pathway are found within the periplasm,

with the exception of the NO_3^- reductase, NarGHI, that is an integral membrane protein with the NO_3^- reducing subunit located on the cytoplasmic side of the membrane. Some denitrification enzymes are integral membrane proteins, such as NO reductase (Nor) which is embedded within the cytoplasmic membrane and reduces NO on the periplasmic side. NO_3^- is reduced to NO_2^- in the cytoplasm, NO_3^- is transported to the cytoplasm via NarK1, a NO_3^-/H^+ symporter, along with NarK2 a NO_3^-/NO_2^- antiporter (Goddard, Moir et al. 2008). NarGHI yields NO_2^- which is then transported to the periplasm by NarK2, NO_3^- is also reduced to NO_2^- by a periplasmic NO_3^- reductase (Nap). The electrons (e^-) involved in the reduction of the intermediates are attained from the oxidation of succinate and Nicotinamide adenine dinucleotide (NADH) the e^- reduce ubiquinone (Q) to ubiquinol (QH_2), the QH_2 is then oxidised by Nar, along with the bc_1 complex releasing protons (H^+) to the periplasm.

In the denitrification pathway NO_2^- is converted to NO by NO_2^- reductase (Nir), a periplasmic protein. This enzyme comes in two forms, there is the haem dependent cd_1, and the Cu dependent (CuNir), these two NO_2^- reductases have never been found to coexist in one organism (Zumft 1997; Baker, Ferguson et al. 1998; Bothe, Ferguson et al. 2007). Ammonification is another means of reducing NO_2^- in gram negative proteobacteria such as, *Escherichia coli,* which is capable of this metabolic pathway, where NO_2^- is reduced to NH_4^+ without forming any intermediates (See Figure 1.1-1). The catalysis of this 6 e^- reduction is carried out by NrfA, a dimeric periplasmic enzyme, each monomer of NrfA contains 5 haems and is approximately 55 kDa (Bamford, Angove et al. 2002; Simon 2002; Clarke, Mills et al. 2008). Formate acts as the e^- donor to NrfA via QH_2 and menaquinol, this is also true for *Wolinella succinogenes, Sulfurospirillum deleyianum* and *Campylobacter sputorum bv. Bubulus* (Eaves, Grove et al. 1998; Simon 2002). As NO is a potent cytotoxin, the conversion of NO to N_2O carried out by NO reductase and is thought to be a detoxification process, currently three forms of NO reductase have been identifies, NorBC, qNor and qCu_ANor (See section 1.4.3). It has been experimentally shown that bacteria deficient in the Nor are not viable under denitrifying conditions (Stouthamer, Boogerd et al. 1983; Boer, Oost et al. 1996; Baker, Ferguson et al. 1998; Butland, Spiro et al. 2001; Richardson, Felgate et al. 2009). The final step in denitrification is the generation of N_2 by the reduction of

N_2O by the periplasmic soluble N_2O reductase, NosZ (Zumft 1997; Baker, Ferguson et al. 1998; Bothe, Ferguson et al. 2007). Some denitrifiers, such as *Neisseria gonorrhoeae*, do not contain this enzyme and so the final step of denitrification is NO to N_2O. Regulation of these enzymes is primarily through oxygen (O_2) limitation. When O_2 is present, e^- flow from the quinone/quinol pool is diverted to one or more haem copper oxidases that form the aerobic metabolic pathway. NO_3^-, NO_2^- and NO sensors are also involved in regulation of the expression of genes essential for denitrification. Thus complete denitrification involves the use of the Nar and/or Nap, Nir or CuNir, Nor and Nos enzymes, as well as the bc_1 complex and the quinol pool, in an intricate network of enzymes and regulators. There are a large number of species of denitrifying bacteria, which range across the prokaryotic kingdom which includes archaea, eubacteria and cyanobacteria. *Paracoccus denitrificans* and *Achromobacter xylosoxidans* are biochemically well characterised denitrifiers, which will be discussed in the following sections.

1.3.1 *Paracoccus denitrificans*

P.denitrificans is a well known α proteobacteria and a model denitrifier. It contains the *nar, nap, nir, nor* and *nos* operons that code for all the enzyme systems necessary to fully reduce NO_3^- to N_2 (Figure 1.1-1 and *Figure 1.3-2*). The denitrification genes are located in clusters across the 3 chromosomes that encode the *P.denitrificans* genome. *P.denitrificans* has been extensively studied and all denitrification enzymes are well characterised making this a useful model bacteria to study denitrification. *P.denitrificans* is capable of utilising many types of carbon sources, ranging from methanol to butyrate and some strains of *Paracoccus* are also capable of utilising inorganic compounds such as carbon disulphide (Jordan, McDonald et al. 1997; Baker, Ferguson et al. 1998). In an environmental context *P.denitrificans* has been found to colonise many ecosystems with fluctuating aerobic and anaerobic conditions. Strains have been isolated from active sludge and are a vital part of the waste water treatment due to the removal of nitrogen oxides (Juretschko, Loy et al. 2002). In

aerobic respiration the electron transport pathway is similar to that of the eukaryotic mitochondrion. 16S rRNA analysis has revealed that *P.denitrificans* is closely related to the eukaryotic mitochondrion, which makes this organism a popular model for aerobic electron transfer and energy conservation (Baumann, Snozzi et al. 1996; Baker, Ferguson et al. 1998; Spiro, Richardson et al. 2006).

In 2006 the genome of *P.denitrificans* was sequenced (Spiro, Richardson et al. 2006) . It has an unusual genomic structure as the genome consists of three distinctive chromosomes which are assigned by the numerals, I (2.852282 Mbp), II (1.730097 Mpb) and III (0.653815 Mbp) (Spiro, Richardson et al. 2006); typically bacterial genomes consist of a single chromosome and a number of plasmids. The genes encoding the denitrification genes are found across the three chromosomes (*Figure 1.3-1*). In other strains of *Paracoccus* the amount of chromosomes and plasmids can vary, for example *P.denitrificans* GB-17 and DSM 65 are reported to both have four chromosomes of 2.2, 1.5, 0.71 and 0.5 Mbp (Baker, Ferguson et al. 1998).

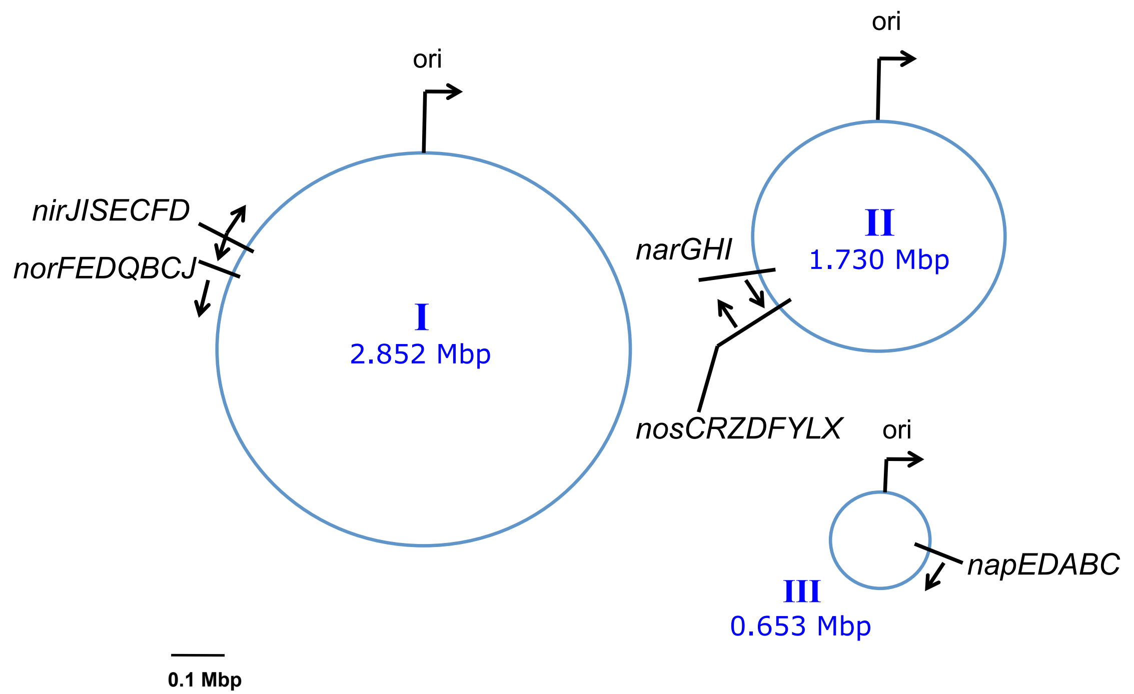


Figure 1.3-1 The organisation of the denitrification genes of *P.denitrificans*. The genes are located on all three chromosomes. The origin of replication is indicated as ori, and direction of gene transcription is indicated by arrows. The length of the chromosomes is indicated by mega base pairs (Mbp).

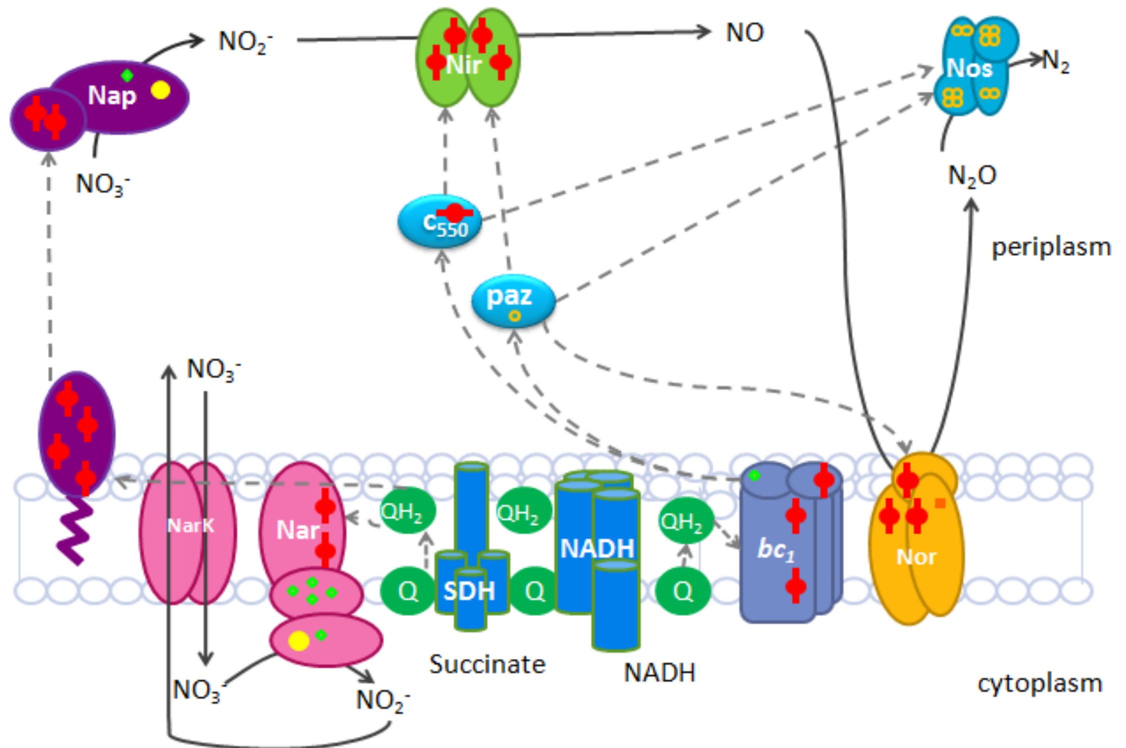


Figure 1.3-2 Schematic of the denitrification pathway in *Paracoccus denitrificans* (Pd1222). Enzyme systems involved are NO_3^- reductase (Nar (pink) and Nap(purple)), NO_3^-/NO_2^- antiporter NarK, NO_2^- reductase (Nir (green)), NO reductase (Nor(orange)) and N_2O reductase (Nos(blue)). Electrons are obtained (dotted lines) from the ubiquinol pool (Q, QH_2), which is reduced by the succinate dehydrogenase (SDH) and NADH dehydrogenase. Electrons from the *bc₁* complex are transferred to pseudoazurin (paz) and cytochromec$_{550}$ (c$_{550}$). The redox cofactors include haem ( ), non haem Fe ( ) Fe-S cluster ( ), copper ( ) and molybdenum ( ).

1.3.2 *Achromobacter xylosoxidans*

Previously known as *Alcaligenes xylosoxidans, Achromobacter xylosoxidans* is a motile β proteobacteria that is an opportunistic pathogen, first isolated from clinical samples. (Yabuuchi, Yano et al. 1974). However, it has also been isolated from the environment in wetland sediments (Wan, Gu et al. 2007). The denitrification pathway in *A.xylosoxidans* uses the Nar, Nor and Nos systems, similar to *P.denitrificans. A.xylosoxidans* reduces NO_2^- using a blue CuNir, encoded by the *nirK* gene, which differs to the *P.denitrificans* cd_1 haem containing Nir (Figure 1.3-3). *A.xylosoxidans* reduces NO to N_2O using a quinol nitric oxide reductase (qNor), which differs from the *c*-type cytochrome Nor system found in *P.denitrificans* (details of the quinol Nor system are discussed in section 1.4.3). In addition to being able to denitrify NO_3^- and use succinate, xylose and mannitol as carbon sources, *A.xylosoxidans* is also capable of using 2,4-dichlorophenoxyacetic acid as a sole carbon source and so has been used in studies in degrading pesticides (Yabuuchi, Yano et al. 1974; Gunalan and Fournier 1993). *A.xylosoxidans* is also capable of mineralising *p*-Nitrophenol (PNP) and utilising it as a sole N source. PNP is a toxin commonly found in pesticides and is a by product of synthesis of medicines, dyes, rubber chemicals, and contaminates waste waters, soils, rivers, (Wan, Gu et al. 2007). Therefore *A.xylosoxidans* has a high bioremediation potential in clearing up contaminated soils and waters.

Currently the genome for *A.xylosoxidans* is being sequenced (by the Department of Clinical Microbiology, University Hospital of Copenhagen, Denmark) various plasmids from *Achromobacter spp.* have been sequenced such as the *A.xylosoxidans* A8 plasmid p*581* which does not encode any denitrification enzymes (Jencova, Strnad et al. 2008). When the genome is sequenced it will provide a great insight in to the metabolic pathways that could be useful for bioremediation and also the denitrification pathway, along with the mechanisms that allow it to infect and cause disease.

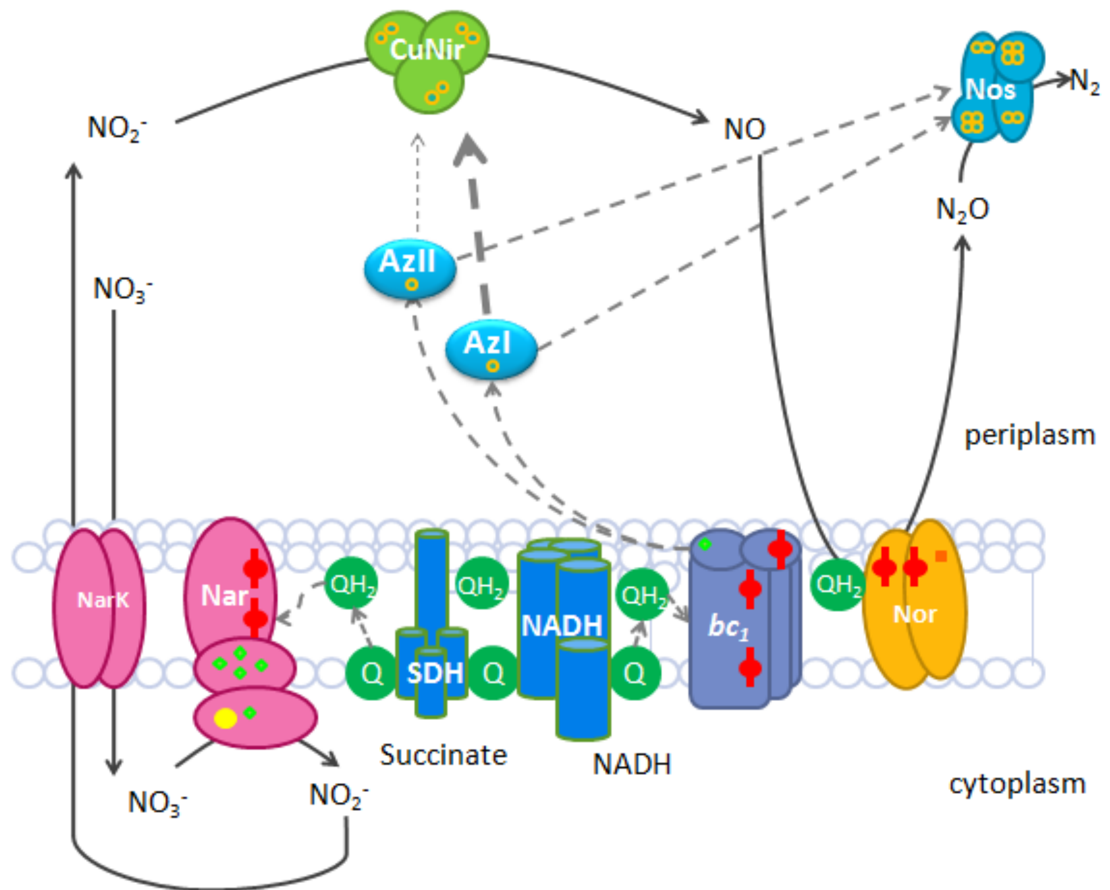


Figure 1.3-3 Schematic of the denitrification pathway in *Achromobacter xylosoxidans*. Enzyme systems involved are NO_3^- reductase (Nar(pink)), NO_3^-/NO_2^- antiporter NarK, NO_2^- reductase (Nir(green)), NO reductase (Nororange)) and N_2O reductase (Nos (light blue)). Electrons are obtained (dotted lines) from the ubiquinol pool (Q, QH_2), which is reduced by the succinate dehydrogenase (SDH) and NADH dehydrogenase. Electrons from the *bc_1* complex are transferred to Azurin I (Az I) and Azurin II (Az II) which transfer e^- to CuNir and NosZ. The redox cofactors include haem ( ), non haem Fe ( ) [4Fe-4S] cluster ( ), copper ( ) and molybdenum ( ).

1.4 Enzyme complexes involved in denitrification

1.4.1 Nitrate Reductase

There are three main types of NO_3^- reductase enzymes found in denitrifying bacteria. One form is a cytoplasmic soluble assimilatory-type enzyme (Nas), the second type is bound to the cytoplasmic membrane (Nar) and the third form is a periplasmic protein (Nap); the membrane bound and periplasmic form are both respiratory reductases (Zumft 1997; Baker, Ferguson et al. 1998) and these are to be discussed in this section.

The membrane bound NO_3^- reductase in *P.denitrificans* is similar to that of *E.coli, t*he *nar* operon is regulated by the fumarate nitrate reduction transcription factor (FNR). NarGHI is a three subunit protein encoded by the *narGHI* genes, the transmembrane γ subunit (NarI) anchors the α and β subunit to the membrane, NarG is a molybdoprotein, the molybdenum redox centre is inserted by a molybdenum cofactor, molybdopterin which binds Mo, however previous to the Mo insertion a Cu ion also binds to this cofactor (see section 1.6) (Zumft 1997; Baker, Ferguson et al. 1998; Morrison, Cobine et al. 2007). NarG contains the Molybdenum-bis-molybdopterin guanine dinucleotide (MGD) cofactor which is the site of NO_3^- reduction (Anderson, Richardson et al. 2001). NarH is the β subunit which contains three [4Fe-4S] clusters and a [3Fe-4S] cluster (Anderson, Richardson et al. 2001). In addition to the genes encoding the catalytic enzyme, the *narKGHJI* operon encodes NarJ, which is involved in the assembly of the entire complex as well as NarK1 which is a NO_3^-/H^+ symporter and NarK2 which is an NO_3^-/NO_2^-antiporter, both of which are embedded in the cytoplasmic membrane (Moir and Wood 2001; Goddard, Moir et al. 2008). NarR is upstream of the *narKGHJI* operon and is transcribed in the opposite direction, it is hypothesised to be a transcriptional regulator, as it shows 70 % homology to NNR of *P.denitrificans*, and is essential for the maximum expression of NarGHI anaerobically (Wood, Alizadeh et al. 2001).

The periplasmic NO_3^- reductase is a molybdoprotein complex (which contains both haem and non haem iron) (Berks, Richardson et al. 1994). In *P.denitrificans* the

napEDABC operon encodes all the structural and biosynthetic peptides required to construct the holoenzyme, *napA* codes for the larger subunit and *napB* encodes the smaller subunit (Zumft 1997; Baker, Ferguson et al. 1998). The large NapA subunit contains a molybdenum centre and a [4Fe-4S] centre and is approximately 80 kDa (Butler, Charnock et al. 1999). The smaller NapB subunit contains two *c*-haems and is 16 kDa (Butler, Charnock et al. 1999), NapC is a tetra haem cytochrome involved in electron transfer from the quinol pool to the periplasmic NO_3^- reductase (Roldán, Sears et al. 1998), NapD acts as a chaperone and is involved with the maturation of NapA, the function of NapE is still unknown (Potter and Cole 1999; Sargent 2007). *nap* expression is present in anaerobic and aerobic cultures. Under anaerobic conditions the expression of *nap* is at a fairly low level. However Nap synthesis is increased under aerobic conditions with a reduced carbon source, such as butyrate. Activity decreases with more oxidised carbon sources such as succinate or malate (Richardson 2000; Sears, Sawers et al. 2000; Ellington, Bhakoo et al. 2002; Ellington, Sawers et al. 2003).

1.4.2 Nitrite Reductase

There are two forms of NO_2^- reductase, a haem dependent NO_2^- reductase, cytochrome *cd_1,* and a copper dependent NO_2^- reductase, which can be either blue or green depending on the oxidation state of the Cu centres. These both carry out the reduction of NO_2^- to NO, the first committed step of denitrification (Zumft 1997; Baker, Ferguson et al. 1998; Tocheva, Eltis et al. 2008).

P.denitrificans encodes the cyt cd_1 NO_2^- reductase which receives electrons from two electron donors, either the haem containing cytochrome c_{550} (CycA) or the copper containing pseudoazurin, Paz (Figure 1.3-2) (Zumft 1997; Baker, Ferguson et al. 1998). Cyt cd_1 exists as a homodimer with a subunit mass of approximately 60 kDa, and contains two haems, a normal *c*-type haem and a unique d_1 haem (See Figure 1.4-1A). Both of these haems are incorporated into the protein either in the periplasm or during the preprotein's translocation to the periplasm (Heikkilä, Honisch et al. 2001).

The gene encoding cytochrome *cd_1* (*nirS*) was sequenced by Boer *et al.* (1994), in *P.denitrificans. T*he *nir* operon is located on chromosome I along with the *nor* operon; however they are transcribed in the opposite direction. The *nir* operon encodes genes essential for haem *d_1* biosynthesis. Upstream from *nirS* are the *nirI* and *nirX* genes, NirI and NirX are transcribed in the opposite direction to the *nir* cluster and are essential for NO_2^- reduction and influence the transcription of the gene, but are not thought to be involved in the biosynthesis of the protein (Saunders, Houben et al. 1999). Another four important genes are *nirE, nirC, nirF* and *nirD*; it is proposed that NirE is involved in the addition of methyl groups to a precursor to form the d_1 haem. NirE shows homology to CysG of *E.coli,* a multifunctional siroheam synthase, but even with this information it is not clear how NirE acts, however when deleted from the operon NO_2^- reductase does not contain a d_1 haem (Spencer, Stolowich et al. 1993; Glockner and Zumft 1996). NirC codes for a low molecular weight class I *c*-type cytochrome, which plays an undefined part in the formation of the d_1 haem and maturation of cytochrome cd_1 (Glockner and Zumft 1996; Baker, Ferguson et al. 1998). NirF and NirN share similar sequences to NirS, they are thought to be more involved in the supporting and positioning of the d_1 haem when it is being assembled, prior being inserted into the NO_2^- reductase. The last gene *nirD* as yet has no known function (Baker, Ferguson et al. 1998). The *nir* operon is regulated by NNR, a transcriptional regulator homologous to FNR, which senses NO (Saunders, Houben et al. 1999).

The copper containing NO_2^- reductase enzyme can be subdivided into two classes, green NO_2^- reductase, found in *Achromobacter cycloclastes* and *Alcaligenes faecalis,* and blue, found in *Achromobacter xylosoxidans* (Ho, Ooi et al. 2003). This periplasmic protein exists as a homotrimer, consisting of 3 identical 36 kDa subunits (Suzuki, Horikoshi et al. 1999) and they contain two types of copper, type I (blue) copper and type II (green) copper. The type I Cu (Cu(I)) is located near the protein surface where electrons are received from electron donors and is coordinated by two histidines, a methionine and a cysteine (Figure 1.4-1B) (Suzuki, Horikoshi et al. 1999; Hough, Ellis et al. 2005; MacPherson and Murphy 2007; Tocheva, Eltis et al. 2008). The electrons are then passed to the type II copper site (Cu(II)), (Suzuki, Horikoshi et al. 1999; Hough, Ellis et al. 2005; Tocheva, Eltis et al. 2008). The two Cu sites are

connected by a cysteinyl ligand from Cu(I) and a histidinyl ligand from the Cu(II), and it is this bridge that ensures efficient electron transport (Tocheva, Eltis et al. 2008). In resting state the two copper ions are oxidised and the Cu(II) is coordinated by three histidines and either H_2O or OH^-, it is then thought that the NO_2^- binds asymmetrically to Cu(II), and the electron from Cu(I) is likely to cause a rearrangement at the active site producing NO and H_2O (Tocheva, Eltis et al. 2008). Cytochrome *cd₁* synthesis requires a complex operon and system of chaperones and proteins, all organised within the *nir* operon. By contrast CuNir is encoded by a single gene, *nirK*, which has been identified in many bacteria, *A.xylosoxidans, A. cycloclastes, Rhodoacter sphaeroides* and *Pseudomonas spp.* (Velasco, Mesa et al. 2001). The expression of *nirK* is proposed to be regulated by a nitrogen oxide species other than NO_3^-, the *nir* operon is regulated by NNR, a NO sensor, in *P.dentirficans* (Velasco, Mesa et al. 2001). There are proposed genes that interact with NirK, *ncgABC,* which are found directly upstream of *nirK* in *Nitrosomonas europaea* (Beaumont, Lens et al. 2005).

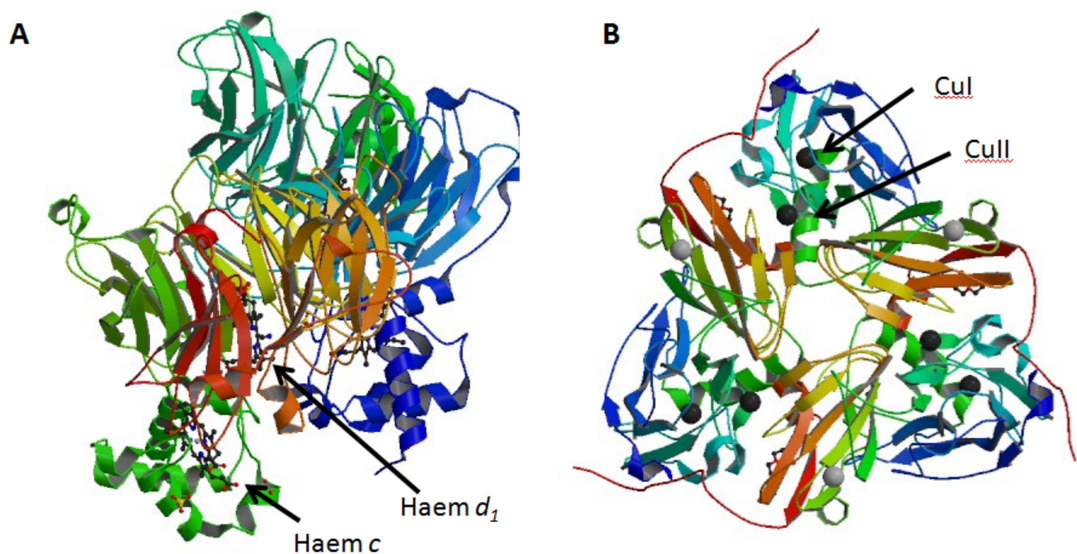


Figure 1.4-1 The crystal structures of cytochrome *cd_1* (A) isolated from *P.pantotrophus* (Sjögren and Hajdu 2001) and NirK (B) isolated from *Achromobacter cycloclastes* (Hough, Antonyuk et al. 2008). Cyt *cd_1* exists as a dimer and contains two haems where as the NirK exists as a trimer and contains a total of 6 Cu ions (black circles) in its trimeric state (2 per monomer).

1.4.3 Nitric oxide reductase

In *P.denitrificans* the *nor* genes are found in a cluster upstream of the *nir* locus and are regulated by NNR, the NO-binding protein (Van Spanning, De Boer et al. 1995 ; Hutchings and Spiro 2000). The *norC* gene codes for the *c* haem subunit, *norB* codes for the larger *b* haem subunit. The *norCB* gene has been found to be cotranscribed with *norQDEF* (found downstream of *norC*), and are essential for the maturation of NO reductase. *norQ* codes for a protein located in the cytoplasm and contains two putative nucleotide binding motifs. NorD is also located in the cytoplasm and it is proposed that NirQ and NirD are involved and influence the transcription of the NO reductase protein (Baker, Ferguson et al. 1998). Mutations in *norQD* render an inactive NO reductase (Boer, Oost et al. 1996). NorE and NorF are vital for the stability and assembly of the Nor complex, NorE is also postulated to be a part of the Nor complex (Boer, Oost et al. 1996). The *norQ* sequence has been found to be homologous to *nirQ* and the product of NorD encodes a protein of 69.7kDa, the sequence is homologous to that of an open reading frame (ORF) downstream of *norB*. The genes *norE* and *norF* have no known function, but when knocked out there is a change in NO reductase activity, even though the expression of the other *nor* genes remain the same (Zumft 1997; Baker, Ferguson et al. 1998).

NO is a cytotoxic radical and therefore rarely accumulates above nanomolar concentrations. NO is quickly reduced to N_2O by the haem containing cytoplasmic membrane bound protein, NO reductase. In *P.denitrificans* NO reductase is a two subunit protein, a larger subunit NorB (approximately of 53 kDa) is a highly hydrophobic *b*-type cytochrome that binds two high spin *b* haems (Figure 1.4-2) (Fujiwara and Fukumori 1996; Zumft 1997; Baker, Ferguson et al. 1998; Hino, Matsumoto et al. 2010). It contains 12 membrane spanning helices, which anchor the whole protein within the membrane, except for the N and C terminus (Fujiwara and Fukumori 1996; Zumft 1997; Baker, Ferguson et al. 1998). The smaller soluble periplasmic subunit NorC is a 17 kDa *c* type cytochrome which binds haem *c*, it is this subunit that obtains e^- from Paz and cyt c_{550} which is essential for NO reduction (Figure 1.4-2). The cytochrome is anchored to the membrane via a single α helix at the N

terminus. The complex also contains a non haem iron at the metal centre which is formed by the histidines from helices 6 and 7 (Fujiwara and Fukumori 1996; Zumft 1997; Baker, Ferguson et al. 1998; Hino, Matsumoto et al. 2010).

There is another type of NO reductase which is not as well characterised as the *c*-type cytochrome containing Nor (cNor). Most work on this novel NO reductase has been done in *Ralstonia eutropha*, this Nor does not encode the NorC subunit, the *c*-type cytochrome subunit, it therefore receives its e^- from quinols, this Nor is known as qNor (Hendriks, Oubrie et al. 2000). The qNor exisits as a single NorB subunit, which includes a 280 amino acid N terminal extension (Cramm, Pohlmann et al. 1999; Hendriks, Oubrie et al. 2000). The N terminal extension shows a low homology to NorC, implying that evolutionary history of Nor involves both NorC and NorB (Hendriks, Oubrie et al. 2000). The NorB of qNor also shows high homology to other cNor NorB subunits (Cramm, Siddiqui et al. 1997). The qNor has been found in several Firmicutes such as *Staphylococcus aureus,* proteobacteria, *Neisseria gonorrrhoeae, N.meningitidis* and cyanobacteria, *Synechocystis spp.* (Hendriks, Oubrie et al. 2000).

Another variant of the NO reductase has been isolated from *Bacillus azotoformans* and is a menaquinol-dependent nitric oxide reductase (qCu_ANor) with a Cu_A centre (Suharti, Heering et al. 2004). qCu_ANor obtains e- from menaquinol and cyt c_{551}, and it is proposed that the use of cyt c_{551} increases activity by 4 fold, which is a possible reason why *B.azotoformans* has a high NO tolerance (Suharti, Heering et al. 2004).

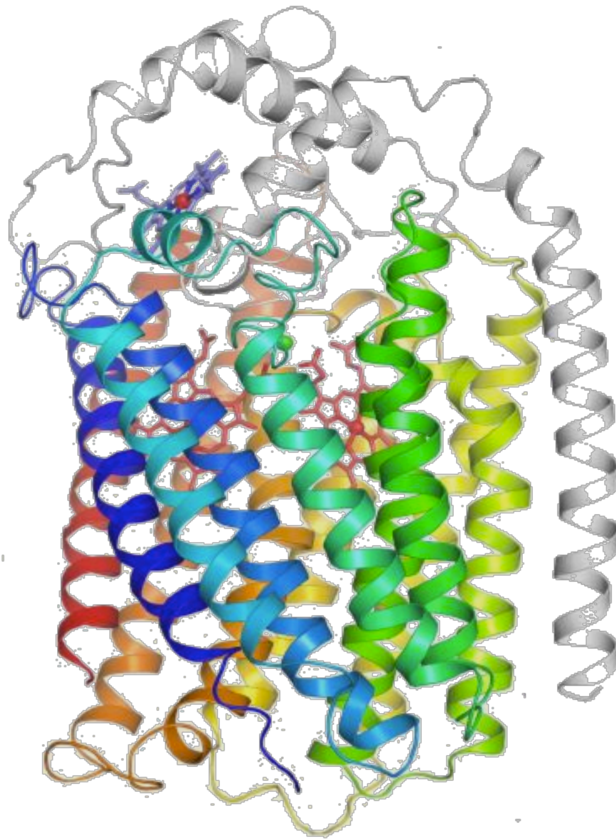


Figure 1.4-2 The crystal structure of NorCB from *Pseudomonas aeruginosa.* NorC (grey) is binds a haem *c* (blue) and NorB (multicolour) binds contains two haem irons *b* and b_3 (red) as well as a non haem iron Fe_B (green sphere). The protein sequence of NorBC shows 95 % homology to NorCB of *P.denitrificans* (Hino, Matsumoto et al. 2010).

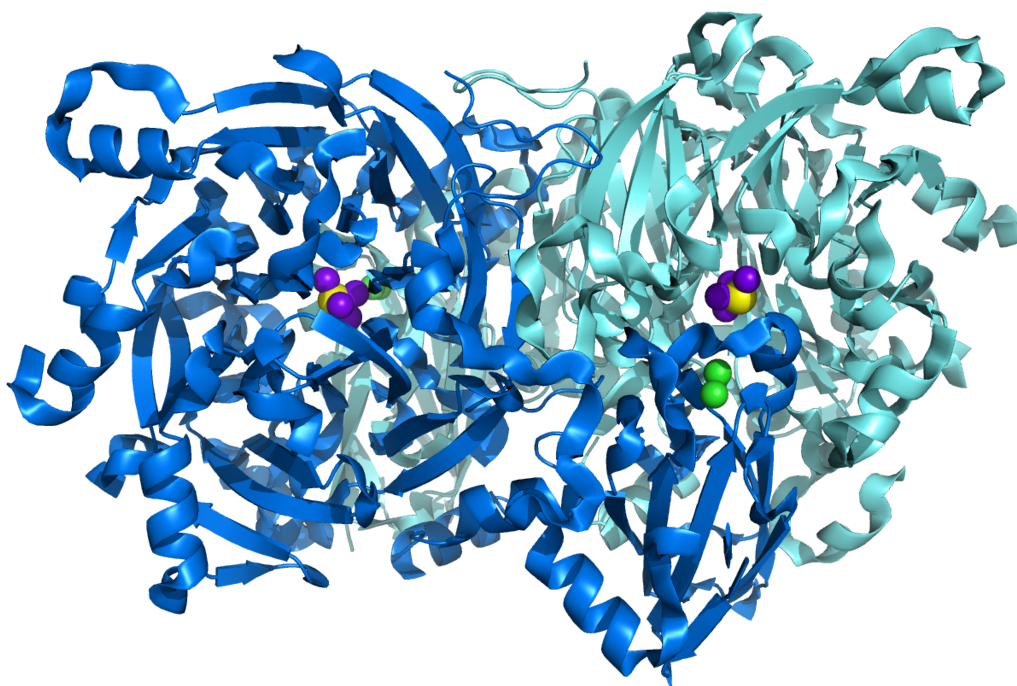
1.4.4 Nitrous oxide reductase

The reduction of N_2O is the final step in the denitrification pathway, N_2O is reduced to N_2 by N_2O reductase. This copper containing periplasmic enzyme occurs as a homodimer with each monomer consisting of a N terminal domain which contains the Cu_z site and the C-terminal domain which holds the Cu_A site (Figure 1.4-3) (Haltia, Brown et al. 2003). The Cu_z site is the catalytic site in the form of a tetranuclear copper cluster which resides in the centre of a seven bladed β-propeller (Haltia, Brown et al. 2003). An inorganic sulphur is also present which bridges the coppers together, however the involvement of the sulphur in the reduction of N_2O is unclear (Haltia, Brown et al. 2003). This copper-sulphide centre is thought to be coordinated by 7 histidine residues residing in the centre of the 7 bladed β-propeller (Zumft 1997; Rasmussen, Berks et al. 2002). The C terminal domain of the enzyme holds the Cu_A site, it also has a cupredoxin fold which is conserved among N_2O reductase (Haltia, Brown et al. 2003). The Cu_A site is very similar to the dinuclear copper site of cytochrome *c* oxidase, which also acts as an electron entry site (Haltia, Brown et al. 2003). It has been thought that the Cu_A was first present in N_2O reductase, then adopted by cytochrome *c* oxidase when the atmosphere became more aerobic (Haltia, Brown et al. 2003). Cu_A has been found in other enzymes, such as a menaquinol-oxidizing NO reductase in *B.azotoformans.* The main function of the Cu_A site in N_2O reductase is to accept electrons from a reducing substrate, which are then fed to the catalytic centre Cu_Z, where N_2O is reduced to N_2 (Haltia, Brown et al. 2003). The two copper ions are ligated by two central cysteines, two terminal histidines and two axial ligands (a methionine sulphur and a main chain carboxyl group)(Haltia, Brown et al. 2003). The Cu_A redox site has a low reorganisation energy which means that this site is well adapted for fast electron transfer (Haltia, Brown et al. 2003). In the N_2O reductase monomer the distance from the Cu_A to Cu_Z is 40Å which is too far for e^- exchange between the two sites to occur at a catalytically relevant rates. Therefore e^- transfer occurs between the Cu cluster in separate monomers across the homodimer interface, which reduces the distance to 10Å (Figure 1.4-3)(Haltia, Brown et al. 2003). The

residues at the dimer interface are highly conserved[1] and interactions are specific. There are two tightly bound calcium ions in each mononer which contribute to dimerisation, along with a chloride and many Van der Waals interactions (Haltia, Brown et al. 2003).

N_2O reductase is encoded by the *nosZ* gene, while the remaining auxiliary genes are also found in the *nos* cluster. In *P.denitrificans* the *nos* operon is organised as *nos CRZDFYLX,* similar *nos* clusters have been found in *B.japonicum* and *Achromobacter cycloclastes* (Cuypers, Viebrock-Sambale et al. 1992; Saunders, Hornberg et al. 2000). Correct expression of NosR is vital for a functional NosZ protein, *nosR* is found immediately upstream of *nosZ* and is co transcribed with *nosZ* (Cuypers, Viebrock-Sambale et al. 1992; Hoeren, Berks et al. 1993; Berks, Baratta et al. 1993 ; Saunders, Hornberg et al. 2000; Haltia, Brown et al. 2003; Wunsch and Zumft 2005). If *nosR* is knocked out or the C terminal is not functional the *nosZ* gene is not transcribed (Wunsch and Zumft 2005). NosR is hypothesised to be a cytoplasmic membrane protein with a periplasmic N terminus and a cytoplasmic C terminus. The C terminus contains cysteine signatures capable of carrying two [4Fe-4S] clusters, similar to patterns of polyferredoxin-like structures (Wunsch and Zumft 2005). Similarities in C terminal of NosR sequence suggest there is a flavin mononucleotide (FMN)- binding domain, and it has been speculated that the metal centres have DNA binding properties (Wunsch and Zumft 2005).

[1] Glu^{125}, (Lys^{129}) Phe^{130}, Ala^{133} are involved in the insertion of the monomer between blades 1 and 2. They interact with residues 618-622. Phe^{618}, Val^{619}, Ala^{620}, Ala^{621}, Ans^{622} interact with the helical insertion in the other monomer (125-133) (Haltia *et al.* 2003).



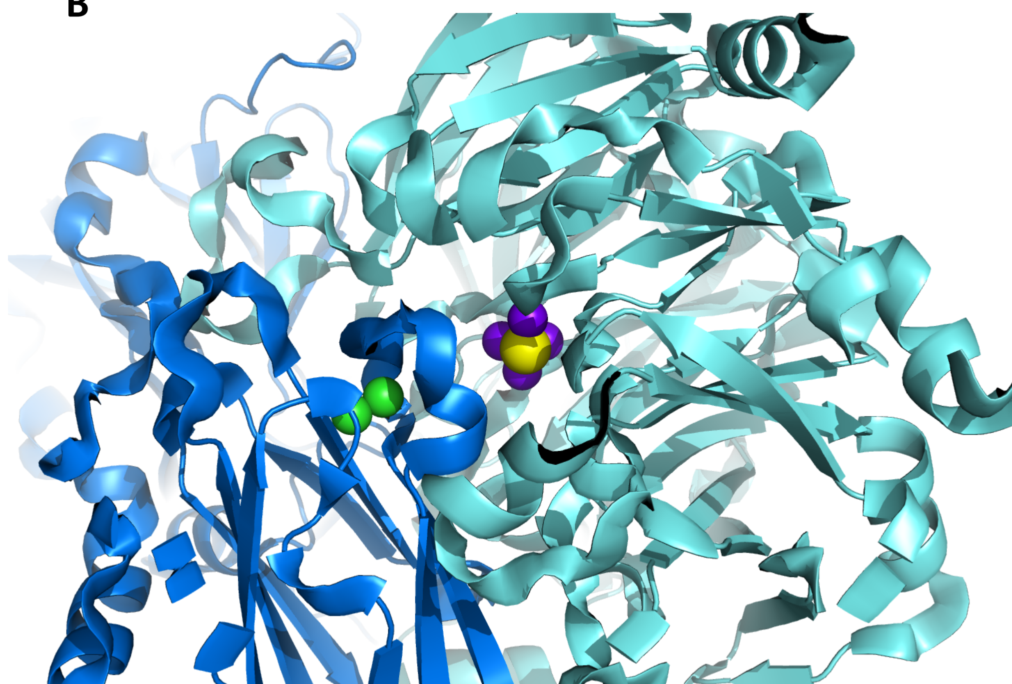


Figure 1.4-3 The crystal structure of NosZ (A) isolated from *P.denitrificans* (Brown, Djinovic-Carugo et al. 2000). NosZ exists as a dimer (light and dark blue), each monomer consists of a Cu_Z (Cu are purple spheres and the S is yellow) and a Cu_A site (green spheres). Figure B shows that the position of the Cu_A and Cu_Z sites are closer across the dimeric interface, where e^- transfer occurs.

1.5 **Dependency on metals**

Denitrification is dependent on many metalloenzymes, and so the availability of metal ions to organisms in the environment are essential for denitrification and many other metabolic pathways. Molybdenum, iron (in the form of *b* and *c* type haems and also [4Fe-4S] clusters) and copper are all involved in the process of reducing NO_3^- to N_2. Metal containing cofactors are also vital in many other smaller proteins involved in electron transfer, such as pseudoazurin and cyt $c_{550,}$ as well as chaperones and other proteins. The bioavailability of copper and iron can fluctuate in an environment; bacteria have therefore evolved mechanisms to generate high affinity binding compounds that can bind specifically to metals such as methanobactin (copper) and siderophores (iron). These small molecules are released when their corresponding metals become limited in the environment. They are capable of extracting ions from insoluble minerals and making them available to the organism, and so overcome metal deficiency (Balasubramanian and Rosenzweig 2008). Many bacteria also express a range of electron donors with different metal centres so that it can maintain various electron transport chains under different metal ion limited conditions (Baker, Ferguson et al. 1998). Denitrification is not the only metabolic pathway that involves metalloenzymes, many pathways such as aerobic respiration heavily depend on metalloenzymes. Copper plays a vital role in many metabolic pathways and is of great interest currently as it is involved in the final steps of denitrification.

1.6 **Copper enzymes**

Copper is an essential component for many organisms and metabolic pathways, however it can also be toxic at high concentrations (Banci, Bertini et al. 2010). Therefore the intracellular availability of Cu is highly regulated in eukaryotes and prokaryotes. Billions of years ago the bioavailability of Cu increased as the atmosphere became oxic creating Cu(II), which is more soluble than Cu(I). As organisms adapted to the new oxic environment they were able to utilise Cu(II) in enzymes and catalytic

centres bringing about a whole new array of enzymes. Hence many copper containing enzymes are found frequently in systems involving oxygen respiring pathways (MacPherson and Murphy 2007). Cu is also found in the proteins of anaerobic respiratory pathways such as denitrification. N_2O reductase, found in *P.denitrificans, Pseudomonas stutzeri* and many others, is a Cu containing enzyme. Cu enters eukaryotic cells via the plasma membrane, and once Cu enters the cell it is bound to Cu chaperones/transporters which are involved in the development of metalloproteins, therefore essentially there is no “free” intracellular Cu as all ions are bound to ligands or proteins (Banci, Bertini et al. 2010). It has been shown in work by Manconi *et al* (2006) and Granger and Ward (2003) that *P.denitrificans, Pseudomonas stutzeri* and an un identified species of bacteria, when cultured in artificial seawater and when the Cu concentration is limited, or sulphide is present, N_2O accumulates. Metal depletion could also have effects on other metabolic and survival systems, since there are a variety of other Cu containing enzymes in organisms including super oxidase dismutase (SOD), amine oxidase and electron transfer proteins such as pseudoazurin and plastocyanin.

Superoxide dismutase’s (SODs) are vital for survival as they protect cells from oxidative stress by catalysing the dismutation of O_2^- to H_2O_2 and O_2 (Whittaker 2003; MacPherson and Murphy 2007). Superoxide (O_2^-) is a potent radical and is a by-product of photosynthesis, aerobic respiration and phagocytosis (Whittaker 2003; MacPherson and Murphy 2007). There are three groups of SODs, CuZnSOD are located extracellularly/in the periplasm, and represent one class, FeSOD, MnSOD and Fe/MnSOD form a second class which are found in the cytoplasm and the third class comprises of NiSOD which are typically found in *Streptomyces* species and cyanobacteria (Kroll, Langford et al. 1995; Bryngelson, Arobo et al. 2003; Whittaker 2003; MacPherson and Murphy 2007). Mutations in SODs can have devastating effects, a mutation in the CuZnSOD in humans can cause familial amylotrophic lateral sclerosis (a neurogenerative disease) (MacPherson and Murphy 2007). SOD’s are not isolated to the eukaryotes as O_2^- is just as toxic to prokaryotes. Both CuZn and Mn SODs are present in *P.denitrificans* (Terech, Pucheault et al. 1983), *B.japonicum* contains Fe/Mn SODs to combat oxidative stress (Cytryn, Sangurdekar et al. 2007).

Copper amine oxidases (CAOs) catalyse oxidative deamination of amines and concomitant reduction of oxygen to hydrogen peroxide. In bacteria these enzymes are also capable of providing carbon and nitrogen (MacPherson and Murphy 2007). CAOs are also found in plants and are involved in defence response and developmental processes, here Cu limitation in soils could have major implications for growth of crops as well as N_2O accumulation. The reduction of methylamine is also dependent on Cu. Amicyanin, from *P.denitrificans*, contains a Cu^{2+} site and is similar to pseudoazurin, however it forms a complex with methylamine dehydrogenase and cyt c_{551} to form a long e^- transfer chain (Chen, Durley et al. 1994).

Cu is not only involved in the catalytic centre of enzymes but is also present in cofactors involved in the development and maturation of enzymes. Molybdenum (Mo) is vital in many metabolic reactions in the nitrogen, sulphur and carbon cycles such as Nar and dimethyl sulfoxide reductase (Llamas, Mendel et al. 2004; Morrison, Cobine et al. 2007). In many prokaryotes and eukaryotes Mo is chelated and activated by Mo cofactors, which covalently binds Mo to a dithiolene unit on a conserved tricyclic pterin, generating a cofactor known as molybdopterin (MPT) (Llamas, Mendel et al. 2004; Morrison, Cobine et al. 2007). The biosynthesis of the MPT Mo cofactor has been found to involve Cu. The Cu binds to the dithiolene unit (coordinated by 4 sulphurs) prior to Mo being inserted (Figure 1.6-1), this is hypothesised to protect the site (Llamas, Otte et al. 2006; Morrison, Cobine et al. 2007). Research suggests that lowering the concentration of Cu has a small effect on the activity of the molybdoenzymes, implying that the Mo is inserted into the enzyme, but that the biosynthesis of the Mo cofactor could be affected (Morrison, Cobine et al. 2007).

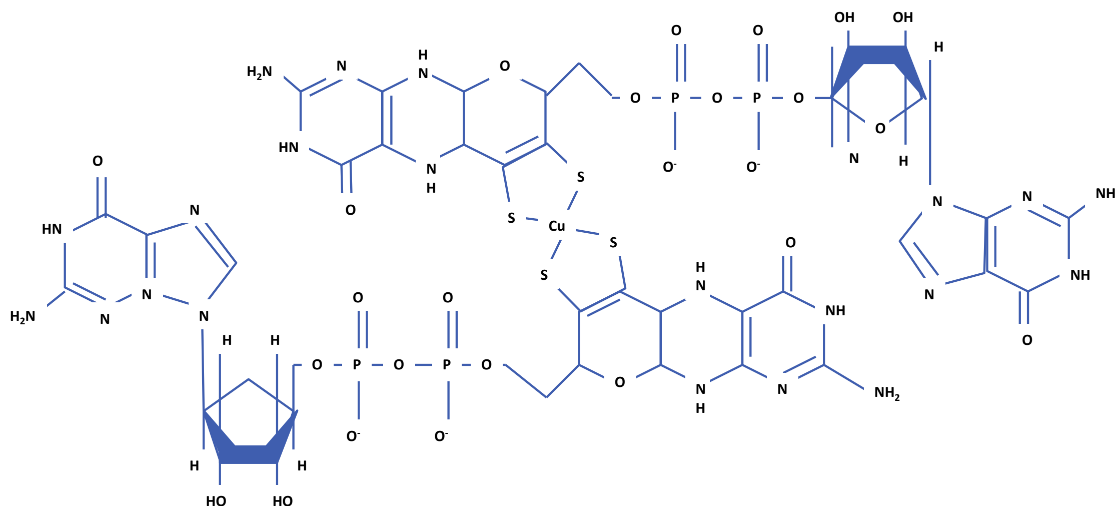


Figure 1.6-1 The Mo cofactor showing the binding of Cu in the dithiolene site prior to the insertion of Mo.

The enzymes involved in the respiration of O_2 are known as the haem-copper (Haem-Cu) oxidase super family. Haem-Cu oxidases are found across the prokaryotic and eukaryotic kingdoms. In prokaryotes they are typically embedded in the cytoplasmic membrane, while in Eukaryotic cells are found in the inner membrane of the mitochondria (Garcia-Horsman, Barquera et al. 1994). All aerobic prokaryotic systems have been found to contain multiple respiratory oxidases, for example *P.denitrificans* contains 3 distinctive haem-Cu oxidases; cyt $ba_{3,}$ cyt aa_3 and cyt $cbb_{3.}$ *P.denitrificans* also contains a fourth oxidase called cyt *bd* which is a quinol oxidase similar to the others, however there it is solely a haem containing enzyme, rather tha a haem-Cu enzyme(See Figure 1.6-2) (Garcia-Horsman, Barquera et al. 1994). The quinol oxidase is involved in oxidising quinol to quinone, which is vital in many respiratory pathways. *B.japonicum* is thought to have four distinctive oxidases cyt cbb_3, cyt ba_3 , cyt aa_3 and the quinol oxidase cyt *bd* (Garcia-Horsman, Barquera et al. 1994).

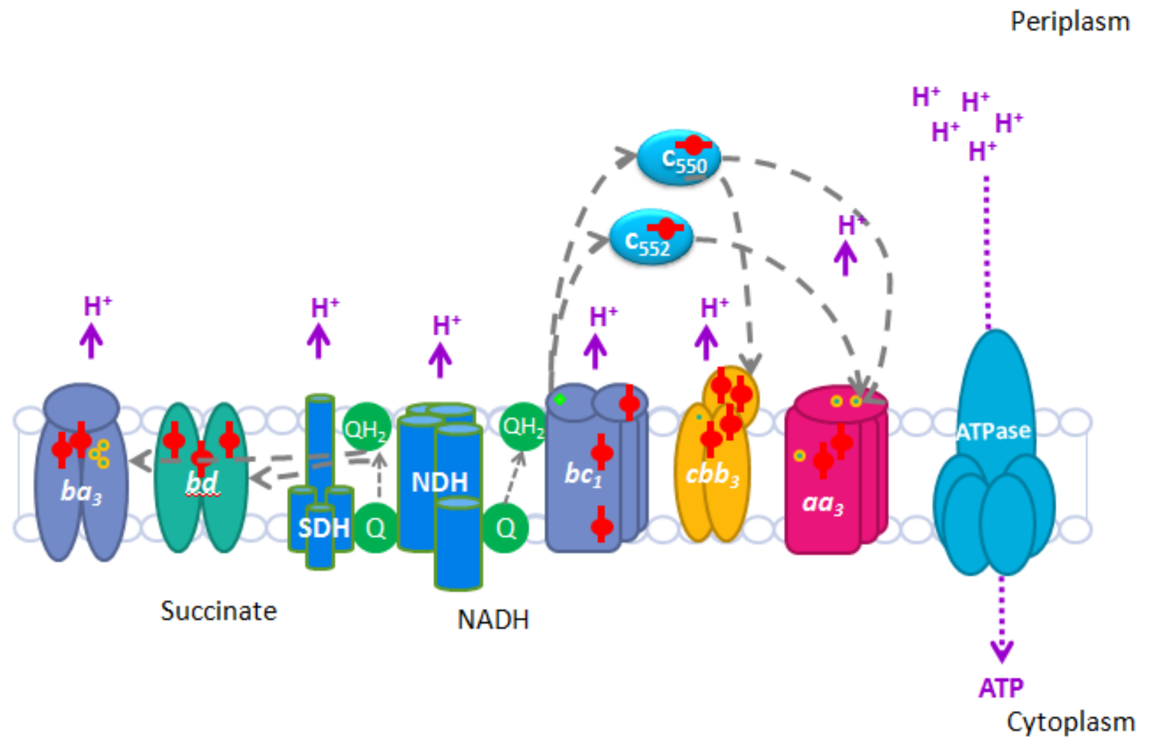


Figure 1.6-2 The aerobic respiratory pathway in *P.denitrificans* (Pd1222) showing the 3 haem-Cu oxidases (cyt *ba₃*, cyt *aa₃* and cyt *cbb₃*) and the quinol oxidase (cyt *bd*). Electrons are obtained (dotted lines) from the ubiquinone/ubiquinol pool (Q, QH_2), which is reduced by the succinate dehydrogenase (SDH) and NADH dehydrogenase. Electrons from the bc_1 complex are transferred to cyt c_{552} or cyt c_{550} which are subsequently transferred to cyt cbb_3 and cyt aa_3. Cyt ba_3 and cyt *bd* obtain e^- directly from the quinol pool. The catalytic centres compose of haem ( ), [4Fe-4S] cluster ( ) and copper ( ).

In the environment bacteria frequently have the capacity to overcome a particular metal deficiency by having two systems that can perform similar tasks. For example pseudoazurin is a Cu containing electron transfer protein and passes electrons to NO_2^- reductase and N_2O reductase, however they can also receive electrons from the cytochrome c_{550} (haem dependent). This ensures that even in the absence of Cu, electrons can still be transported and denitrification can continue. This also occurs in the aerobic pathway in some prokaryotes. *P.denitrificans* contains cyt *bd*, a haem only oxidase, and so a limited Cu concentration therefore may not affect aerobic respiration, although this has never been assessed. Methanotrophs have also overcome the possibilities of metal deficiency. The enzyme responsible for oxidising methane to methanol is methane mono-oxygenase. This enzyme is typically a Cu containing enzyme, however some methanotrophs also contain an Fe dependent methane mono-oxygenase (Knapp, Fowle et al. 2007; Balasubramanian and Rosenzweig 2008). The two forms are regulated differently in response to Cu availability (Knapp, Fowle et al. 2007). The synthesis of methanobactin occurs in response to low Cu availability. This protein acts as a Cu uptake system, it is proposed to act similarly to the Fe uptake system which uses siderophores (Balasubramanian and Rosenzweig 2008).

1.7 Denitrification and Global Warming: The Problems of Greenhouse Gas

There is great concern about our environment and how it will be affected due to global warming. One of the main culprits for the Earth's ever increasing temperature is the greenhouse gas carbon dioxide (CO_2), however methane (CH_4) and nitrous oxide (N_2O) also play a major factor. The global atmospheric concentration of these three gases have greatly increased since 1750 due to anthropogenic activities (Bates, Kundzewicz et al. 2008). Agricultural activities are responsible for the significant amounts of these greenhouse gases being released into the atmosphere (Bates, Kundzewicz et al. 2008; Smith, Martino et al. 2008). CO_2 is released by burning organic matter and microbial decay. CH_4 is produced from decomposition of organic matter in oxygen deprived

environments; this process occurs in ruminants and accounts for 18% of the global anthropogenic emissions of CH_4 (Smith, Martino et al. 2008). CH_4 is also emitted from manures that are stored such as slurry pits, also flooded rice fields are a major contributor (Smith, Martino et al. 2008). N_2O accumulates in the environment due to denitrification, whereby the final step, N_2O to N_2, is not carried out. Soils rich in NO_3^- and denitrifying bacteria often accumulate NO_2^- and N_2O (Betlach and Tiedje 1981). N_2O and CH_4 have greater terrestrial emissions in the tropics as N_2O emissions are sensitive to temperature, soil moisture and the rates of nitrogen cycling as well as carbon cycling (Ehhalt, Prather et al. 2001). Similarly CH_4 emissions are effected by increase in temperature, as well as excessive flooding in wetlands which affects the rates of carbon and nitrogen cycling (Ehhalt, Prather et al. 2001).

An increase of anthropogenic N_2O release has been occurring since the late 1800's and early 1900's due to an increase in the use manures and fertilisers in agriculture (Figure 1.7-1) (Ehhalt, Prather et al. 2001). Before the 1900's atmospheric N_2O increased steadily as the population and agricultural activity increased (Leigh 2004). In 2005 88 teragrams of N in synthetic fertiliser was used in agricultural soils globally, which directly resulted in 3.77 teragrams of N_2O being released to the atmosphere (Del Grosso, Wirth et al. 2008).

Other greenhouse gases that are focused on in the Kyoto Protocol and Montreal Protocol are hydrofluorocarbons (HFCs), perfluorocarbons (PFCs), sulphurhexafluoride (SF_6),chlorofluorocarbons (CFCs) and hydrochlorofluorocarbons (HCFCs) and halons, these gases were not present in our atmosphere before the twentieth century (Ehhalt, Prather et al. 2001). All greenhouse gases undergo a chemical process whereby they are removed from the atmosphere, except CO_2 and N_2O. H containing gases react with hydroxyl radicals (OH·), this process occurs in the troposphere (Ehhalt, Prather et al. 2001). The remaining greenhouse gases N_2O, PFCs, SF_6, CFCs and halons do not undergo this process and are destroyed in the stratosphere by ultra violet light (Ehhalt, Prather et al. 2001).

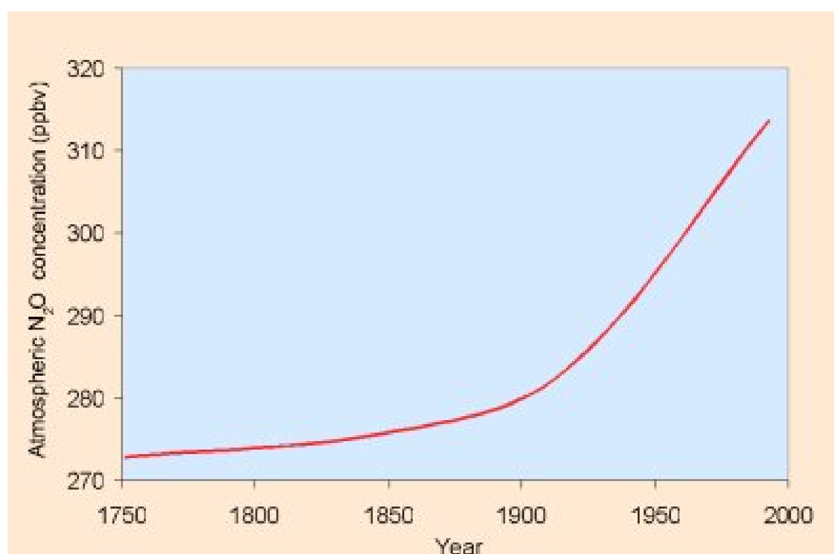


Figure 1.7-1 Average levels of atmospheric N_2O (ppbv) in the atmosphere from 1750 to 2000 (IPCC, Qin et al. 2007).

1.8 Nitrous Oxide

N_2O, commonly known as laughing gas, is a colourless non toxic gas that has been used for medicinal purposes for years (Lassey and Harvey 2007). However, N_2O is a potent greenhouse gas and Ozone depleting gas. N_2O is involved in the catalytic destruction of the stratosphere as it is oxidised to NO ($O^* + N_2O \rightarrow 2NO$) which depletes the ozone layer, $NO + O_3 \rightarrow NO_2 + O_2$ (Granger and Ward 2003; Bothe, Ferguson et al. 2007; Crutzen and Oppenheimer 2008). Due to the stability of N_2O its atmospheric life time is 115 years before it is broken down by ultraviolet radiation in the stratosphere (Rontu Carlon, Papanastasiou et al. ; Okereke 1993; Zumft 1997; Granger and Ward 2003; Lassey and Harvey 2007; Crutzen, Mosier et al. 2008). The concentration of N_2O in the atmosphere is a thousandth of carbon dioxide, however on a molecule by molecule basis the radiative potential of N_2O is 300 times more than CO_2 (Zumft 1997; Kimochi, Inamori et al. 1998; Haltia, Brown et al. 2003; Lassey and Harvey 2007; Crutzen, Mosier et al. 2008; Smith, Martino et al. 2008). N_2O therefore contributes to approximately 10 % of the total global warming effect.

Globally more than two thirds of natural N_2O emissions come from soils mainly due to denitrifying and nitrifying bacteria (Kimochi, Inamori et al. 1998; Skiba and Smith 2000; Lassey and Harvey 2007). The other third of N_2O emissions come from the ocean, where material is broken down by bacteria and aquatic denitrification (Granger and Ward 2003; Lassey and Harvey 2007; Smith, Martino et al. 2008). As global population has increased so has agricultural activity which has consequently lead to a rise in the application of artificial fertilisers and manures as to boost crop yields. Consequently as agricultural activity has increased the concentration of nitrogenous compounds, such as NO_3^- and NH_4^+, in soils has also increased, promoting the growth of nitrifying and denitrifying bacteria. An increase in denitrification in agricultural soils can lead to an increase in N_2O emissions, due to incomplete denitrification rendering N_2O rather than N_2. Wastewater treatment could also potentially be a contributor to N_2O emissions as it is vital for NO_3^- and NH_4^+ to be removed, promoting denitrification and nitrification (Zumft 1997; Skiba and Smith 2000; Lassey and Harvey 2007). Other anthropogenic sources of N_2O include the production of nylon, nitric acid, power plants (fossil fuelled) and a small amount emitted from vehicle emissions (Ehhalt, Prather et al. 2001). These anthropogenic emissions are responsible for an 18% increase of atmospheric N_2O over the past three centuries (Lassey and Harvey 2007), and are still increasing at a rate of 0.25% per year (Kimochi, Inamori et al. 1998; Ehhalt, Prather et al. 2001) (Figure 1.7-1).

1.9 Denitrification and agriculture

Agriculture is an essential human activity, as the population has increased so has the amount of land used for agricultural activities. The 1700's brought about an argument that sooner or later the population will outgrow food supply. As the population of Britain and Europe escalated, at approximately 0.3-0.5 % per annum (Humphrey and Stanislaw 1979), so did agricultural activity and demand for fertilisers. To maintain the increasing demand for agriculture in England and Europe new sources of fertilisers were needed. In the early 18th century Europe imported its first fertiliser outside of

Europe in the form of guano, bird droppings, from Peru. Britain started importing guano in the 1820's, by 1858 they were importing 300,000 tons per annum (Leigh 2004). Even though other guano deposits were discovered around the world, sources became exhausted within fifty years and by 1885 guano trade from Britain had practically disappeared. It was discovered, by Alexander von Humboldt, that guano contained high concentrations of nitrogen and phosphorus (Leigh 2004). Knowing that nitrogen, as well as other nutrients, was essential for growing crops, fixing nitrogen at an industrial level was of great importance. In 1903 it was demonstrated by Fritz Haber and Carl Bosch that ammonia (NH_3) can be formed from N_2 and H_2, in a process known as the Haber-Bosch process ($N_2 + 3H_2 \leftrightharpoons 2NH_3$) and was introduced commercially in 1907 (Leigh 2004). The Haber-Bosch process is the most significant form of nitrogen fixing this side of the 20th century, worldwide 10^7 tonnes per annum of NH_3 is made via the Haber-Bosch industry (Leigh 2004).

To cope with current demand for agriculture vast amounts of land are used for crops, and to get them to a high quality standard nitrogenous compounds are needed in the soils. The presence of NO_3^- and NH_4^+ in soils promotes plant growth, but also supply many microorganisms with alternative electron donors used in nitrification, denitrification and ammonification. These organisms are found in many environments from fresh and marine water, soils and waste water treatment systems and due to N_2O emissions there has recently been much interest in agricultural soils and denitrification.

In agriculture the main sources of nitrogenous compounds, NH_4^+ and NO_3^-, are from fertilisers, organic and artificial. NH_4^+ fertilisers provide substrate for nitrification, whereby NH_4^+ is converted to NO_3^- (Skiba and Smith 2000). NO_3^- derived from nitrification and NO_3^- from fertilisers all drive denitrification in soils (Skiba and Smith 2000) and cause N_2O to be released. The type of crops, compaction and water logging all influence N_2O accumulation which can vary greatly in soils. Crops such as potatoes, sugar beet and broccoli show an increased N_2O release from soils compared to that of barley, wheat and rape seed (Skiba and Smith 2000). Soil compaction thatncan be caused by tractor traffic, for example, has an effect on N_2O accumulation. Tractor traffic is a major problem in agriculture, especially in areas that have fine soil and a

humid climate as this increases anaerobic environments which promote nitrification and denitrification (Sitaula, Hansen et al. 2000). Soil compaction results in an increase of N_2O emissions with the effect being more prominent in fertilised (mineral fertiliser) soils, increasing emissions up to 170 %. Even in unfertilised soils, N_2O accumulation increases by 40 % when soil is compacted (Sitaula, Hansen et al. 2000). Compaction of soil results in the total pore volume being reduced, which consequently increases anaerobic microenvironments and also reduces diffusion of N compounds, potential creating hot spots for N_2O accumulation (Sitaula, Hansen et al. 2000; Skiba and Smith 2000). N_2O emissions are also promoted in soils with poor drainage and a fine texture, as these all promote anaerobic environments. High organic carbon also promotes denitrification (Russow, Sich et al. 2000; Skiba and Smith 2000; Bothe, Ferguson et al. 2007). Soils prone to water logging, such as paddy fields and the fens, have also been found to have increased N_2O accumulation compared to that of aerated and dry soils. This is again due to increased anaerobic microenvironments (Conrad 1996; Russow, Sich et al. 2000).

Arable soils are not the only contributors in N_2O emissions. Grassland also releases N_2O to the atmosphere, due to animals causing soil compaction and hot spots. N compounds from animal excrement are deposited onto soils and are then unable to diffuse throughout the compacted soils thus creating hot spots of N_2O mitigation. The input of NH_4^+ into soils from urine and faeces promotes nitrification, which in turn promotes denitrification, N_2O has been found to peak from 10 days to 4 months after deposition (Russow, Sich et al. 2000; Sitaula, Hansen et al. 2000; Skiba and Smith 2000).

1.10 Copper in agriculture

It has been previously discussed that copper plays a vital role in denitrification. Cu is an essential trace element for plants, as well as microorganisms and higher organisms (Alloway 2008). In plants Cu is involved in photosynthesis due to a Cu containing e^- transfer protein, similar to pseudoazurin in *P.denitrificans*, called

plastocyanin. Cu is also heavily involved in chlorophyll production, respiration, protein and carbohydrate metabolism and pollen formation (Solberg Elston, Evans Ieuan et al. 1995; Alloway 2008). Cu deficiency has major affects on crops such as cereals, spinach, carrots, onions and citrus fruit (Solberg Elston, Evans Ieuan et al. 1995; Alloway 2008; Shorrocks and Alloway 2011). Plants suffer from Cu deficiency when Cu content in soils is at <2-5 mg kg^{-1} (<30-80 µM), Cu being sufficient at 3-30 mg kg^{-1} (50 -500 µM). Soils with excessive Cu, 20-100 mg kg^{-1} (300-1600 µM), can become toxic to plants (Mortvedt 1991; Alloway 2008). Deficiency in cereals can go unnoticed until the ears, or spikes, develop; the ears become deformed in appearance, known as “rat tailing” (Alloway 2008). The main symptoms of Cu deficiency are wilting, blackening/melanism, white twisted tips (known as rat tailing), yellowing of young leaves and interrupted cell wall lignin formation and pollen formation which consequently decreases the fertility (Alloway 2008). Crops that have been found to cope well in Cu deficient soils are potatoes, rice and soybean (Alloway 2008).

Cu concentration is vital in agriculture to maximise the quality and yields of crops. In Europe up to 1.2 million km^2 of soil are Cu deficient. Cu deficient soils tend to be sandy and acutely leached soils, high pH and saline soils, organic and peaty soils, reclaimed heathland and shallow chalk soils with 6-12% organic matter (Solberg Elston, Evans Ieuan et al. 1995; Alloway 2008). Clay soils also absorb/chelate Cu and therefore only a fraction of the total Cu content in biologically available in such soils for plant uptake. Some Cu is also fixed by bacteria and fungi (Alloway 2008). In England the main areas with Cu deficient soils are the South-West, South-East and East Anglia, due to the high chalk and clay content of these areas (Alloway 2008).

Treatment of Cu deficient soils involves adding Cu supplements. Copper sulphate (25 % w/v Cu) and copper oxychloride powder (52 % w/v Cu) can be applied directly to the soil, a typical application being 2.5-5 kg Cu.ha^{-1}, which can last up to 10 years (Alloway 2008). Foliar application (spraying crops) either consists of copper oxychloride (~25 % w/v Cu in liquid) or chelated Cu (9 % w/v). Application rate is between 200 – 500 g Cu ha^{-1} and 70 g Cu ha^{-1} respectively. Many waste fertilisers such as sewage sludge, pig slurry and manures have a high Cu content (Alloway 2008). Poultry manures contain on average 80 mg Cu/kg dm, cattle manures have a lower Cu content and approximately contain 50 mg Cu/kg dm (Nicholson, Chambers et al. 1999).

The concentration of available Cu does not only affect crops but has the potential to affect metabolic pathways in soil denitrifying bacteria. N_2O reductase is unable to reduce N_2O to N_2 if the Cu centres are not formed. N_2O is not toxic or vital to bacterial survival and so the last step of the denitrification pathway can potentially be sacrificed. Cu is also involved in NO_2^- reduction in some bacteria such as *A.xylosoxidans,* therefore Cu can have a variety of effects on various denitrifying bacteria. With 20-40 % of Europe's arable soil being Cu deficient, along with Southern and Western Australian soils and Brazil, the potential impact on N_2O production is high.

1.11 **Aims**

The impact of Cu on denitrification is of great topical importance and is the subject of this Doctoral thesis. The aim of this thesis is to investigate how Cu concentration affects two denitrifying soil bacteria, *P.denitrificans* and *A.xylosoxidans,* both of which are capable of fully reducing NO_3^- to N_2. Incomplete denitrification can lead to the release of N_2O, and with the Cu dependent nitrous oxide reductase it is possible that the Cu concentration in an environment could potentially have a significant effect on the release of this potent greenhouse gas.

Chapter 2: Methods and Techniques

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2.1 Growth of bacteria

P.denitrificans (Pd1222) and NosZ mutant (Pd10221) were grown in *Paracoccus* minimal medium (29 mM Na_2HPO_4; 11 mM KH_2PO_4; 10mM NH_4Cl; 0.4 mM $MgSO_4$) with either 5 mM succinate and 20 mM NO_3^- (carbon limited, NO_3^- sufficient) or 20 mM succinate and 5 mM NO_3^- (carbon sufficient, NO_3^- limited); and supplemented with 2 ml/L Vishniac trace element solution (130 mM ethylenediaminetetraacetic (EDTA); 7.64 mM $ZnSO_4$; 25 mM $MnCl_2$; 18.5 mM $FeSO_4$; 0.89 mM $(NH_4)_6Mo_7O_{24}$; 6.4 mM $CuSO_4$; 6.72 mM $CoCl_2$; 37.4 mM $CaCl_2$) (Vischniac and Santer 1957), media was pH 7.5 unless stated otherwise. The final copper concentration of the *Paracoccus* minimal media, including the Vishniacs was measured at 18 µM (for method see 2.4). To reduce the Cu concentration $CuSO_4$ was omitted from the Vishniacs, this reduced the total Cu concentration of the *Paracoccus* minimal media to 0.8 µM. To further reduce the Cu concentration in the *Paracoccus* minimal media 1 mM Ascorbate and 0.2% (w/v) Bicinchoninic Acid solution (Sigma cat no. B9634) was added to the media supplemented with the Vishniacs (with $CuSO_4$ emitted) and incubated at 37°C for 1 h (Moody, Mitchell et al. 1997), then sterilised. To increase the Cu concentrations 1 ml/L of 3mM $CuSO_4$ solution was added to media then sterilised, the Cu concentration was measured at 49 µM. *A.xylosoxidans* was also grown on *Paracoccus* minimal media supplemented with 2ml/L Vishniacs and 0.01 % (w/v) Yeast Extract.

Typical batch culture experiments were inoculated with 1 % of an over night culture and were grown at 37 ^{o}C over 12 h and for each condition the cultures were repeated 3 times.

2.2 Chemostat Cultures

Chemostat experiments were performed in New Brunswick Scientific Co Bioflo 3000 chemostats, where a waste port was added at the liquid vessel volume (1.5 L). Chemostats were calibrated for pH and dissolved oxygen (DO) prior to sterilisation, pumps were also calibrated for each run. The inoculum for the chemostat cultures

were grown aerobically at 37 ^{o}C in 100 ml of *Paracoccus* minimal media in 250 ml conical flasks. Chemostat inoculations (0.6% innocula) were performed under aerobic conditions at 100 % DO, 37 ^{o}C, pH 7.5. After 24 h of aerobic growth the air supply was removed and feed was added at a dilution rate (D) of 0.06 h^{-1} ($D = \frac{F}{V}$ where F represents the flow rate and V the vessel volume) for *P.denitrificans* and *A.xylosoxidans* grown in both NO_3^- sufficient carbon limited and NO_3^- limited carbon sufficient media. Throughout the chemostat culture experiment the temperature was maintained at 37 ^{o}C and pH 7.5 using 1 M NaOH and 0.1 M H_2SO_4. After 3 vessel volumes the culture becomes steady state, the cultures were harvested at 120 h. Throughout the chemostat culture cell and supernatant samples were attained and stored at -20 ^{o}C, and 12 ml gas samples were stored in 12 ml exetainers (Labco) at room temperature, samples were taken 2 – 3 times a day. Chemostat cultures were repeated 3 times for each separate condition.

2.3 **Cell Fractionation**

Cells from either chemostat cultures or batch cultures were centrifuged at 3743 x g for 15 min. The pellets were resuspended in sphaeroplastic buffer (500 mM sucrose; 100 mM Tris; 3 mM EDTA; pH 8), 1 mg/ml of lysozyme. Cells were agitated and incubated at 37 ^{o}C for 1-2 h then centrifuged at 14972 x g for 15 min and the supernatant (periplasmic fraction) was removed and frozen (-80°C). The remaining pellet was resuspended 20 ml 10 mM Tris-HCl pH 8 and vigorously stirred at 4 ^{o}C until the pellet was completely resuspended and the solution was homogenous (method adapted from Paul Mills). The mixture was then centrifuged at 25000 x g for 1 h, the supernatant (cytoplasmic fraction) was frozen at -80 ^{o}C and the remaining pellet was resuspended in 2 ml of 10 mM Tris-HCl pH 8. The fractions were then concentrated using an amicon (Millipore Stirred Ultra Filtration System) with a 3 kDa cut off membrane (Millipore YM3) to a final volume of 1 ml per litre of culture. All samples were analysed for protein concentration using the BCA method (See section 2.5), then samples were diluted to 100 mg/ml of protein and stored at -80 ^{o}C until needed.

2.4 Copper Assay

A colourimetric test was carried out to determine the copper concentration in minimal media used for growing *P.denitrificans* and *A.xylosoxidans*. Aliquots of 500 µl media were added to 400 µl copper determination solution (1 M NaOH; 730 mM HEPES; 2.7 % bicinchoninic acid (Sigma cat no. B9634) dissolved in MQ H_2O) and 100 µl of 64 mM ascorbic acid. Samples were incubated at room temperature for 5 min so that the ascorbic acid can reduced Cu(II) to Cu (I) so that the total Cu can be measures, the absorbance was measured at 562 nm (Shimadzu UV-160 UV-visible recording spectrophotometer). The absorbance readings were converted to µM Cu using a standard curve of 10 – 50 µM $CuCl_2 2H_2O$ (Figure 0-1) (method adapted from Kihlken, Leech et al. 2002). For measuring the Cu content of *Paracoccus* media a 10 x *Paracoccus* media solution was made, including the addition of Vishniac solution. All measurements were replicated 3 times.

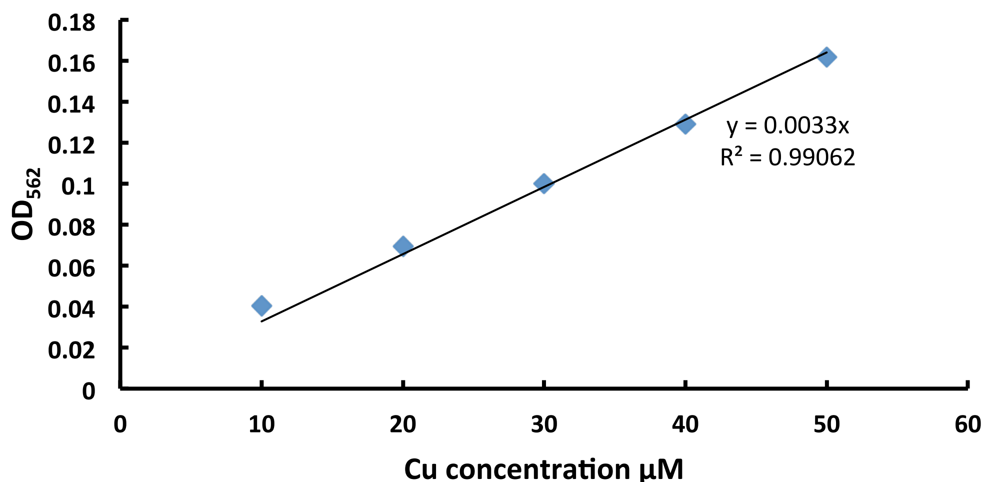


Figure 0-1 Standard curve of the copper assay

2.5 Protein determination

Protein concentration was determined using the bicinchoninic acid (BCA) method: 100 µl samples from chemostat cultures or cell fractions were added to 2 ml of protein determination solution (1 part 4% copper (II) sulphate solution to 50 parts bicinchoninic acid (Sigma cat no. B9634)). Samples were incubated at 37 ^{o}C for 30 mins and protein concentration was determined by measuring the absorbance at 562 nm (Shimadzu UV-160 UV-visible recording spectrophotometer) and comparing it to a standard curve prepared using 20 – 100 µg bovine albumin protein as the standard (Smith 1987). Each sample analysed was repeated 3 times.

2.6 Compilation for Henrys Law Constants

Henry's law was formulated in 1803 and is a gas law whereby at a constant temperature the concentration of a given gas in a volume of liquid is directly proportional to the partial pressure or concentration of that gas in equilibrium with the liquid. Through analysing gas samples taken from the head space of chemostat cultures we can identify the concentration of trace gases in the gas phase of the chemostats vessels. Using Henry's law literature values and rearranging the equations the concentration of gas dissolved in the liquid phase of the vessel can be calculated, which can be used to identify the total net production of a gas (all equations in this section have been attained from Sander 1999).

Typically Henry's law constant, k_H, is defined as Equation 2-1

$$k_H = c_a / p_g$$

Equation 2-1

Where c_a refers to the concentration of a trace gas in the liquid phase and the p_g is the partial pressure (101325 Pa (Nm^{-2})) of the trace gas in the gas phase, at a temperature.

Standard temperature (T^{Θ}) is referred to as 298.15 K (25 °C) and the constant is shown as T_{H}^{θ}. Henry's law constant can also be expressed as a dimensionless ratio between liquid and gas phase concentration, k_{H}^{cc}, however this is dependent on temperature and therefore a series of calculations have to be undertaken as to find the k_{H}^{cc}.

Henry's law constant as a function of temperature can be expressed as seen in Equation 2-2. Where k_{H}^{θ} represents the k_H at 25 °C, exp is the 1/natural log, $\Delta_{\mathrm{soln}}H$ is the enthalpy of solution (Kj/mol), R is the gas constant (8.3141 JK^{-1} mol^{-1}).

$$k_H\left(\frac{mol}{atm \times dm^3}\right) = k_H^{\Theta} \exp\left(\frac{-\Delta_{so\ln}H}{R}\left(\frac{1}{T} - \frac{1}{T^{\Theta}}\right)\right)$$

Equation 2-2

The temperature dependence refers to enthalpy of the solution being equal to that as the change in the k_H which is dependent on temperature (T) (Equation 2-3):

$$\frac{-\Delta so\ln}{R} = \left(\frac{d\ln k_H}{T}\right)$$

Equation 2-3

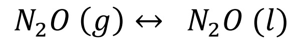
The k_{H} can then be used to express Henry's law constant as a dimensionless ratio, Equation 2-4. The constant 12.2 is determined by the relation between K_{H} and K_{H}^{cc}

$$\frac{T}{[K]} \; x \; \frac{k_H}{[\frac{M}{atm}]} = 12.2 \; x \; k_H^{cc}$$

$$k_{\mathrm{H}}^{cc} \overset{\mathrm{def}}{=} \frac{k_{\mathrm{H}}T}{12.2}$$

Equation 2-4

Equation 2-4 represents the K_{H}^{cc} as the liquid over gas form. The liquid phase concentration over gas phase concentration form of the Henry's law constant is the equilibrium constant for the reaction:



$$k_H^{cc} = \frac{C_{N_2O}\ (l)}{C_{N_2O}\ (g)}$$

Equation 2-5

For example, N_2O has a $k_{\mathrm{H}}^{\theta} = 2.5 \text{ x } 10^{-2}$ M/atm and $\frac{\mathrm{d\ In}\ K_{\mathrm{H}}}{\mathrm{d\ (1/T)}} = 2600$ (data obtained from Sander 1999). Equation 2-6 describes the steps taken to find the K_{H} of N_2O at 310.15 K (37 °C).

$$k_{\mathrm{H}}\left(\frac{\mathrm{mol/dm^3}}{\mathrm{atm}}\right) = k_{\mathrm{H}}^{\theta}\ exp\left(\frac{-\Delta_{\mathrm{soln}}H}{\mathrm{R}}\left(\frac{1}{T} - \frac{1}{T^{\theta}}\right)\right)$$

$$k_{\mathrm{H}} = k_{\mathrm{H}}^{\theta} \text{ x } exp^{\left(2600\left(\frac{1}{310.15} - \frac{1}{298.15}\right)\right)}$$

$$= 0.0178\ \mathrm{M/atm}$$

Equation 2-6

Equation 2-7 expresses the dimensionless constant ($\mathrm{K}_{\mathrm{H}}^{cc}$) of N_2O at 310.15 K (37 °C)

$$k_{\mathrm{H}}^{cc} = \frac{k^{\mathrm{H}} \text{ x T}}{12.2}$$

$$k_{\mathrm{H}}^{cc} = \frac{0.0178 \text{ x } 310.15}{12.2}$$

$$k_{\mathrm{H}}^{cc} = 0.4525\ (37\ ℃)$$

Equation 2-7

Table 2-1 Henry's law K_H^{cc}, $\frac{d \ln k_H}{d(1/T)}$ constants attained from Sander (1999) and the dimensionless ratio K_H^{cc} at 37 °C calculated using the Henry's law equation.

	k_H^{θ} (M/atm)	$\frac{d \ln k_H}{d(1/T)}$	K_H^{cc}
NO	1.9×10^{-3}	1500	0.05868
N_2O	2.5×10^{-2}	2600	0.4525
N_2	6.5×10^{-4}	1300	0.0237

2.7 Nitrate/Nitrite Reductase Activity

A colourimetric methyl viologen (MV+)-dependent assay was used to measure the NO_3^- and NO_2^- reductase activity of the cells grown in chemostat cultures. Glass cuvettes (10 mm lightpath) containing 10 mM Hepes buffer; 2 mM EDTA, pH 7.5, 1 mM Methyl viologen (MV+) and chemostat cell sample (75 µl/ml of reaction) were sealed with rubber septa and sparged with oxygen free nitrogen for 1 min per 1 ml of reaction. Sodium dithionite (100 mM) was titrated into the anaerobic cuvette until the absorbance (Abs) at 600 nm reached 0.7 – 1. The reaction was initiated by the addition of 1 M NO_3^- or NO_2^- to give the final concentrations of 10 mM NO_3^-or 1 mM NO_2^-. The change in Abs_{600} was monitored using a Shimadzu UV-160 UV-visible recording spectrophotometer. The change in Abs_{600} was used to calculate the NO_3^-or NO_2^- reductase activity using the Beer Lambert law $A = \varepsilon c l$, where A = Abs_{600}, ε is the absorbance coefficient of reduced MV+ which is equal to13 $mM^{-1}cm^{-1}$, c = concentration of NO_3^- or NO_2^- and l = lightpath length. The assay was repeated in duplicate for each chemostat sample.

2.8 Measurement of Nitrate and Nitrite

Supernatant samples from chemostat cultures were diluted 1 in 10 and filtered (0.2 µm RW prefilters, Millipore). Samples were measured on a ICS-900 Dionex HPLC system with an IonPac AS9-SC Carbonate Eluent Anion-Exchange Column (Dionex) and a Anion Self-Regenerating Suppressor (ASRS® 300, Dionex). The eluent (1x HPLC Buffer 1.8 mM Na_2CO_3; 1.7 mM $NaHCO_3$) and regenerant (10 mM H_2SO_4) were also sterilised using 0.22 µm millipore membrane filters. The flow rate was set at 250 µl.min^{-1} with a 20 µl injection volume. Standards of 0.1 – 2 mM NO_3^- and NO_2^- were used to determine the NO_3^- and NO_2^- concentration in the chemostat samples. The samples analysed were replicated twice.

2.9 Measurement of Nitrite (Griess reaction)

A 100 µl sample of supernatant from chemostat cultures was added to 0.9 ml of 1 % (w/v) sulphanilamide in 1 M HCl, 100 µl of 0.2 % (w/v) N-napththylethylenediamine hydrochloride, dissolved in H_2O, was then added. Samples were incubated at room temperature for 25 mins and the amount of NO_2^- was determined by measuring the absorbance of each sample at 540 nm, the final NO_2^- concentration was determined using a standard curve constructed with known concentrations of NO_2^- ranging from 20-100 µM (Nicholas and Nason 1957). Samples analysed were replicated in triplicate.

2.10 Measurement of Nitric Oxide

A method for measuring the concentrations of NO was developed using a modified Griess assay adapted from van Wonderen, J. H. *et al*. (2008). To dissolve the NO into solution, a 2 ml gas samples from the head space in chemostat cultures were taken and immediately transferred into 3 ml exetainers (Labco) scrubbed with 0.1 M NaOH and containing 1 ml of 2 mM $CaCl_2$, 50 mM Hepes buffer, pH 7.4, sparged with N_2 for 5

mins. The samples were vortexed and left to agitate at room temperature for 10 minutes. The buffer was then used in a modified Griess reaction. 50 µl of sample was added to cuvettes (10 mm lightpath) containing 1.9 ml 17 mM sulphanilic acid, 0.4 mM *N*-(1-naphthyl) ethylenediamine dihydrochloride. After 1 min 50 µl of phosphoric acid (85 %) was added, and the cuvettes were incubated at room temperature for 15 min and the absorbance was measured at 540 nm (van Wonderen, Burlat et al. 2008). A standard curve was constructed using 10-50 µM NO.

The NO concentration determined from the modified Griess reaction was for gas phase. Henry's law was used to determine the concentration of NO in liquid phase (See

Table 2-1)

2.11 N_2O electrode

An O_2 Clarke electrode was modified to have a Ag cathode and anode. This electrode was optimised for the detection of N_2O, however O_2 was also detected and so the vessel had to be anaerobic before any experiments were started. All media and solutions were sparged with N_2 prior to the experiment. The electrode was set up according to Figure 2.1. The electrolyte used consisted of 1 M KOH and 100 mM KCl, to minimise electrolyte breakdown at the relatively high polarising voltage required for N_2O reduction (Alefounder and Ferguson 1982). Polarising voltage was set to -1.2 V. A stock solution of N_2O was made by sparing 10 ml H_2O with N_2 for 10 min, and then cooled to 4^oC then the H_2O was N_2O was sparged for 10 min. The N_2O concentration was determined by GC analysis (Section 2.12) as 36 ± 1.76 mM. As the electrode reacts with O_2 as well as N_2O so to attain an anaerobic vessel an O_2 scrubbing system composing of glucose oxidase (5 mg/ml), glucose (16 mM) and catalase (0.3 mg/ml) was added to the reaction vessel containing 2 ml of *Paracoccus* minimal media, without NO_3^-. When the electrode signal (mV) became steady 3.6 µmols of N_2O was added from the stock solution causing the mV to increase. Cell samples were

centrifuged at 13 000 rpm in a desk top centrifuge. The cells were washed and resuspended in minimal media not containing NO_3^- as this can interfere with the electrode signal. The washed cells were added to the vessel and the change in mV corresponded to the reduction of N_2O to N_2.

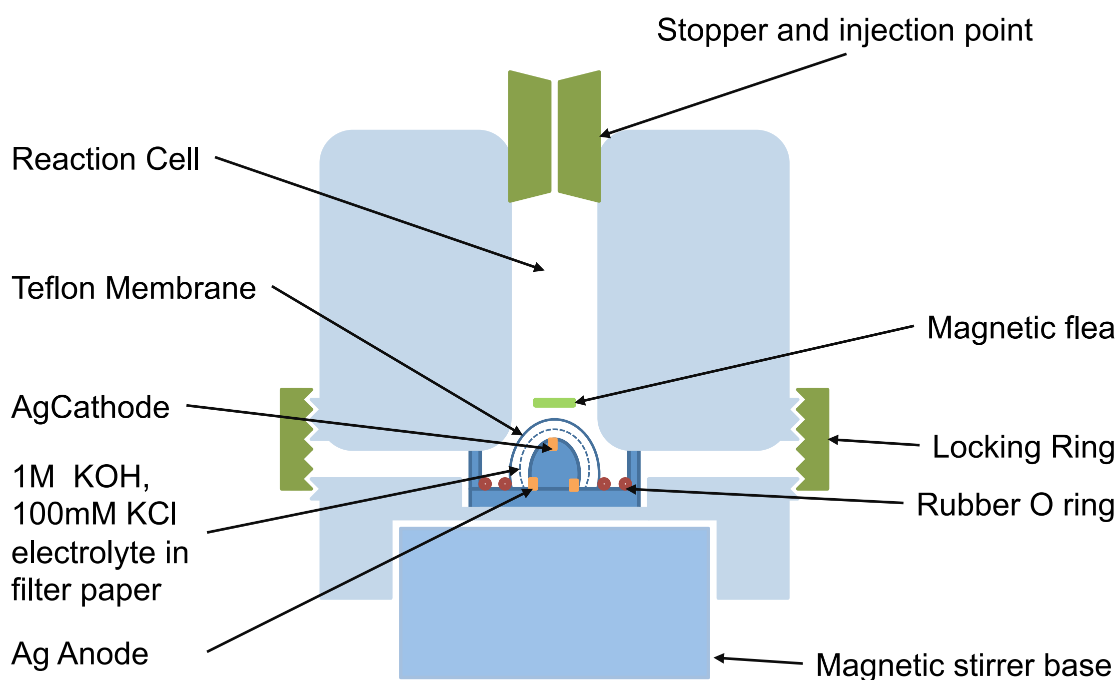


Figure 2.1 The set up of the Ag/Ag Clarke electrode. The light blue areas represent the Oxytherm system which controls temperature and contains the electronics. The dark blue represents the epoxy resin electrode base which the anode and cathode are set in.

2.12 Measurement of Nitrous oxide

Evacuated exetainers (737W Labco) with Telfon/silicon seals were used to store gas samples taken from the head space of chemostat cultures. The samples were analysed using a Perkin Elmer Clarus® 500 Gas Chromatography with an Electron Capture Detector (ECD) and Elite-PLOT Q (DVB Plot Column, 30 m, 0.53 mmID). The carrier gas consisted of zero N_2, and the make up gas consisted of 95 % argon and 5 % methane The temperature of the ECD was maintained at 350 ^{o}C until ≤ 2 mV, the column

temperature was maintained at 90 ^{o}C, and the injector at 115 ^{o}C. The N_2O concentration in the gas samples were determined by peak area and comparing the areas to a standard curve, using known gas standards of N_2O (0.4, 100, 1000 ppm (m), supplied from StGas). The ppm was converted to mM using the following calculation:

2.13 Conversion of N_2O ppm (m) to μM

Calibration gas cylinders contain N_2O in N_2.

1 ppm (m) = 1/1000000 g/g

so in 1g N_2O/N_2 mixture:

$$\frac{1\text{x}10^{-6}\ \text{g}}{\text{N}_2\text{O (44.013 g/mol)}} = 2.272 \text{ x } 10^{-8} \text{ mols N}_2\text{O}$$

assume the air in the experiment = 100% N_2:

per 1g N_2 (28.013 g/mol)

$$\frac{1\ \text{g}}{\text{N}_2\ (28.013)} = 3.57 \text{ x } 10^{-2}$$

$$\frac{\text{N}_2\text{O}}{\text{N}_2} = \frac{2.2\text{x}10^{-8}}{3.57\text{ x }10^{-2}} = 6.36 \text{ x } 10^{-7} \text{ mole/mole}$$

Volume of 1 mole of ideal gas:

$$pV = nRT$$

$$V = \frac{nRT}{p}$$

$$V = \frac{8.3141 \text{ x } 310.15}{101325} = 0.0254 \frac{\text{m}^3}{\text{mole}}$$

$$= 25.4 \text{ L/mole}$$

Convert concentration to μM:

$$= \frac{6.36 \text{ x } 10^{-7} \text{mole/mole}}{25.4 \text{ L/mole}}$$

1 ppm (m) = 2.504×10^{-8} moles/L

= 2.504×10^{-2} μM

Due to the solubility of N_2O Henry's law was used to calculate the concentration of N_2O in liquid phase.

2.14 ^{15}N analysis

Chemostat cultures of *P.denitrificans* and *A.xylosoxidans* were set up as described in section 2.2, except for the NO_3^- was labelled with ^{15}N, rendering ^{15}N-NO_3^- (20-30 % atom enriched). The cultures were run until steady state was entered, as previously described. At 102 h a 120 ml gas sample was taken along with a 250 ml/Liquid sample. The gas samples were analysed for $^{15}N_2$ and liquid samples were measured for $^{15}NH_4^+$, by colleagues at the University of Aberdeen, on a isotope ratio mass spectrometer (SerCon Ltd) following cryofocusing in an ANCA TGII gas preparation module. The data received, % ^{15}N, was converted to ppm and μM , 1 ppm = 0.3937 μM (section 2.13).

2.15 Conversion of N_2 ppm to μM

For N_2 to be converted into μM from ppm using the calculation from section 2.13. The constant attained from the calculation ;

1 ppm (m) = 3.94×10^{-8} moles/L

= 3.94 x 10^{-2} µM

2.16 SDS PAGE

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) was used to separate and detect proteins from cellular fractions. Gels were prepared using a Hoefer Mighty Small Dual Gel Castor, with 12.5 % polyacrylamide resolving gel [12.5 % polyacrylamide (30 % acrylamide/Bis 37.5:1, (BioRad);0.1 % SDS (w/v); 375 mM Tris HCl, pH 8.8, 0.1 % N,N,N',N;-tetramethylethylenediamine (BioRad); 0.1 % Ammonium persulphate (Sigma)) and a 4 % polyacrylamide stacking gel [4 % polyacrylamide (30 % acrylamide/Bis 37.5:1,(BioRad); 0.1 % SDS (w/v); 125 mM Tris HCl, pH 6.8; 0.1 % N,N,N',N;-tetramethylethylenediamine (BioRad); 0.1 % Ammonium persulphate (Sigma)) were run on a Hoefer Mighty Small Gel system at 15 mA through the stacking gel and 30 mA through the resolving gel , using a running buffer of 25 mM Tris HCl, 192 mM glycine; 0.1 % SDS (w/v). Cell fractions and protein samples were incubated at 100 ^{o}C for 15 min with protein loading buffer (100 mM Tris HCl, pH 6.8; 200 mM Dithiothreitol; 4 % SDS (w/v); 0.2 % bromphenol blue; 20 % glycerol) (10:4 ratio respectively). The gels were stained with InstantBlue Coomassie stain (Expedon) for 15-20 mins or gels were removed and Western-blotted, see section 2.18, or haem stained, see section 2.17.

2.17 Haem Stained SDS PAGE Gels

A 12.5 % SDS PAGE gel was run as described above. The gel was removed from the glass plates and placed in a container containing 70 ml of 0.24 mM sodium acetate, pH 5. The container was incubated in darkness at room temperature with agitation for 10 min. A 30 ml solution of 3 mM 3,3',5,5'-Tetramethylbenzidine dihydrochloride Hydrate dissolved in methanol was added and incubation continued at room temperature with agitation for a further 10 min. While remaining in darkness 300 µl of 10 % H_2O_2 was added. Blue bands present on the SDS PAGE gel represent haem containing proteins.

2.18 **Western Blot**

A 12.5 % SDS PAGE gel was run as described above. The Trans-Blot SD Cell (BioRad) was prepared with six layers of 10 x 6 cm blotting paper soaked in transfer buffer [10 mM CAPS, 11 % methanol pH 11] were stacked with one 10 x 6 cm PVDF membrane (Amersham Hybond™-P, GE Healthcare) washed in methanol and transfer buffer, the 12.5 % polyacrylamide gel washed in transfer buffer and a further six 10 x 6 cm layers of blotting paper soaked in transfer buffer. The Trans-Blot SD Cell was run for 45 mins at 20 mA per full gel (10 x 6 cm). The membrane was removed from the stack and blocked for 1 h with the blocking solution (5 % w/v skimmed milk powder in PBS-T 0.05 % TWEEN 20 (8 g NaCl, 0.2 g KCL 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4, 1 ml TWEEN in 1 litre of H_2O)). The primary antibodies (Abs), specific to the protein of interest, was added (volume dependent on antibody) and incubated at 4 ^{o}C overnight with agitation (Paz Abs were raised in Rabbit and the NarGH Ab, NirS Ab, NorB Ab and NosZ Ab were raised in sheep). The membrane was washed in PBS-T 0.05 % TWEEN 20, then blocked again for 1 h with blocking solution. The secondary antibody was then added (volume dependent on antibody), which was a horseradish peroxidase conjugate, and agitated at 4 ^{o}C for 1 h. The membrane was washed with PBS-T 0.05 % TWEEN 20 and incubated in darkness for 5 mins with 100 µl per 1 cm^2 of membrane Chemoluminescent Substrate – Super Signal® West Pico Chemoluminescent Substrate kit (PIERCE), with agitation. The membrane was exposed to Kodak Bio max XAR film (Sigma) in a dark room and then developed using an exograph film developer. Exposure time depended on the antibodies. Controls were carried out using purified protein (Figure 2.2).

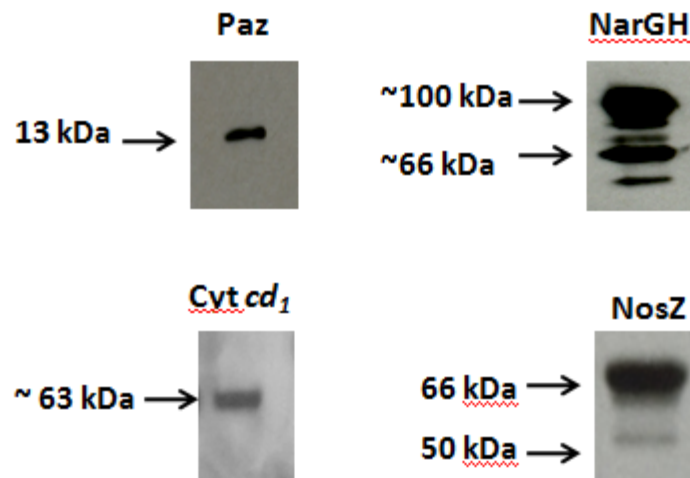


Figure 2.2 Western blotted SDS PAGE gels probed with Abs against Paz, ~ 20 µg loaded, NarGH, ~ 30 µg loaded, cyt cd_1, ~ 30 µg loaded and NosZ, ~ 20 µg loaded.

2.19 RNA extraction and Reverse Transcription

RNA was extracted using RiboPure™-Bacteria Kit (Ambion), isolation of RNA was carried out as instructed. The RNA was checked for degradation using the Experion™ RNA High Sens system (BIO RAD). The RNA High Sens chips were loaded with 1 µl of RNA samples, which had been denatured at 90 °C for 2 min. The chips were loaded on the Experion™ and run according to manufactures instructions. Degraded RNA shows a smear, RNA that has not been degraded shows 2 distinct bands on the gel, one representing 16s RNA and the other 23s RNA. Samples that did not show degradation were used for Real Time PCR.

2.20 **Reverse Transcription**

For the reverse transcription 200 ng of RNA (stored as 10 µl aliquots) was added to a sterile and RNase free eppendorf with 200 ng random hexamers (Invitrogen) and incubated at 70 ^{o}C for 10 mins. The samples were incubated on ice, to each sample the following were added: 4 µl of 5 x superscript buffer; 2 µl DTT; 1 µl superscript 11 (Invitrogen); 1 µl dNTP's (Roche) and 1 µl RNase inhibitor, RNasin® (Promega). The samples were incubated at 42 ^{o}C for 1 hour, the final concentration of cDNA after the reaction is 10 ng/µl. Samples were stored at -20 ^{o}C until required for Real Time PCR

2.21 **Real time PCR**

For each set of primers used, a standard curve was needed. This was set up by using cDNA from the chemostat cultures. A 1/10 dilution was carried out and this was then used in a serial dilution where by 8 µl of the diluted DNA were added to 12 µl of H_2O; 10 µl where transferred to 10 µl of H_2O, then 10 µl of the second dilution was diluted into another 10 µl of H_2O and so on. This standard curve rendered 4, 2, 1, 0.5, 0.25 and 0.125 ng samples, these were then loaded in a 96 well plate. For the samples cDNA from the reverse transcription were diluted 1/10 and 10 µl were loaded onto the 96 well plate.

For each PCR reaction 12.5 µl of Precision 2 x qPCR Master Mix with low ROX (Primer design), 1.25 µl primers (designed by primer design (Table 2.1), quencher dye is TAMARA^{TM}) 1.25 µl H_2O and 10 µl cDNA sample were added to a well on a Micro Amp® fast optical 96 well plate (Applied Bioscience).

Once all the wells were filled the plates was placed into a 7500 Fast Real Time PCR system (Applied Biosciences) and the run cycle was started (50 ^{o}C for 2 min, 95 ^{o}C for 10 min, 40 x (95 ^{o}C for 15 s, 60 ^{o}C for 1 min)) and the fluorescence from the report dye, TAMARA^{TM} was monitored. To analyse the data obtained standard curves were constructed using the cycle number (ct) plotted against the log input (Log (µg RNA in

sample)). The *x* and *y* intercepts of the standard curve, obtained from the line of best fit (R^2= > 0.98) are also used in calculating the gene expression (in arbitrary units). The following calculation was used to identify the input:

$$input = 10 \wedge \frac{(ct - x)}{y}$$

The input of the gene of interest, *nosZ,* was normalised by dividing the *nosZ* input by the housekeeping gene input, *RpoB.*

Table 2.1 Primers used for Real Time PCR. Primers were designed by Primer Design Ltd. with double dye SYBRgreen. Tm represents melting temperature in ^{o}C.

		Primer sequence	Tm
Pd1222 RpoB	Sense Primer	GGTCACCGTCGGGATGAAATA	58.1
	Anti-sense Primer	GGGTCACCAGGCTGTAGGG	59.4
Pd1222 NosZ	Sense Primer	GCCCATTTGCCCTCGTAATC	57.4
	Anti-sense Primer	ATGGAGGACGTGGCGAATTA	57.2
A.xylo RpoB	Sense Primer	CCGTGAAGCTGGCGAAACC	59.7
	Anti-sense Primer	GTCGTCGTGCTCCAGGAAC	58.5
A.xylo NosZ	Sense Primer	GTGGCTGGTGTCGCTGAAC	59.4
	Anti-sense Primer	GGAAATGTCGATGAGCTGGTC	57.4

Chapter 3: The effect of copper on *Paracoccus denitrificans* chemostat cultures

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3.1 **Introduction**

3.1.1 *Paracoccus denitrificans*

There has been much research into the production of N_2O from denitrifying bacteria. A study by Granger and Ward (2003) looked into *Pseudomonas stutzeri, P.denitrificans* and an unknown bacterial species, into how copper concentration affected N_2O production in an aquatic type environment. Two thirds of the atmospheric N_2O comes from the soil, especially from NO_3^- and NH_4 rich soils. *P.denitrificans* is a model denitrifying organism that contains a variety of metalloproteins, the one of most interest being the copper containing N_2O reductase. N_2O reductase has been well characterised in many bacteria, however some denitrifiers do not contain N_2O reductase, such as A*grobacterium tumefaciens* (Wood, Setubal et al. 2001). The reduction of N_2O to N_2 is not essential for bacterial life as N_2O is not cytotoxic. As yet there has only been one protein identified capable of efficiently reducing N_2O to N_2, the Cu containing N_2O reductase. Therefore in *P.denitrificans* cultures copper limitation has an effect on the release of N_2O, as found by Granger and Ward (2003). When Cu was removed from an aquatic environment (artificial sea water) N_2O was found to accumulate, they also found that NO_2^- accumulated. In one of the species they examined N_2O was not produced. So how does copper affect the denitrification pathway? Using *P.denitrificans* as model denitrifier we aim to find how the accumulation of intermediates and regulation of N_2O reductase is affected by copper limitation.

Using carefully defined chemostat culture conditions *P.denitrificans* can be grown in NO_3^- sufficient and carbon limited media with various copper concentrations. By monitoring the intermediates formed during denitrification it can be determined whether Cu concentration has a direct impact on N_2O production.

3.1.2 Batch and Continuous Cultures

To grow bacteria in liquid cultures there are two main methods, batch culture and continuous culture. A batch culture is a closed system where the volume of media is constant with a fixed substrate such as carbon eg. succinate, and a nitrogen source eg. NO_3^-, and growth is defined by Equation 3.1-1, where x represents drymass (mg.ml), t represents time (h) and μ represents the growth rate (h^{-1})

$$\frac{dx}{dt} = \mu x$$

Equation 3.1-1

In batch cultures growth typically shows a lag phase followed by an exponential phase, stationary phase and then finally the death phase (Figure 3.1-1). The lag phase can vary significantly due to changes in oxygen concentration and also adapting to new pH, carbon or nitrogen source. The exponential phase is where the bacteria start to double, the gradient of this phase is equal to μmax, the maximum growth rate. The stationary phase is where nutrients are becoming limited and so bacteria are unable to replicate, or toxins begin to accumulate. Once all nutrients are exhausted or the toxin concentration is too high the bacteria start to die, the death phase (Figure 3.1-1).

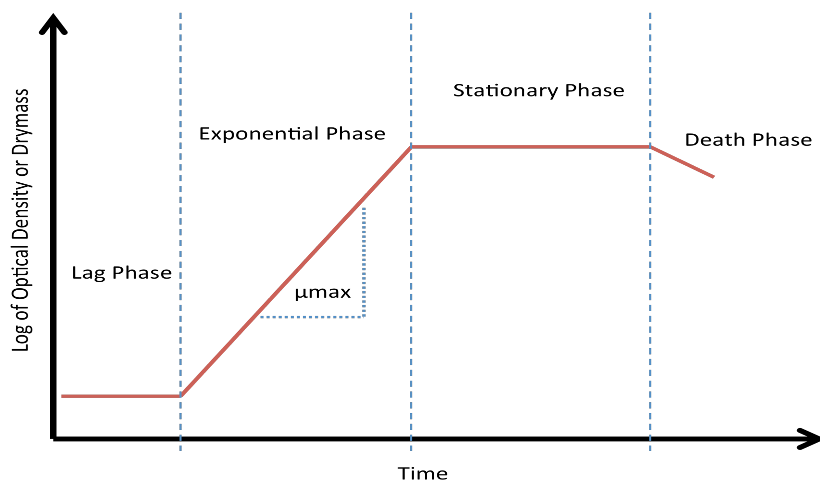


Figure 3.1-1 Typical batch culture growth kinetics showing the 4 phases of growth. μmax is maximum growth rate which is derived from the exponential phase gradient.

Continuous cultures are far different from batch cultures as they are open systems, in which medium flows in and out as a function of time. A form of continuous culture is chemostat culture in which growth is limited by the availability of a key metabolic substrate, such as the carbon source. Bacteria (or other microorganisms) are maintained at a “steady state”, where by drymass or the optical density (OD) is maintained at a constant level. In batch cultures the drymass and OD continually change over time, going through the exponential phase, stationary phase and the death phase (Figure 3.1-1). In the environment bacteria do not form batch cultures as there are a variety of parameters that change over time, such as carbon source, as the environment is an open system. Bacteria are capable of surviving in the environment by changing their metabolic pathways if a carbon source becomes depleted or if the O_2 concentration is low. Therefore continuous cultures, such as chemostat cultures, can be used to study bacteria/microorganisms in an environment where by all parameters remain constant, typically with one limiting nutrient that prevents the culture from growing at µmax. As drymass (x) remains constant over time, theoretically cultures can remain indefinitely in this open system. The steady state revolves around the dilution rate of the culture. To sustain a chemostat culture feed is added to the vessel at a designated flow rate (F), and waste is removed at the same rate (F_{out}), the dilution rate (D) is calculated using the flow rate (F) and vessel volume (V), D = F/V (Figure 3.1-2). In a chemostat culture to account for the removal of biomass from the open system Equation 3.1-1 becomes Equation 3.1-2. Under steady state there is no net change in biomass over time, Equation 3.1-3. When there is no net change, Equation 3.1-4, the µ is equal to the D, Equation 3.1-5.

$$\frac{dx}{dt} = \mu x - Dx$$

Equation 3.1-2

$$\frac{dx}{dt} = 0$$

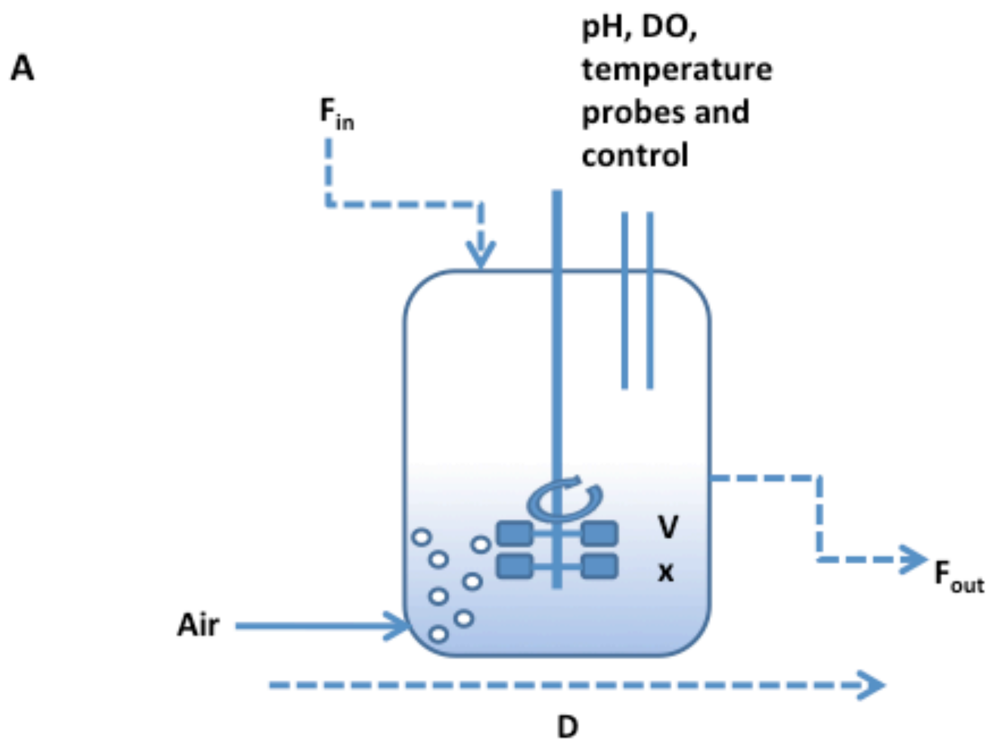
Equation 3.1-3

$$0 = \mu x - Dx$$

Equation 3.1-4

$$\mu = D$$

Equation 3.1-5



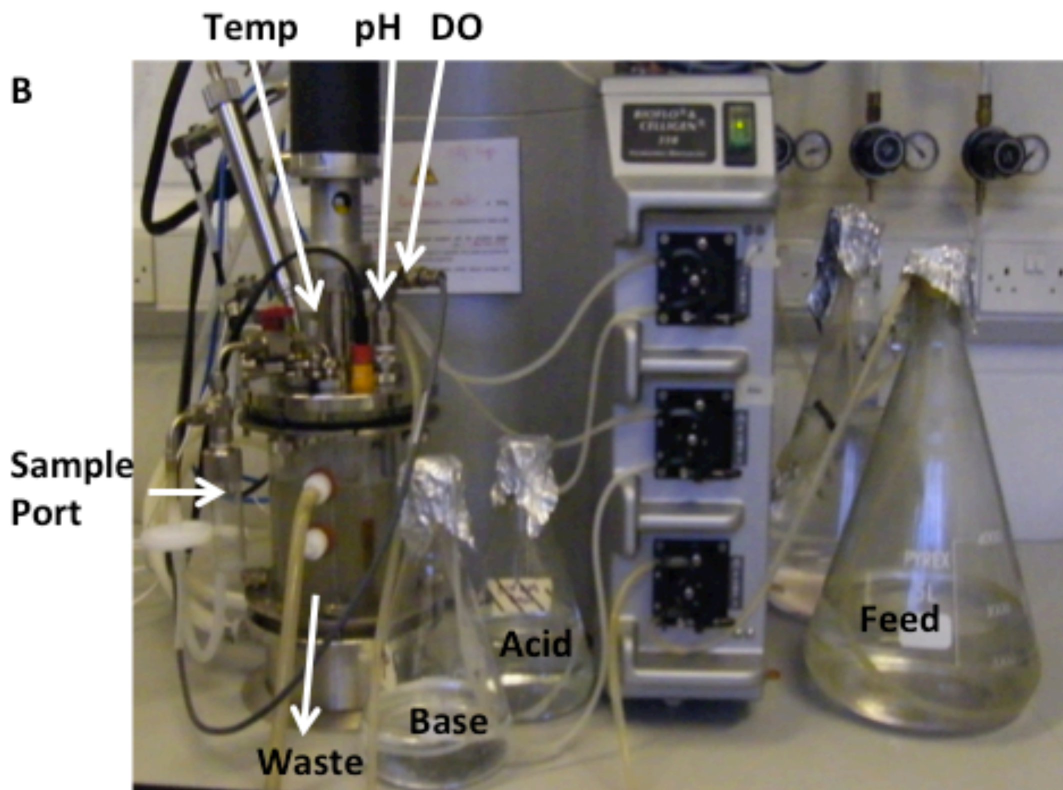


Figure 3.1-2 Schematic of continuous culture (Panel A), F = Flow rate, D= Dilution rate, V = Volume, *x* = drymass. Bioflo 300 New Brunswich Scientific continuous culture system (Panel B) used in this thesis. Temperature (Temp), pH and dissolved oxygen (DO) are monitored by probes. pH is maintained using acid (0.1 M H_2SO_4) and base (1 M NaOH).

In batch cultures environmental parameters constantly change and toxins can accumulate. Using chemostats rather than batch cultures has many advantages, toxins do not accumulate and the dissolved oxygen (%) and pH can be kept constant and critically it is possible to compare steady state under a range of clearly defined conditions, for example Cu concentration. Chemostats are also useful in culturing bacteria that have long doubling times as they (theoretically) can be run indefinitely due to feed being added and so carbon sources and electron acceptors are constantly being added to the cultures. Due to these advantages chemostat cultures have become very popular in research involved with greenhouse gases and biofuels. As oil prices increase and greenhouse gas emissions from fuels become more of a problem in our environment, the search for biofuels is of great significance. Biohydrogen is a biofuel, the biological production of hydrogen gas are now of great relevance in the development of biofuels. Anaerobic fermentation has been vital in the development of this biofuel, previous research has shown that thermophilic and mesophilic fermentations can produce hydrogen (Turcot, Bisaillon et al. 2008). The growth of enteric bacteria also produce hydrogen, and twice as much in *Clostridia spp* when grown on sucrose (Kim, Han et al. 2006). The use of chemostat cultures has lead to developing optimum conditions for hydrogen production, such as temperature ($35^{o}C$) (Lin and Chang 2004).

Other continuous culture work has looked into greenhouse gas emissions from waste water treatment processes where denitrifying bacteria are numerous and reduce NO_3^- to N_2O. There has been much research into this by Tseunda *et al.* 2005, and many others including Tallec *et al.* 2008. The research by Tallec (2008) shows that denitrifiers are the major contributor of N_2O emissions from activated sludge, which can amount to 155 Tons N_2O-N per year, in Paris alone (Tallec, Garnier et al. 2008). N_2O emissions vary greatly, especially between continuous and batch cultures, as well as N_2O measurements from different waste water treatment plants. Working with continuous cultures has greatly increased our knowledge of what conditions increase N_2O emissions in waste water treatments.

Paracoccus spp. have been previously grown in carbon limited chemostat cultures to investigate the expression of denitrification enzymes and nitrogen removal

from waste water (Moir, Richardson et al. 1995; Baumann, Snozzi et al. 1996; Rezic, Santek et al. 2006). Work by Moir *et al* (1995) show that aerobic nitrate reduction can occur and was due to the periplasmic Nap system, however aerobic denitrification could not occur due the absence of the nitrite reductase, cyt cd_1, activity in aerobically grown cells. The activity and expression of nitric oxide reductase and nitrous oxide reductase were both repressed in aerobically grown cells compared to anaerobically grown cells. Baumann *et al.* (1996) ran carbon limited chemostat cultures of *P.denitrificans* and looked at the expression of the denitrification genes after switching the cultures from aerobic to anaerobic during steady state (Baumann, Snozzi et al. 1996). From switching an anaerobic culture to aerobic respiration denitrification stopped immediately, however cyt cd_1 was still present for short period of time. The synthesis of NarGHI and NosZ occurred immediately after the cultures became anaerobic, the synthesis of NirS occurred after NarGHI and NosZ suggesting that the genes are regulated differently (Baumann, Snozzi et al. 1996).

Neither study examined the impact of copper on the denitrification process. Therefore the aims of the work described in this chapter were to grow *P.denitrificans* in carbon limited chemostat cultures and observe how changes in Cu concentrations affected the denitrification pathway, by monitoring the accumulation of intermediates.

3.2 Results: The effect of copper concentration on *P.denitrificans* grown in NO_3^- carbon limited chemostat cultures.

3.2.1 Batch Cultures: The effect of NO_3^- and carbon concentration on the anaerobic growth of *P.denitrificans*

P.denitrificans is capable of utilising many carbon sources, such as the 4 carbon fatty acid butyrate to the 1 carbon alcohol methanol. Cultures of *P.denitrificans* growing on mixtures of succinate and butyrate show that the succinate, which contains 4 carbons, is the preferred carbon source over more reduced carbon source butyrate (Ellington, Bhakoo et al. 2002). Succinate metabolism occurs in aerobic and anaerobic environments; the enzyme succinate dehydrogenase plays a vital role in the reduction of the ubiquinone (Q) to ubiquinol (QH_2). For denitrifying bacteria to grow anaerobically they therefore require a carbon source e.g. succinate, and the presence of e^- acceptor, such as NO_3^- or NO_2^-.

In order to identify conditions to run chemostat cultures under carbon or NO_3^- limitation, batch culture experiments were undertaken to identify the concentrations of NO_3^- and succinate that limited growth yield and growth rate. Batch cultures inoculated with *P.denitrificans* were grown in various concentrations of succinate (sodium succinate) and NO_3^- ($NaNO_3$), in *Paracoccus* minimal media at 37°C, pH 7.5, anaerobically. The first set of batch cultures were grown on 20 mM NO_3^- and the succinate concentration varied between 1 - 60 mM (See Figure 3.2-1). The cultures were inoculated with 0.5 % v/v *P.denitrificans* grown aerobically over night on LB broth, cultures were set up on different days and so a slight variation in the initial OD_{600} is sometimes observed. Cultures with a higher inoculation may have a slightly elevated final OD_{600}. In all cultures a small lag phase was seen of up to about 2 h. The semi log graph (Figure 3.2-1B) shows that the exponential phase lasts for approximately 5 h in 5, 10, 15 and 20 mM succinate cultures. The exponential phase is only 2 h in the 1 mM succinate culture, and the final OD_{600} is also significantly lower suggesting that *P.denitrificans* growth yield is limited. From the semi log graphs the

growth rates (μ) were obtained in the exponential phase. There was no significant difference in the μ derived from the succinate culture semi log graphs, μ = 0.2 h^{-1}. Succinate can be seen as sufficient at $\geq$ 15 mM as the final OD_{600} plateaus, therefore another substrate becomes a limiting factor (Figure 3.2-1).

A second set of anaerobic batch cultures was carried out to examine the effect of NO_3^- concentration on the growth rate and final OD_{600} of *P.denitrificans*. The cultures were set up to be succinate sufficient (20 mM) with NO_3^- at 1 – 60 mM. As observed in the NO_3^- sufficient cultures there was a lag phase present of about 2 hours, followed by the exponential phase, which is indicated by a straight line in the log v OD_{600} graph (Figure 3.2-2 B). The exponential phase is observed in all cultures between 3-8 h of growth, the μ derived from the semi log graphs (Figure 3.2-2 B) shows that growth rates in all the cultures are similar, approximately 0.2 h^{-1}. The growth of *P.denitrificans* become limited once the NO_3^- concentrations is ≥ 30 mM, suggesting that the NO_3^- concentration is sufficient and growth is now being limited by another factor or substrate (Figure 3.2-3 B). When the NO_3 concentration is reduced the final OD_{600} decreases, up to 50 % of growth is lost when the NO_3^- concentration is decreased to ≤ 15 mM (

Table 3-1). The growth of *P.denitrificans* is severely limited in the 1 mM NO_3^- as there is only a ~0.1 OD_{600} units increase over 12 h.

Table 3-1 The final OD_{600} of *P.denitrificans* grown on *Paracoccus* minimal media with 20 mM NO_3^- and succinate 1, 5, 10, 15 and 20 mM.

Succinate mM	Final OD_{600}
60	1.64
50	1.63
40	1.75
30	1.75
20	1.32
15	1.33
10	1.11
5	0.77
1	0.38

Table 3-2 The final OD_{600} of *P.denitrificans* grown on *Paracoccus* minimal media with 20 mM NO_3^- and succinate 1, 5, 10, 15 and 20 mM.

Nitrate mM	Final OD_{600}
60	2.07
50	2.37
40	2.04
30	2.1
20	1.37
15	1.11
10	0.89
5	0.45
1	0.22

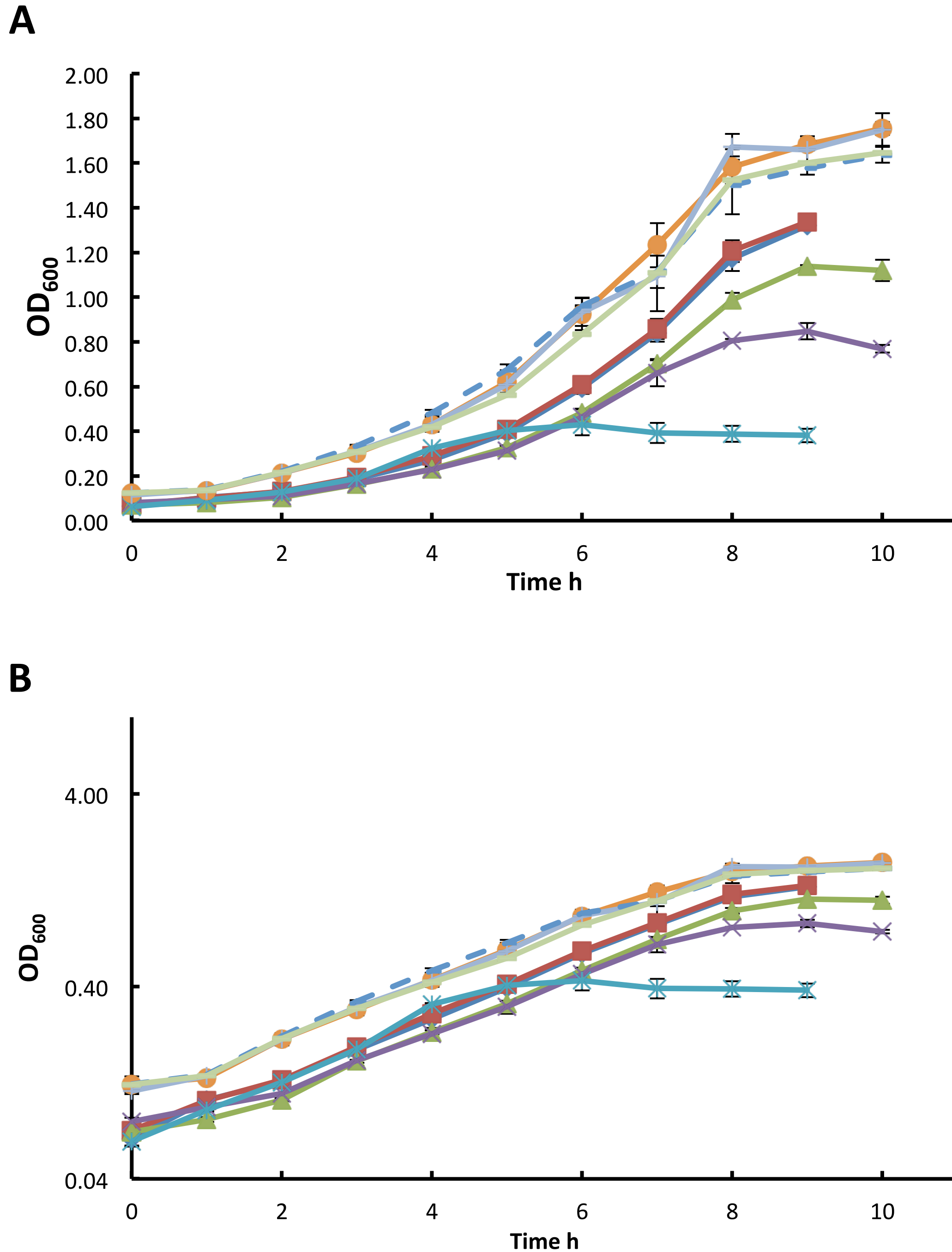


Figure 3.2-1 Batch culture experiments with *P.denitrificans* with varying succinate concentrations, monitored over time by observing the OD_{600} (A) or Log/OD_{600} (B). The cultures were grown anaerobically in *Paracoccus* minimal media containing 20 mM NO_3^- and either 60 mM (—), 50 mM (- -), 40 mM (+), 30 mM (●), 20 mM (◆), 15 mM (■), 10 mM (▲), 5 mM (×) and 1 mM (✳) succinate. For each condition there were 3 repeat cultures, standard error was calculated and is represent by the y axis error bars.

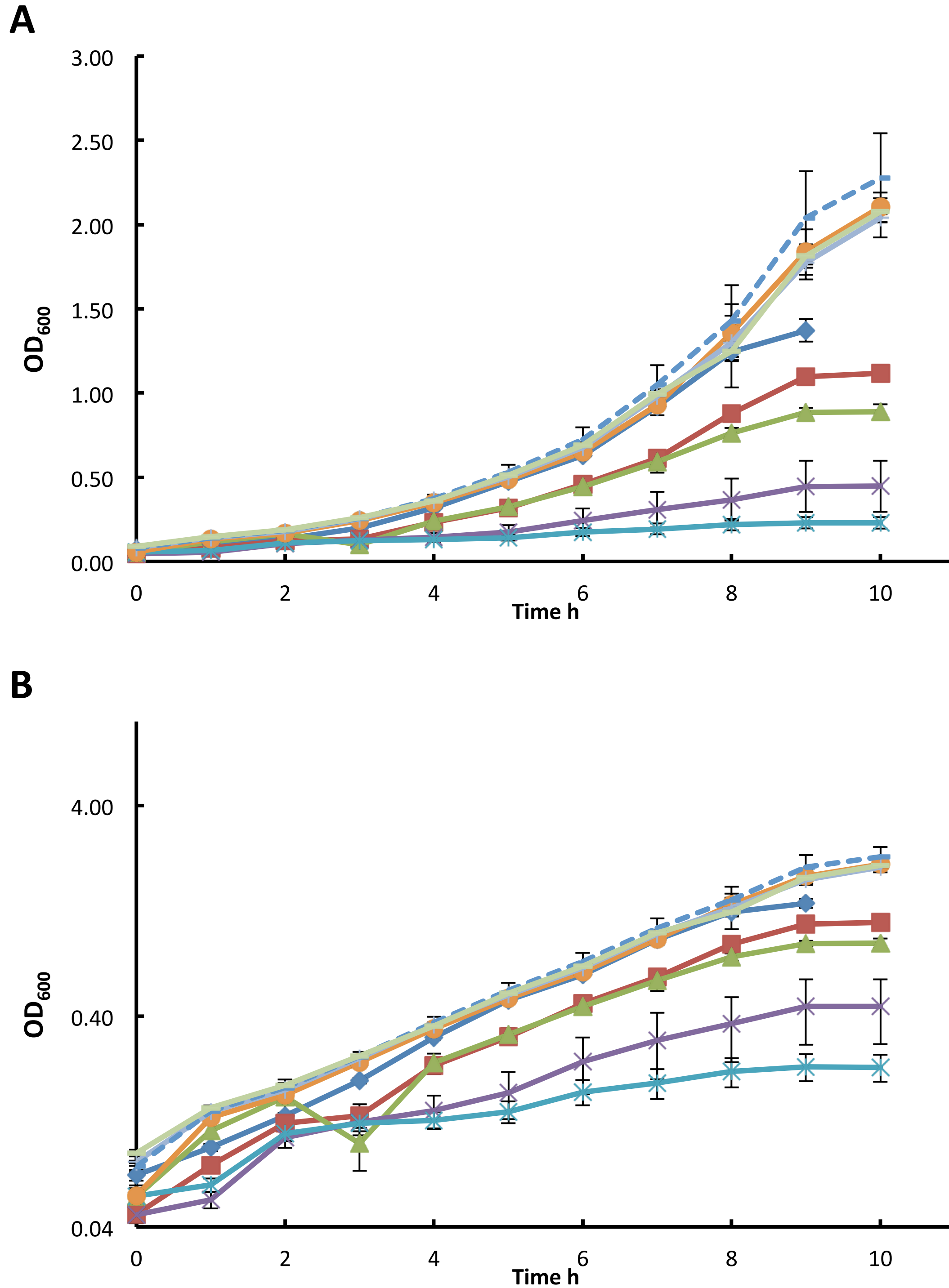


Figure 3.2-2 Batch culture experiments with *P.denitrificans* with varying NO_3^- concentrations, monitored over time by observing OD_{600} (A) or Log/OD_{600} (B). The cultures were grown anaerobically in *Paracoccus* minimal media containing 20 mM succinate and 60 mM (—), 50 mM (--), 40 mM (+), 30 mM (●), 20 mM (◆),15 mM (■), 10 mM (▲), 5 mM (✕), and 1 mM (✳) NO_3^-. For each condition there were 3 repeat cultures, standard error was calculated and is represent by the y axis error bars.

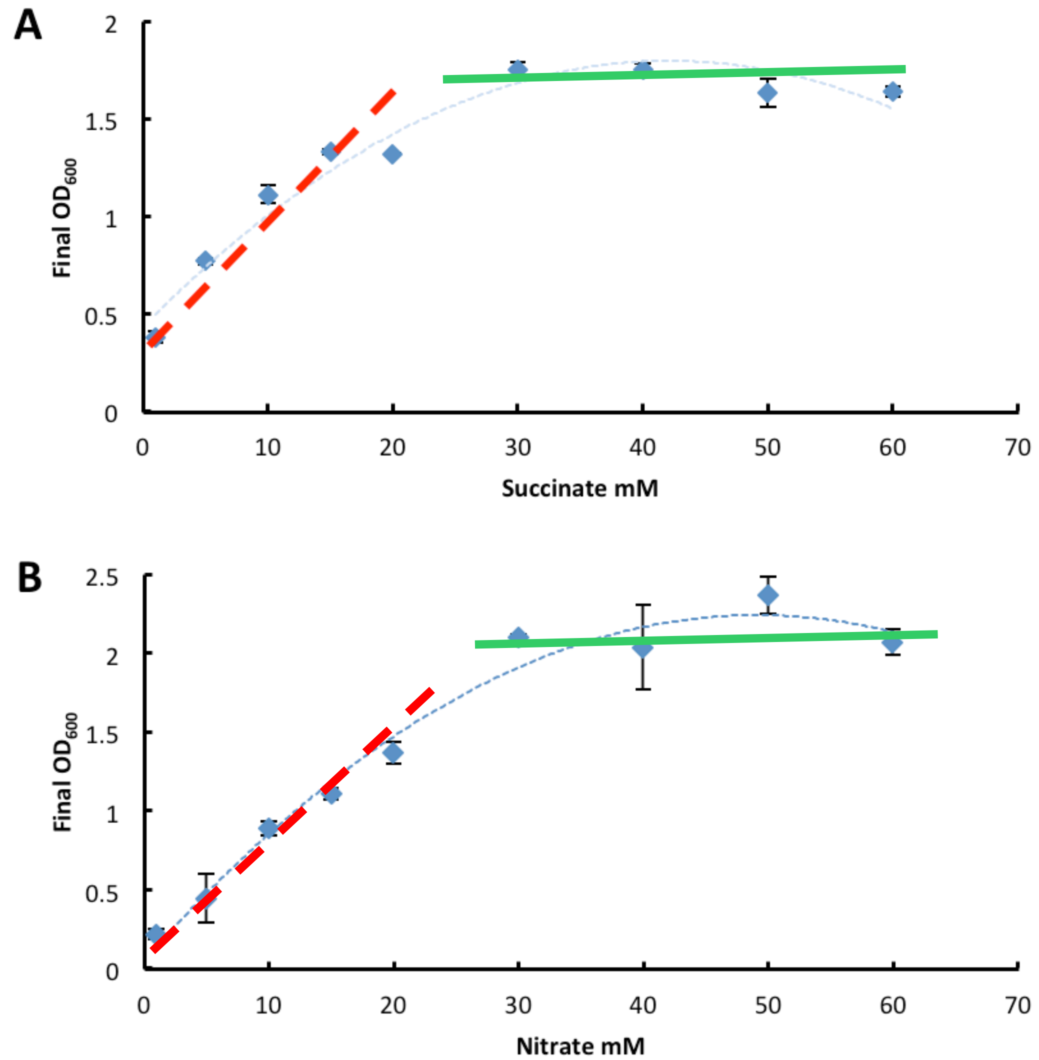


Figure 3.2-3 Final OD_{600} plotted against 20 mM NO_3^- and varying succinate concentrations (A) and 20 mM succinate and varying NO_3^- concentrations (B) . The trend is indicated by the dotted line (-------), the line can be split into two separate phases, the substrate limited phase (------) and the substrate sufficient phase (—) where by growth in being limited by another substrate. The error bars represent the standard error.

3.2.1.1 The effect of the copper concentration on the growth of *P.denitrificans* in batch culture

Copper plays a vital role in aerobic and anaerobic metabolism in *P.denitrificans*; Cu is found in three of the four oxidases, cyt *aa₃,* cyt *ba₃* and cyt *cbb₃* and in denitrification present in Paz and NosZ. A set of NO_3^- -sufficient succinate-limited batch cultures undertaken as to see if *P.denitrificans* was capable of anaerobic growth under low Cu concentrations. To inoculate the cultures *P.denitrificans* was grown overnight aerobically in NO_3^- sufficient *Paracoccus* minimal media containing either 18 µM or <0.5 µM Cu. *P.denitrificans* was capable of growing in <0.5 µM Cu aerobically. growth was sustainable due either trace amounts of Cu present in the medium, and the haem-Cu oxidases were active, or if no Cu was present then the haem containing oxidase, cytochrome *bd*, could potentially be capable of sustaining aerobic growth (Garcia-Horsman, Barquera et al. 1994). The aerobic cultures were used to inoculate the anaerobic cultures (0.5 % v/v innocula). The presence or absence of Cu had no significant effect on the growth of *P.denitrificans* anaerobically in NO_3^- sufficient, succinate limited cultures. The µ for both cultures were 0.2 h^{-1}, which was using the semi log graph (Figure 3.2-4 B), and there was no significant difference in the final OD_{600} (Figure 3.2-4). Anaerobic growth under low Cu concentrations was sustainable due to the denitrification pathway consisting of only two Cu proteins, and NosZ. It is therefore plausible that NO_3^-, NO_2^- and NO reduction can still conserving enough energy to maintain growth. Also there is an alternative non Cu e^- donor to NosZ, cyt c_{550}, which also transfers e^- to Nir and Nor. To determine if grown in NO_3^- sufficient, carbon limited <0.5 µM Cu accumulated N_2O, gas samples from the batch cultures were taken and analysed using the GC method. The cultures grown in <0.5 µM Cu had a 30 fold increase in N_2O release compared to the 18 µM Cu culture (Figure 3.2-5). The accumulation of N_2O under low Cu concentration suggests that the capacity of the NosZ step is lower compared to the Cu sufficient cultures. To see if the NosZ activity was completely knocked out under low Cu concentrations, three anaerobic cultures of *P.denitrificans* were set up in 20 mM NO_3^-, 5 mM *Paracoccus* media with 18 µM, 0.8 µM and <0.5 µM Cu and were grown at 37 °C for h. After 12 h of growth the cells were harvested and washed, to remove traces of NO_3^- and NO_2^- as these could interfere with the electrode. The N_2O electrode was set

up, see methods, and 3.6 µmols of N_2O was injected into the electrode chamber and then the washed cells were injected into the chamber and the consumption of N_2O was monitored. The rate of N_2O consumption was determined from the gradients obtained from the traces (

Figure 3.2-6 B). No rate of N_2O consumption in the cells grown in <0.5 µM Cu was detectable. The cells grown in 18 µM Cu were capable of reducing N_2O at 3.3 $nmol.min^{-1}$ mg $drymass^{-1}$. The activity decreased by 3 fold in cells grown in 0.8 µM Cu. This suggests that as Cu is removed from the environment cellular NosZ activity is reduced, possibly due to the Cu centres not properly being inserted into the NosZ protein or due to decreased levels of *nosZ* transcription.

Since it is not known whether the activity of NosZ is lost at low Cu concentrations due to transcription regulation or failure to insert the Cu centres, further experiments using chemostat cultures are used in the later sections of this thesis, to investigate whether the effects of Cu are manifested at a protein or transcriptional level.

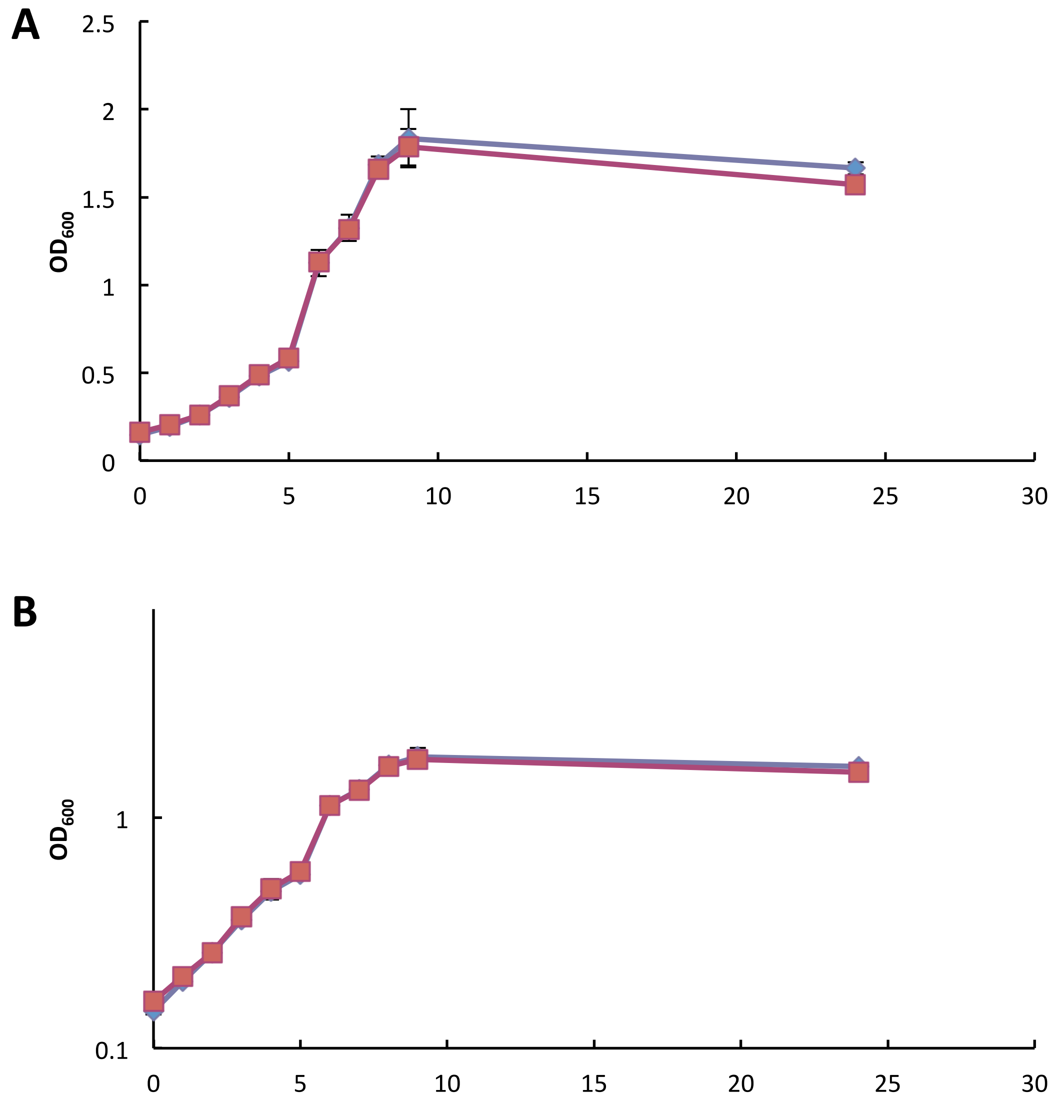


Figure 3.2-4 Batch culture experiments with *P.denitrificans* grown in different Cu concentrations, the OD_{600} (A) or Log/OD_{600} (B) were monitored over time. *P.denitrificans* was grown in 5mM succinate and 20 mM NO_3^- *Paracoccus* minimal media containing either 18 µM Cu (◆) or <0.5 µM Cu (■). The batch cultures were replicated three times, the error bars represent standard error.

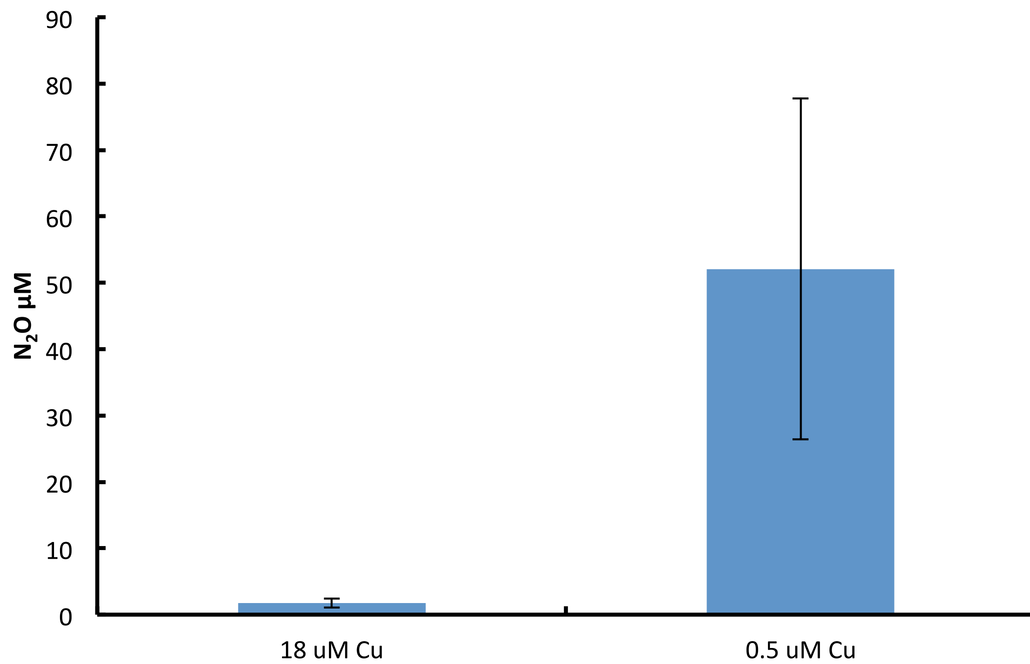


Figure 3.2-5 The effect of Cu concentration on the accumulation of N_2O by *P.denitrificans* in batch cultures grown on minimal media with 20 mM NO_3^-, 5 mM succinate and Cu 18 µM or <0.5 µM Cu. Samples were taken after 24 h. This data represents the average of two replicates for each batch culture (total of 6 samples) measured by gas chromatography, the error bars represent the standard error.

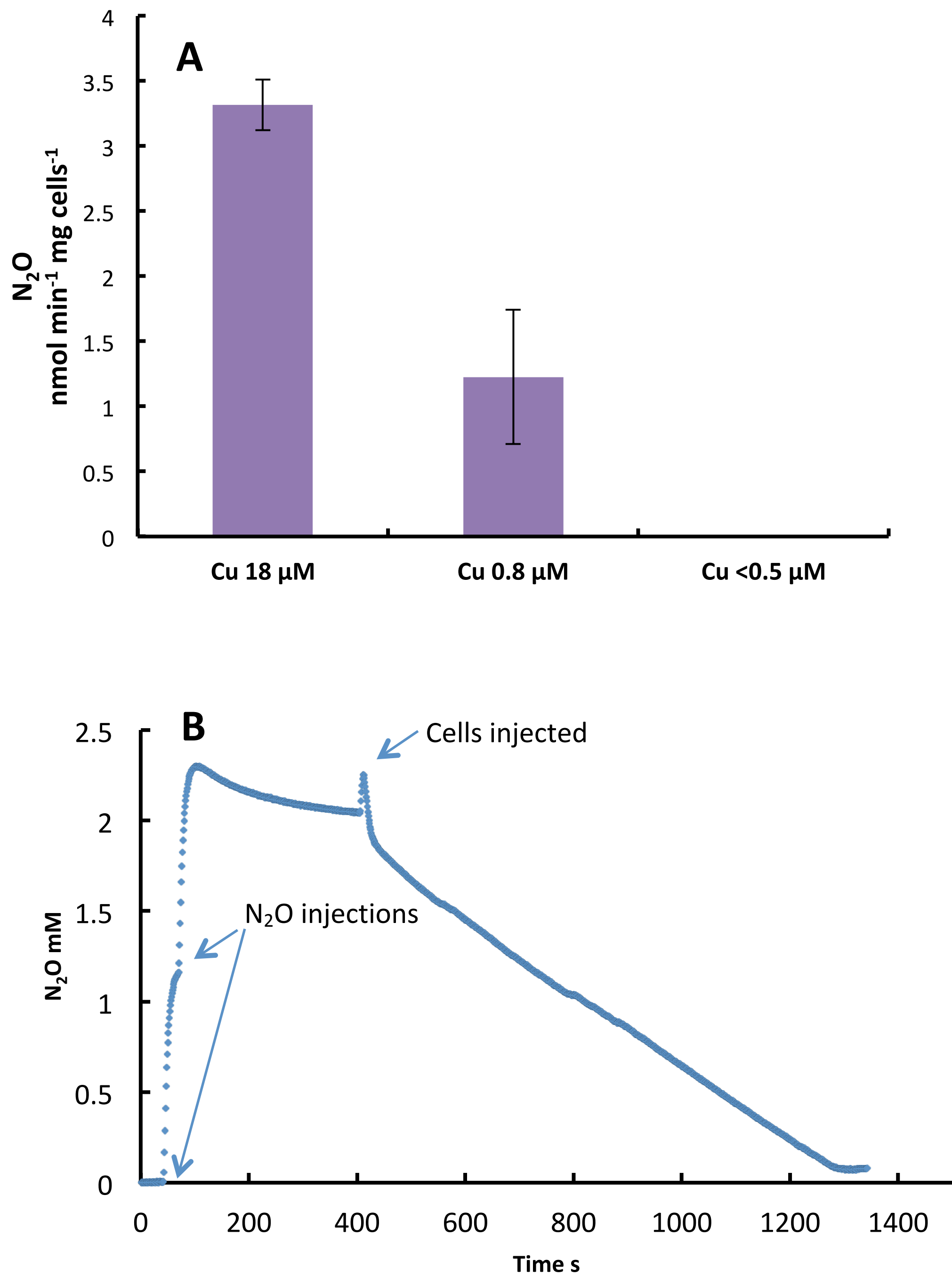


Figure 3.2-6 The rate of N_2O consumption by *P.denitrificans* grown at different Cu concentrations (A), using the N_2O electrode. Consumption rates were determined from the gradients of the traces obtained from the electrode (B). Figure B shows a trace attained from injecting 34.5 mg of cells grown in 18 µM Cu NO_3^- sufficient media. The data in panel A is represents the average rate of consumption over 3 batch cultures, the error bars represent the standard error

3.2.2 The characteristics of *P.denitrificans* in NO_3^- -sufficient succinate-limited chemostat cultures

The previous section established that N_2O was released from Cu deficient batch cultures. To study the production of N_2O in more detail *P.denitrificans* was grown under NO_3^- sufficient (20 mM) succinate limited (5 mM) conditions in chemostat cultures.

3.2.2.1 Drymass production in NO_3^- sufficient chemostat cultures of *P.denitrificans*

P.denitrificans growth was sustainable under low Cu concentrations aerobically and anaerobically in batch cultures where parameters such as pH and substrate concentration, are constantly changing (See section 3.2.1). In the environment bacteria act far differently, parameters maybe more constant or slower acting and so bacterial numbers may remain fairly constant, unless there are sudden changes in the environment, which the bacteria cannot adapt to. Chemostat cultures of *P.denitrificans* were set up aerobically in either 18 µM Cu, 0.8 µM or <0.5 µM Cu containing NO_3^- sufficient *Paracoccus* minimal media (5 mM succinate, 20 mM NO_3^-). The temperature was maintained at 37 ^oC throughout the experiment and the pH was regulated with 1 M NaOH and 0.1 mM H_2SO_4 to maintain the culture at pH 7.5.

The first 24 h of growth in the bioreactor was as an aerobic batch culture during which the drymass increased to ~0.35 – 0.39 mg ml^{-1} (Figure 3.2-7). After 24 h of batched growth the air supply was removed and the feed was started. Oxygen was completely consumed by 30 min in all cultures, therefore growth was anaerobic from 24.5 h. The flow rate in the chemostats was 87.5 ml h^{-1}, the vessel volume was 1.5 L and so the D was 0.06 h^{-1}, as soon as the feed was started the drymass decreased (24-50 h, Figure 3.2-7). After 3 vessel volumes, approximately 70 h (48 h of anaerobic growth) the drymass stopped decreasing and entered steady state, where the drymass

remained between 0.2- 0.25 mg ml^{-1}. This steady state can theoretically be maintained indefinitely, however there is a risk that mutations will build up during prolonged continuous cultures so the experiments were stopped at 120 h.

Figure 3.2-7 shows that there is no significant difference in drymass amongst the three cultures at steady state. The only slight difference is that the 0.8 μM Cu and the <0.5 μM Cu cultures grow faster in the first 6 h of growth, therefore by 24 h the batch culture has begun to fall into the death phase, causing a decrease in drymass. This is corrected once the feed is added and the cultures go into steady state at the same time. The yield of drymass, calculated at 102 h, represents the amount of drymass produced per hour (Table 3-3).

At 102 h a sample of cells was taken from the chemostats to assess how much Cu was taken up by the cells. The cells were spun, washed and then sonicated, the fractions were then analysed for Cu content. As expected the cells grown at 18 μM Cu contained more Cu than the 0.8 μM Cu cells. The cells grown in the 18 μM, 0.8 μM and <0.5 μM Cu *Paracoccus* media contained 0.16 ± 0.02 μM.g drymass, 0.045 ± 0.018 μM.g drymass and 0.016 ± 0.0007 μM.g drymass Cu respectively. Therefore even though there is a Cu chelator (BCA) in the <0.5 μM Cu chemostat cultures the cells have scavenged a small amount of Cu either from the environment or from lysed cells that contained Cu in the inoculum. Even though there is a very small amount of Cu present *P.denitrificans* is capable of transporting Cu into the cell and incorporating it into enzymes that are essential for growth. Sco1 and Sco2 are involved in Cu uptake in *P.denitrificans*, and delivering Cu to cytochrome *c* oxidases (Leary, Kaufman et al. 2004).

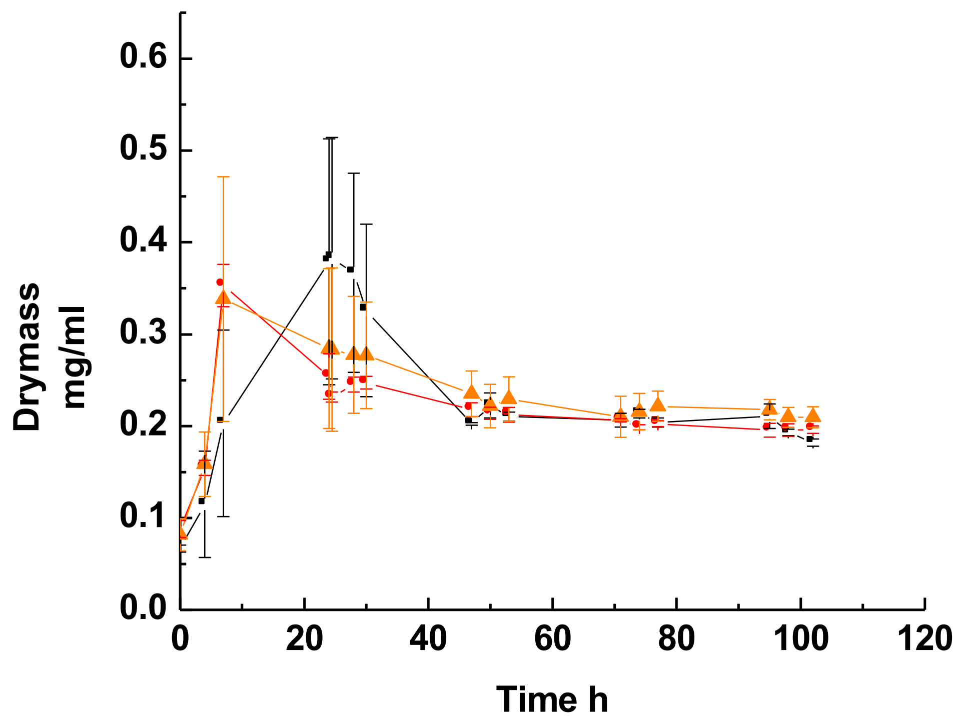
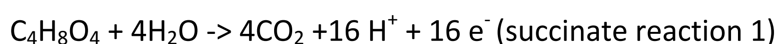


Figure 3.2-7 The drymass of *P.denitrificans* chemostat cultures grown in NO_3^- -sufficient succinate-limited 18 µM Cu (■), 0.8 µM Cu (●) and < 0.5 µM Cu (▲) *Paracoccus* minimal media. At 24 h the air supply was removed and the feed was added (D= 0.06 h^{-1}). Chemsotat cultures were repeated three times for each condition, the error bars represent the standard error.

3.2.2.2 Nitrate consumption kinetics in chemostat cultures of *P.denitrificans*

The first step of denitrification is the reduction of NO_3^- to NO_2^-, therefore to establish if denitrification was occurring supernatant samples were collected and analysed. Significant levels of NO_3^- consumption only began when the cultures became anaerobic at 24.5 h, there was no NO_3^- consumption detected during the aerobic phase (Figure 3.2-8). NO_3^- assimilation did not occur during the aerobic phase of the culture, as the addition of NH_4^+ represses the assimilatory nitrate reductase, and there was little aerobic NO_3^- respiration.

Complete catabolism of one molecule of succinate to CO_2 maximally yields 16 electrons so that there are maximally 80 mM electrons available for anaerobic respiration in the NO_3^--sufficient, succinate-limited cultures (although it will be less than this because some of the succinate is used for anabolic reactions):

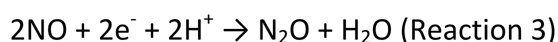


$C_4H_8O_4 + 4H_2O \rightarrow 4CO_2 + 16\ H^+ + 16\ e^-$ (succinate reaction 1)

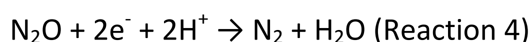
The complete reduction of 2 molecules of NO_3^- to 1 molecule of N_2 consumes 10 electrons:

$2NO_3^- + 4e^- + 4H^+ \rightarrow 2NO_2^- + 2H_2O$ (Reaction 1)

$2NO_2^- + 2e^- + 4H^+ \rightarrow 2NO + 2H_2O$ (Reaction 2)



$2NO + 2e^- + 2H^+ \rightarrow N_2O + H_2O$ (Reaction 3)



$N_2O + 2e^- + 2H^+ \rightarrow N_2 + H_2O$ (Reaction 4)

Thus the 20 mM nitrate available in the reservoir-feed provides a potential sink for the consumption of 100 mM electrons. This is greatly in excess of that required for complete catabolism of 5 mM succinate and illustrates the succinate-limited culture conditions and the consequent detection of a high level of NO_3^- (6 -10 mM) in the bioreactor of the steady-state cultures. Significantly though, from Figure 3.2-8, it is apparent that more NO_3^- was being consumed in the 0.8 µM Cu and <0.5 µM steady state cultures compared to the 18 µM Cu culture. The 18 µM Cu culture shows a net

consumption in steady state of approximately 10 mM NO_3^- compared to 15 mM in the 0.8 µM and <0.5 µM Cu cultures (Figure 3.2-8). The specific rate of nitrate consumption ($qcNO_3^-$) in steady state can be calculated from:

$$qcNO_3^- = ([NO_3^-]_R - [NO_3^-]_r)\, D / x; \quad \textbf{(Equation 3.2-1)}$$

where $[NO_3^-]_R$ is the NO_3^- concentration in the reservoir, $[NO_3^-]_r$ is the residual NO_3^- concentration in the reactor vessel, D is the dilution rate and x is the drymass concentration in the reactor vessel. Table 3-3 shows that the specific rate of NO_3^- consumption was approximately 50 - 60 % higher when Cu concentration was reduced to 0.8 µM and <0.5 µM compare to the copper-sufficient cultures.

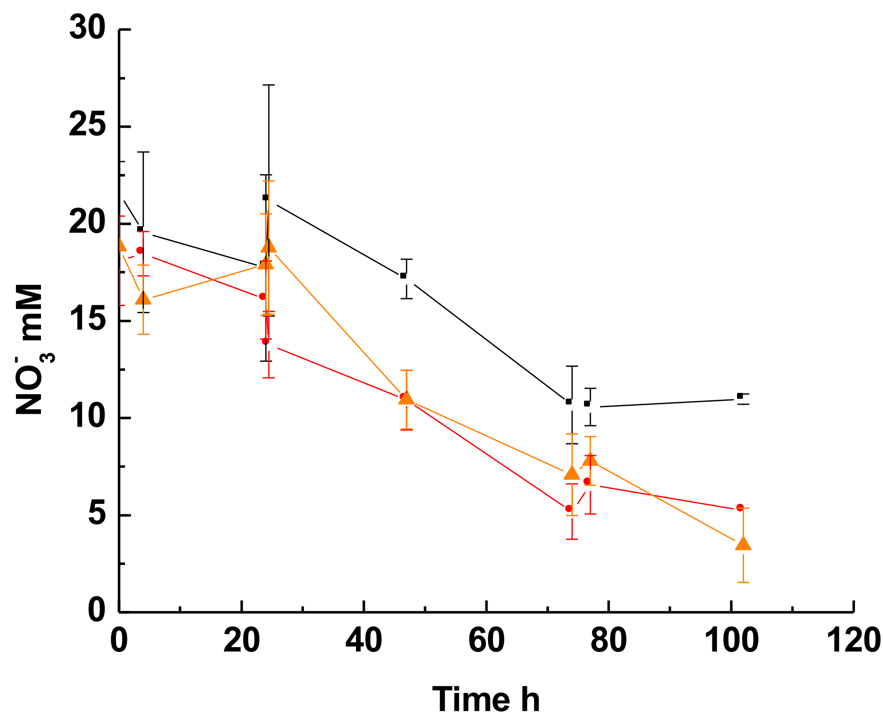


Figure 3.2-8 The consumption of NO_3^- in *P.denitrificans* chemostat cultures grown in NO_3^- -sufficient succinate-limited 18 µM Cu (■), 0.8 µM Cu (●) and < 0.5 µM Cu (▲) *Paracoccus* minimal media. At 24 h the air supply was removed and the feed was added (D= 0.06 h^{-1}). Samples were analysed twice from each chemostat culture, the error bars represent the standard error.

3.2.2.3 Nitrite production kinetics in chemostat cultures of *P.denitrificans.*

The reduction of NO_2^- is the second step of denitrification; in *Paracoccus pantotrophus* batch cultures no more than 50 µM NO_2^- accumulates in NapA mutants (Wood, Alizadeh et al. 2002). In the chemostat cultures of *P.denitrificans* the first 24 h of growth was aerobic and little NO_3^- was consumed and therefore little NO_2^- was produced. Figure 3.2-9 shows that there is a small amount of NO_2^- at 0 h, this is due to carry over from the inoculum which had accumulated a small amount of NO_2^- and also from NO_2^- contamination in the growth medium. When the air is removed at 24 h the culture becomes anaerobic by 24.5 h, a small peak of NO_2^- was seen at 27 h in all 3 cultures (Figure 3.2-9). Figure 3.2-9 shows that NO_2^- does not accumulate above 80 µM in the <0.5 µM the NO_2^- and approximately 10 times more NO_2^- is produced in the <0.5 µM Cu culture than in the 18 µM and 0.8 µM Cu chemostats, at 102 h (Figure 3.2-9). The total amount of NO_2^- accumulated in the <0.5 µM Cu chemostat culture was 68 ± 2 µM which accounts for 0.4 % of the total NO_3^- consumed.

The specific rate of nitrite accumulation ($qpNO_2^-$) at steady state can be calculated from:

$$qp\ NO_2^- \ = \ [NO_2^-\ ]\ r\ D\ /\ x \quad \textbf{(Equation 3.2-2)}$$

where $[NO_2^-]_r$ is the NO_2^- concentration in the reactor vessel – the initial/background NO_2^- concentration. The specific rate of NO_2^- consumption ($qcNO_2^-$) is a function of the rate of nitrate reduction minus the rate of nitrite production and can be calculated from:

$$qc\ NO_2^- = qcNO_3^- - \ qp\ NO_2^- \quad \textbf{(Equation 3.2-3)}$$

The NO_2^- accumulation (Table 3-3) shows no significant difference between the different Cu cultures. The 18 µM and the 0.8 µM Cu cultures consume more NO_2^- than they produce, whereas the <0.5 µM Cu culture accumulates NO_2^- but at a low rate of 0.009 mmol .g drymass. h^{-1}.

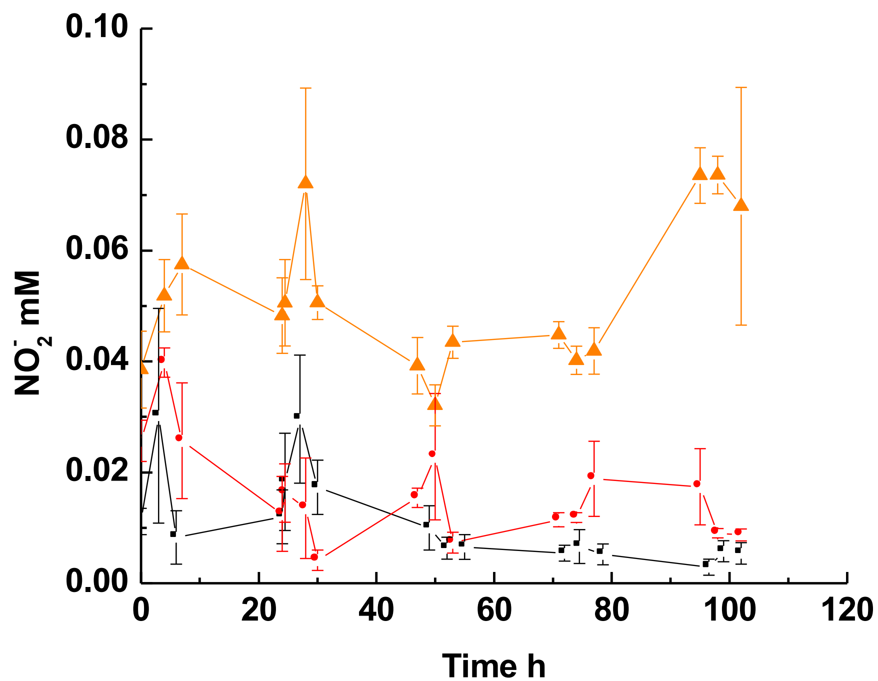


Figure 3.2-9 The accumulation of NO_2^- in chemostat cultures of *P.denitrificans* grown in NO_3^- -sufficient succinate-limited 18 µM Cu (■), 0.8 µM Cu (●) and < 0.5 µM Cu (▲) *Paracoccus* minimal media. At 24 h the air supply was removed and the feed was added (D= 0.06 h^{-1}). Samples were analysed twice from each culture, the error bars represent the standard error.

3.2.2.4 Nitrous Oxide production in chemostat cultures of *P.denitrificans.*

NO_2^- is reduced to NO by NirS in *P.denitrificans*, NO is cytotoxic and therefore does not tend to accumulate above nanomolar levels. Throughout the experiment NO was not detected extracellularly (detection limit <10 µM), therefore NO_2^- was being directly reduced to NO and then N_2O, i.e. the rate of Reactions 2 and 3 were closely matched. The concentration of Cu has a major effect on the accumulation of N_2O, due to NosZ containing 12 Cu atoms per dimer. Figure 3.2-10 B shows that during the first 2 h of aerobic growth there is no net N_2O production. Atmospheric N_2O is currently about 320 ppb or $3x10^{-3}$ µM (IPCC, Qin et al. 2007), this corresponds to the chemostat N_2O levels during the aerobic phase. In the 18 µM Cu cultures as soon as the vessel became anaerobic N_2O increased to 0.2 µM after 3 h. N_2O production, similarly to the drymass, is in steady state from approximately 48 - 102 h, ranging from 0.5 – 0.6 µM (Figure 3.2-10 B). The 0.8 µM and <0.5 µM Cu chemostat cultures showed a significant increase in N_2O accumulated (Figure 3.2-10 A). In the 0.8 µM culture N_2O accumulated up to 832 ± 327 µM, which accounts for 10.7 ± 6.4 % of the total NO_3^- being consumed at steady state. The <0.5 µM Cu culture also accumulated N_2O and it increased by 3 fold compared to the 0.8 µM Cu culture, the net N_2O accumulated reached 2275 ± 70 µM, which accounts for 40.4 ± 7.25 % of the total NO_3^- being consumed at steady state.

The specific rates of N_2O produced, qpN_2O (Table 3-3), also reflect the significant effect that Cu has on the *P.denitrificans* cultures. The qpN_2O is a function of the rate of NO_3^- reduction through NO_2^- and NO (Reactions 1,2 and 3) to form N_2O minus the rate of nitrous oxide consumption (Reaction 4) and can be calculated from:

$$qp\ N_2O = [\,N_2O\,]\,r\,D\,/\,x; \quad \textbf{Equation 3.2-4}$$

where $[N_2O]_r$ is the N_2O concentration in the reactor vessel – the initial/background N_2O concentration. The qpN_2O in the <0.5 µM Cu culture increased by 4 times more than in the 0.8 µM Cu culture (Table 3-3). The 0.8 µM and <0.5 µM Cu cultures showed a lag in the net accumulation of N_2O, compared to the 18 µM Cu culture. The N_2O increases in the 18 µM Cu culture immediately after the vessel has turned anaerobic,

however the 0.8 µM and <0.5 µM Cu cultures N_2O accumulates after an initial 32 h lag phase. After 56 h the N_2O increases dramatically, this is also when the drymass enters into steady state. The N_2O increases until about 96 h where it begins to also get into a steady state. Also when the culture enters steady state 90 % of the cell culture has been generated in the anaerobic phase. The Biomass is washed out of a continuous culture bioreactor exponentially, as defined by $xt/x0 = e^{-Dt}$ (where x0 = biomass at time 0 and xt = biomass at time t). Thus at $D = 0.06\ h^{-1}$ around 50 h (or 3 vessel volumes) is required before the bioreactor is dominated by newly generated cells, rather than cells that were present in the reactor when the culture was switched from aerobic to anaerobic.

The increase in N_2O produced in the <0.5 µM Cu culture suggests that there is a loss of function of NosZ, however it is not known whether this is at a protein or transcriptional level. If the Cu centres are not inserted correctly then the enzyme is not active; however the *nosZ* gene could be Cu regulated, and therefore not be transcribed at all. Further work into the protein and transcriptional expression is later discussed in Chapter 4.

The total amount of N_2O accumulated accounts for 40.4 ± 7.3 % of net NO_3^- consumed in the <0.5 µM Cu chemostat, and the NO_2^- accounts for 0.42 ± 0.2 %. Therefore about 41 % of the total consumed NO_3^- is accounted for. In the 0.8 µM Cu culture only 10 % of the NO_3^- is accounted for in the form of N_2O, and only 0.01 % is accounted for in the 18 µM cultures. As NO was not detected in any of the cultures the remaining NO_3^- must be converted to N_2. To detect N_2 the cultures were run using ^{15}N-$NaNO_3$, therefore the ^{15}N could be traced either at ^{15}N-N_2 or as any other N containing intermediate.

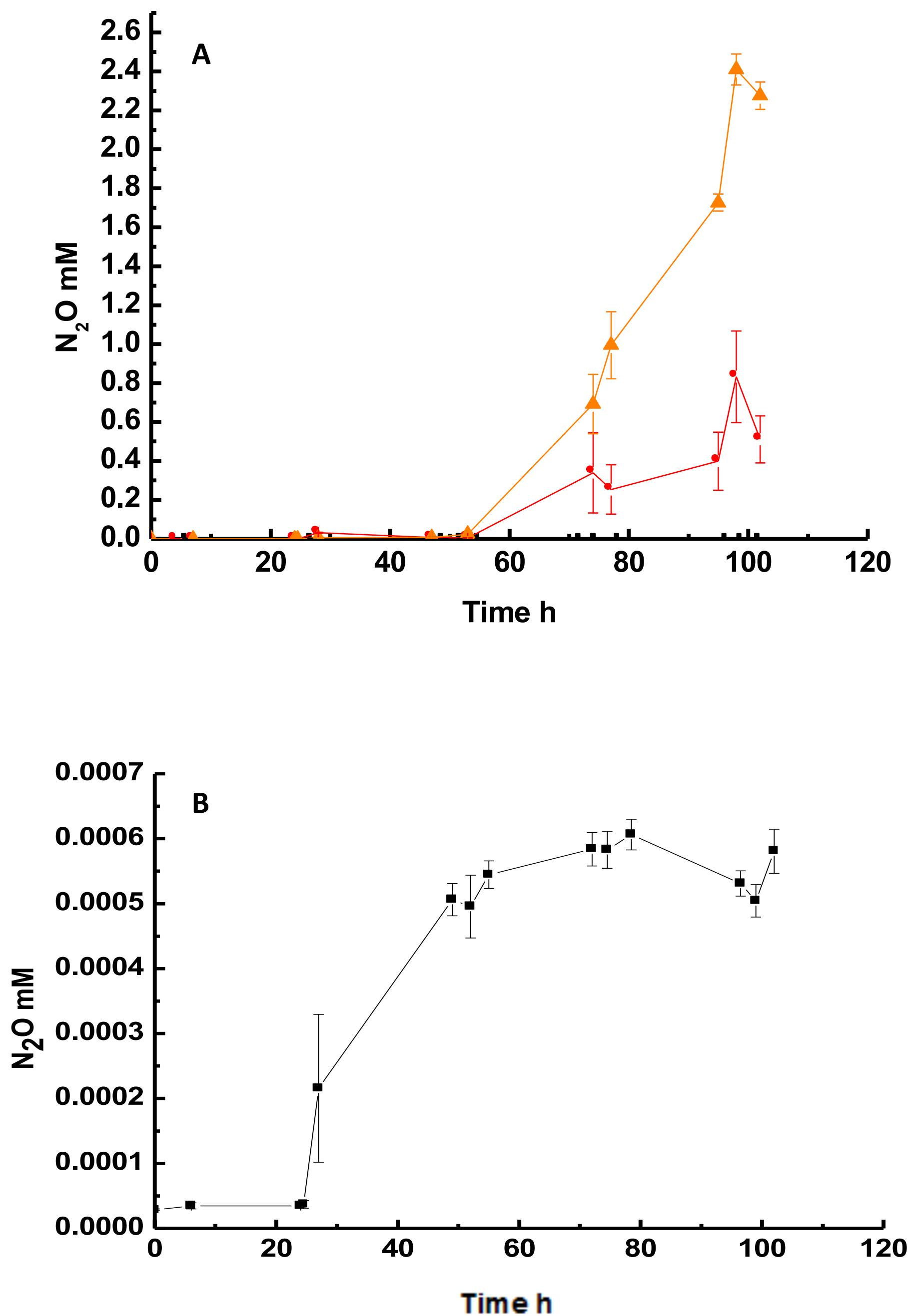


Figure 3.2-10 The accumulation of N_2O in chemostat cultures of *P.denitrificans* grown in NO_3^- -sufficient succinate-limited 18 µM Cu, (B ■) 0.8 µM Cu (●) and < 0.5 µM Cu (▲) *Paracoccus* minimal media. Graph A shows all 3 chemostat cultures and B shows an expansion of the 18 µM Cu chemostat culture. At 24 h the air supply was removed and the feed was added (D= 0.06 h^{-1}). Gas samples were analysed twice for each chemostat culture, the error bars represent the standard error.

3.2.2.5 Dinitrogen production in chemostat cultures of *P.denitrificans*

P.denitrificans was grown in 18 µM, 0.8 µM and <0.5 µM Cu *Paracoccus* minimal media with 30 % enriched ^{15}N-$NaNO_3$ (20 mM). With the addition of ^{15}N labelled NO_3^- when the NO_3^- is reduced to N_2 it can be distinguish from atmospheric N_2 as it is labelled as ^{15}N-N_2. From identifying the concentration of ^{15}N-N_2 present in the gas phase of the chemostat culture using Henrys law the total concentration of N_2 in liquid phase can be determined, therefore the total net of N_2 can be calculated.

At steady state in the 18 µM Cu culture a total of 3.05 ± 0.5 mM N_2 was detected. When the Cu concentration was reduced to 0.8 µM the N_2 detected was slightly elevated compared to the 18 µM Cu culture. At steady state in the 0.8 µM Cu culture the N_2 detected reached 3.94 ± 0.64 mM. It was hypothesised that when the Cu was reduced to <0.5 µM the activity of NosZ would be reduced, or possibly completely inhibited. At steady state in the <0.5 µM Cu chemostat culture a total of 1.33 ± 0.18 mM of N_2 was detected. Currently NosZ is the only known enzyme capable of reducing N_2O in *P.denitrificans* and therefore in the <0.5 µM Cu chemostat cultures the NosZ protein must therefore still be active as N_2 is being produced. The fact that the concentration of N_2 produced in the chemostat cultures decreased as the Cu concentration is lowered implies that the cellular activity of NosZ is affected by the Cu concentration, but is not completely absent in low Cu containing environments.

The specific rate of dinitrogen production could be calculated from:

$$qN_2 = \left[{}^{15}N - N_2\right] D/x$$

Equation 3.2-5

and is inversely proportional to the rate of N_2O production (Table 3-3). The rate of N_2 production is 3 times higher in the 18 µM Cu culture compare to the <0.5 µM Cu culture.

Table 3-3 Rates for NO_3^- consumption and NO_2^-, N_2O and N_2 accumulation in *P.denitrificans* chemostat cultures at steady state (102h). *P.denitrificans* was grown in *Paracoccus* minimal media with 20 mM NO_3^- and 5 mM succinate with either 18 µM , 0.8 µM or <0.5 µM Cu

	Growth Yield	qcNO$_3^-$	qpNO$_2^-$	qcNO$_2^-$	qpN$_2$O	qpN$_2$
	mg.ml.h^{-1}	mmol.g drymass^{-1}h^{-1}				
Cu 18 µM	0.011 ±0.000	2.010 ±0.098	-0.006 ±0.001	2.016	0.0001 ±0.000	0.902 ±0.219
Cu 0.8 µM	0.011 ±0.000	3.166 ±.790	-0.004 ±0.001	3.170	0.126 ±0.043	0.723 ±0.102
Cu <0.5 µM	0.012 ±0.002	3.284 ±0.362	0.009 ±0.005	3.275	0.527 ±0.007	0.297 ±0.039

Growth Yield -biomass production = Dx; where x = the biomass at steady-state.

NO_3^- consumption $(qcNO_3^-) = ([NO_3^-]_R - [NO_3^-]_r)\,D\,/\,x$; where [$NO_3^-$]R is the NO_3^- concentration in the reservoir, [NO_3^-]r is the residual NO_3^- concentration in the reactor vessel, D is the dilution rate and x is the drymass concentration in the reactor vessel.

NO_2^- production $(qp\,NO_2^-) = [NO_2^-]\,r\,D\,/\,x$ where [NO2-]r is the NO_2^- concentration in the reactor vessel – the initial/background NO_2^- concentration

NO_2^- consumption $(qc\,NO_2^-) = qcNO_3^- - qp\,NO_2^-$

N_2O production $(qp\ N_2O) = [\,N_2O\,]\,r\,D\,/\,x$; where [$N_2O$]r is the N_2O concentration in the reactor vessel – the initial/background N_2O concentration

N_2 production $(qpN_2) = [N_2]r\,D/x$; where [N_2]r is the net N_2 concentration in the reactor vessel, as determined by ^{15}N-N_2 analysis

‘ - ’indicates net consumpti

The increased release of N_2O from the chemostat cultures is directly linked to the concentration of Cu available to *P.denitrificans.* As Cu concentration is reduced the flux through the NosZ step of denitrification is decreased resulting in N_2O accumulating, rather than being converted to N_2. Throughout the experiment NO was not detected, previous research has shown *P.denitrificans* to accumulate extracellular NO at a maximum of 30 nM, when grown on NO_3^- nutrient rich media (this level is below the detection limit of the assay)(Goretski, Zafiriou et al. 1990). As NO did not accumulate to a µM level, it suggests that NO reductase activity remained unaffected by Cu concentration, and so the only intermediate of denitrification that accumulated was N_2O. This then brings about the question, is N_2O reductase synthesised, or does the Cu affect transcription of *nosZ*? And also what is happening to the other denitrification enzymes, Nar, Nir and Nor? This will be explored in the Chapter 4.

3.3 Discussion

Copper plays a major role in denitrification, as well as aerobic respiration in many organisms. In chemostat cultures of *P.denitrificans* Cu does not affect aerobic growth, possibly due to the presence of cytochrome *bd*, which is a haem dependent oxidase. The Cu concentration also has no major effect on anaerobic growth of *P.denitrificans* in NO_3^- -sufficient succinate-limited cultures. It was hypothesised under low Cu concentrations that the NosZ would not be functional, and therefore all the consumed NO_3^- would accumulate as N_2O. However *P.denitrificans* only converted 40 % of the NO_3^- consumed to N_2O, the fact that N_2 is produced therefore means N_2O is being reduced by NosZ, as probably there is no other enzyme in *P.denitrificans* that can turnover N_2O.

The Cu concentration directly affects *P.denitrificans* and cellular N_2O release, so how does this affect our environment? *P.denitrificans* is common in many agricultural soils along with many other denitrifiers that contain *nosZ* in their genome (Baumann, Snozzi et al. 1996). Copper is vital in many metabolic processes whether it be in

bacteria, plants or higher organisms. Plants, especially crops such as rice, cereals, spinach, carrots and citrus fruit when grown in copper deficient soils can greatly suffer from disease and the quality of crops decreases dramatically (Solberg Elston, Evans Ieuan et al. 1995; Dawson 2006; Shorrocks and Alloway 2011). Therefore if the soils are Cu deplete microorganisms are going to be in direct competition with the crops and plants. If Cu becomes limited then not only will it affect crop quality or yield, but also in fields with high NO_3^- and NH_4, the N_2O release will be increased. Other microorganisms also contain other Cu enzymes in the denitrification pathway and so they might be affected differently.

N_2O release has also been found to occur in sewage, where Cu is not limiting N_2O is still accumulating and so there maybe another reason to why N_2O is accumulating in some mixed cultures (Cervantes, Monroy et al. 1998). If the reduction of N_2O is slower than the reduction of NO_2^- and NO (Reaction 1-3) then N_2O (Reaction 4) would also begin to accumulate. In cultures of waste water sludge if Cu was limited then NO_2^- would also accumulate due to other microorganisms containing the CuNir, NirK. However NO_2^- is not found to accumulate and hence the rate of N_2O is the rate limited step of denitrification (Schulthess and Gujer 1996; Cervantes, Monroy et al. 1998). To investigate this further another set of chemostats were set up with another denitrifier that contains the NirK, *Achromobacter xylosoxidans* (See Chapter 7).

Taken together the results of the current continuous culture study are significant because they show for the first time that under NO_3^--sufficient, Cu-depleted, conditions a denitrifying culture can grow and divide stably for a sustained period, maintaining a steady-state biomass that was identical to that of a Cu-replete culture, but releasing the potent greenhouse gas N_2O at >1000 times that rate of Cu sufficient cultures. It is notable that the N_2O electron acceptor 'lost' under these conditions, which is equivalent to a ~20% of the input of NO_3^- electron sink, is compensated for by the increased consumption rate of nitrate in steady-state in 18 µM Cu culture compared to the <0.5 µM Cu culture. Thus the denitrification system adjusts to the 'shut-down' of the last step and compensates for bioenergetic consequences of this. From an environmental point of view this is significant because it leads to nitrous oxide being produced more quickly, but being consumed more slowly. The denitrification enzymes are associated with the

proton-translocating electron transport network of the energy-conserving cytoplasmic membrane of bacteria. The complete reduction of NO_3^- to N_2 gas requires 10 electrons (Reactions 1-4). The partial reduction to N_2O requires 8 electrons (Reactions 1-3). If these electrons originate from NADH then there is an associated translocation of 30 protons across the energy conserving cytoplasmic membrane that creates a protonmotive force to drive ATP synthesis (~3.3 protons / ATP). If denitrification does not proceed to completion, stopping at N_2O release, then this becomes 24 protons. Our results show that this 20 % difference makes very little difference to the growth of *P.denitrificans* in an electron acceptor-rich environment, since the bacterium can compensate for loss of N_2O reduction by consuming more of the available electron acceptor, NO_3^-. However, denitrification originally evolved around 2 billion years ago when the Earth's sub-surfaces were not nitrate-rich. Thus in such a nitrate-limited environment it would be to a significant competitive advantage to be able to consume all of the electron acceptor available from nitrate-reduction i.e. the nitrite, nitric oxide and nitrous oxide.

Since the advent of the Harber-Bosch process and more intensive arable farming agricultural lands have become nitrate-rich environments, leading in turn to nitrate-rich water systems, including sub-surface aquifers. This is perhaps reflected by there being a number of denitrifying bacteria that can reduce NO_3^- to N_2O, but do not have a gene encoding for a N_2O reductase and so denitrification terminates in N_2O release. It would be a serious concern for the environment if this represented a trend towards evolving out this terminal denitrification step in NO_3^--rich environments. As arable lands become more intensively exploited Cu-deficiency is becoming more acute, for example it was recently estimated that around ~20 % of arable lands in Europe are biologically copper deficient, with concentrations of < 1 mg kg^{-1} (~ 1.5 μmol kg^{-1}), which is in the concentration domain explored in this study (Alloway 2008). Such lands can be rich in NO_3^- from added fertilizers and our data from a model laboratory culture shows that denitrifying bacteria can thrive in such a NO_3^--rich Cu-deficient environment by partially shutting down the last step of denitrification and increasing consumption of the readily available NO_3^-. The impact on N_2O release is very pronounced with 40 % of nitrate consumed being released as this potent greenhouse

gas, significantly above the 1 % currently assumed in IPCC models for nitrate added to fields.

Chapter 4: The effects of copper concentration on the transcription and translation of denitrification proteins in nitrate-sufficient chemostat cultures of *Paracoccus denitrificans*

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4.1 Introduction - The regulation of the genes involved in denitrification

The regulation of the denitrification genes is very complex, there are many regulators involved in the transcription of the various genes encoding proteins involved in the reduction of NO_3^- to N_2. One of the main families of regulators involved in the global regulation of anaerobic respiration is the Fumarate and Nitrate Reductase Regulator, FNR. FnrP is an FNR family member encoded in *P.denitrificans* genome, and it binds to the FNR box (signal motif TTGATCCAGATCAA), FnrP contains the [4Fe-4S] cluster which senses O_2, which is also observed in other FNR family members (Van Spanning R.J.M., De Boer A.P.N. et al. 1997; Bouchal, Struhárová et al. 2010). A paralogue of FNR family of regulators is the Nitric oxide Reductase Regulator, NNR, family which contains a haem and detects NO rather than O_2, in *P.denitrificans* NnrR is the NNR family member. Another FNR paralogue regulatory protein is NarR, which is thought to be a nitrite sensor (Wood, Alizadeh et al. 2001; Bouchal, Struhárová et al. 2010). FnrP is involved in the regulation of the *nar* operon and cbb_3- type oxidase, an FNR box is also located upstream of the *nos* operon (Van Spanning, De Boer et al. 1995 ; Van Spanning R.J.M., De Boer A.P.N. et al. 1997; Bouchal, Struhárová et al. 2010). NnrR specifically regulates the expression of the *nir* and *nor* genes and is thought to also be involved in the regulation of the *nos* genes, NarR is a NO_3^- or NO_2^- sensor and is required for the transcription of the *nar* operon, and interacts with FnrP, however the mechanism is yet unknown (Van Spanning R.J.M., De Boer A.P.N. et al. 1997; Wood, Alizadeh et al. 2001; Bouchal, Struhárová et al. 2010). The three regulators, FnrP, NnrR and NarR are not active during aerobic growth, and therefore the synthesis of NarGHI, NirS, NorCB, cbb_3-type cytochrome is largely confined to anaerobic metabolic growth conditions.

The *nosZ* gene has an FNR box upstream, however previous research has reported that mutants deficient in FnrP and NnrR show no significant change in NosZ activity (Van Spanning R.J.M., De Boer A.P.N. et al. 1997). A proposed method of the regulation for the *nos* operon can be seen in Figure 4.1-1. NnrR is thought to bind to the FNR-box which activates transcription by RNA polymerase. NosR is directly

involved with the transcription of *nosZ*, however the mechanism of the interaction is not known. The *nosZ* gene is not transcribed if *nosR* is knocked out, or if *nosZ* is not immediately transcribed after *nosR* (Cuypers, Viebrock-Sambale et al. 1992; Wunsch and Zumft 2005). The other genes in the *nos* operon are involved in the maturation processes of NosZ (Chapter 1.3.4). The proteins are located across the inner and outer membranes as well as the periplasm (Figure 4.1-2). The location of these accessory proteins are vital for the activity of NosZ since if the Cu centres are not inserted the enzyme will be rendered inactive. NosA is thought to be involved in importing Cu into the periplasm where the Cu centres are inserted (Wunsch, Herb et al. 2003). NosDFY could potentially be a sulphide exporter, as a sulphur ion is incorporated into the Cu_Z centre and is vital for catalytic activity, the proteins have been found to show some homology to the ATP Binding Cassette (ABC transporter) family of proteins, which transport small molecules (Zumft, Viebrock-Sambale et al. 1990). NosL has been found to bind Cu(I) and is thought to be involved in incorporating Cu into the NosZ centre, acting similarly to a metallochaperone (McGuirl, Bollinger et al. 2001).

Another set of Cu binding proteins that are encoded in the *P.denitrificans* genome are the Sco proteins. These metallochaperones have been previously located imbedded in the inner membrane of human and yeast mitochondria and they are capable of transporting Cu to cytochrome *c* oxidase in eukaryotic cells (Leary, Kaufman et al. 2004). ScoP is a putative copper-binding protein in *P.stutzeri*, and in *P.denitrificans* the putative Cu-binding proteins are Sco1 and Sco2 which are potentially located on the inner membrane, due to the location of Sco1 and Sco2 in mitochondrial cells (Figure 4.1-2) (Leary, Kaufman et al. 2004), and Scop1 and Scop2 in yeasts show high homology to the ScoP system in *P.stutzeri* (32 % identical and 49 % similarity calculated by blastp suite-2 sequences). These proteins are found across prokaryotes and eukaryotes and they are directly involved in Cu transport (Chinenov 2000). Deletion in Scop1 in yeast cells causes rapid degradation of cytochrome *c* oxidase (Lode, Kuschel et al. 2000). Scop1 transports Cu(II) across the inner membrane, the Cu is then bound by Scop2 which can then be taken up by cytochrome *c* oxidase (Lode, Kuschel et al. 2000).

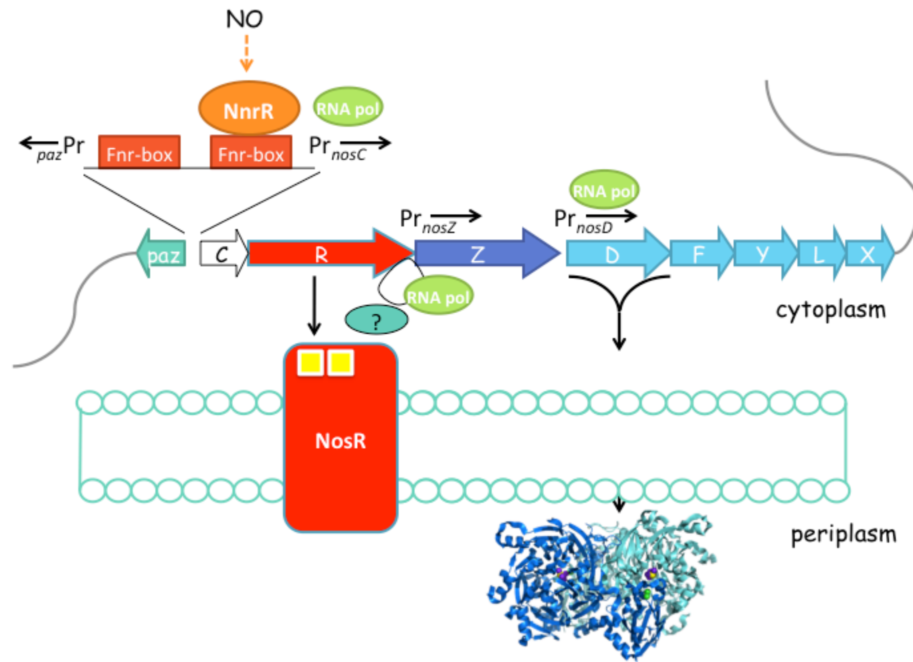

Figure 4.1-1 A proposed model of the regulation for the *nos* operon and pseudoazurin, *paz,* in *P.denitrificans*. Promoter sites (Pr) are located upstream of the genes to which RNA polymerase (RNA pol) binds to. NosR is directly involved in the transcription of *nosZ* and it is a local event. NosR contains two [4Fe-4S] clusters ( ) which may be involved.

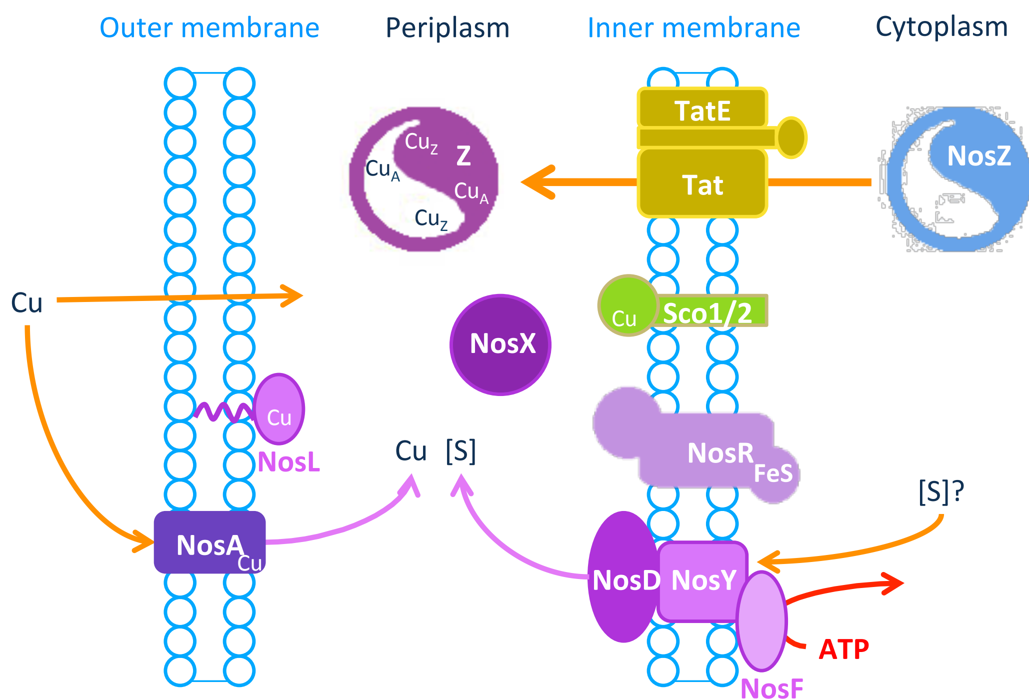

Figure 4.1-2 The subcellular distribution of the Nos proteins (NosRZDFLYX) in *P.denitrificans*. NosZ is exported to the periplasm from the cytoplasm via the Tat pathway. Sco1 and Sco2 are potentially involved in Cu transportation.

In the previous Chapter it was shown that changing the concentration of Cu, NO_3^- -sufficient succinate-limited chemostat cultures had a significant impact on the production of N_2O. However we have not yet established whether these changes are seen at a protein or transcriptional level. The aim of this Chapter is to investigate the of the synthesis of enzymes involved in denitrification, and also the transcription of *nosZ* effect under Cu limited and sufficient concentrations.

4.2 Results: The effect of copper on the synthesis of denitrification enzymes in chemostat cultures of *P.denitrificans* grown in nitrate-sufficient succinate-limited media

4.2.1 Detection of Paz, c_{550}, Nar, Nir and Nor in *P.denitrificans* chemostats grown in nitrate sufficient media

Periplasmic, cytoplasmic and membrane fractions were isolated from the *P.denitrificans* chemostat cultures at 120 h. NirS, NosZ and Paz are located in the periplasm, whereas NarGHI and NorCB are bound to the membrane. A series of Western-blotted SDS PAGE gels were probed with antibodies (Abs) raised against specific *P.denitrificans* denitrification enzymes. The secondary Abs were a horseradish peroxidise (HRP) conjugated Abs. Using a chemoluminescent substrate the HRP catalyses a oxidation of luminol by hydrogen peroxide forming 3-aminophthalate. This product releases light (425 nm) as it decays to a lower energy. Using X-ray film it was therefore possible to quantifiably detect small quantities of protein which the Abs had attached to.

4.2.1.1 Pseudoazurin

Pseudoazurin (Paz) is an e^- transfer protein that contains one Cu ion per monomer and shuttles e^- from the cytochrome bc_1 complex to NosZ, NorBC and NirS (Paes de Sousa, Pauleta et al. 2007). Paz occurs as a dimer in the periplasm (26 kDa), each monomer being approximately 13 kDa. Figure 1.3-1 in Chapter 1 shows the interactions of Paz with the other enzymes involved with denitrification. Probing Western-blotted SDS-PAGE gels of periplasmic fractions of the 18 µM Cu chemostat with Paz-Abs revealed both the monomeric and dimeric form of Paz at 13 kDa and 26 kDa, respectively (Figure 4.2-1). As the Cu is reduced to 0.8 µM and <0.5 µM, the amount of Paz is dramatically reduced (Figure 4.2-1), the protein is therefore either degraded or transcription of *paz* is inhibited. Despite Paz being absent from the 0.8 µM and <0.5 µM Cu periplasmic fractions e^- transfer to the Nir, Nor and Nos systems still occurs, which is confirmed by cyt cd_1 activity and NosZ activity (comnfirmed by N_2 production, see Chapter 3). Therefore e^- transfer to Nir, Nor and Nos is potentially due to cytochrome c_{550} which is a haem containing e^- transfer enzyme. The fact that Cu concentration directly affects the synthesis of the Cu containing protein Paz suggests that Cu will also affect the synthesis of other Cu containing enzymes such as NosZ.

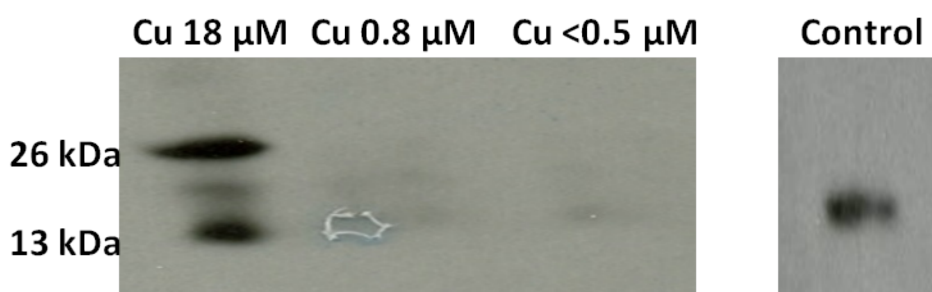

Figure 4.2-1 Western-blotted SDS-PAGE gels probed with Abs raised against Pseudoazurin from *P.denitrificans* periplasmic fractions isolated at steady state chemostat cultures grown on NO_3^- -sufficient succinate-limited media with either 18 µM, 0.8 µM and <0.5 µM Cu. Approximately 10 µg of protein were loaded in each well and2 µg of Paz was loaded as the control.

4.2.1.2 Cytochrome c_{550}

Cytochrome c_{550} is a haem dependent e^- transfer protein and is capable of transferring e^- to the NirS, NorCB and NosZ (Figure 1.3-2). It was hypothesised that when Cu is at 0.8 µM and <0.5 µM the presence of Paz is reduced and therefore for denitrification to continue c_{550} synthesis might be increased to compensate for the loss of Paz. A haem stain was carried out on SDS PAGE gels to identify c_{550} in the periplasmic fractions, the c_{550} was identified as the blue band seen at approximately 15 kDa which was confirmed by running purified c_{550} (Figure 4.2-2). Other haem proteins in the gel all showed similar intensity apart from bands that represent c_{550} and cyt cd_1, the intensity of the band is marginally increased in the <0.5 µM Cu culture (lane 2) compared to the 18 µM Cu culture (lane 1), contrary to Paz (Figure 4.2-1). However SDS PAGE gels and haem stains are very qualitative way of observing protein concentrations. Despite the protein loaded onto the gels being the same in each lane, the process where by the periplasms are isolated involves the addition of lysozyme and so the protein loaded onto each gel may not be completely accurate. Therefore the fact that c_{550} is present in the <0.5 µM periplasmic fraction shows that the synthesis is not affected in response to low Cu concentrations or in response to the redox state of the electron transport chain which might be perturbed under these conditions. However the marginal increase in presence of c_{550} does not immediately show that the synthesis is significantly increased. The regulation of c_{550} is unclear, RegR is a two component regulatory system in *B.japonicum*, that is involved in the regulation of 250 genes and is especially important in the expression of oxygen- or redox-regulated gene expression (Bueno, Richardson et al. 2009). Using BLAST a homologue of RegR was identified in *P.denitrificans*, which is the two component Fis system which could potentially be involved in the regulation of many genes involved in denitrification including cyt c_{550}.

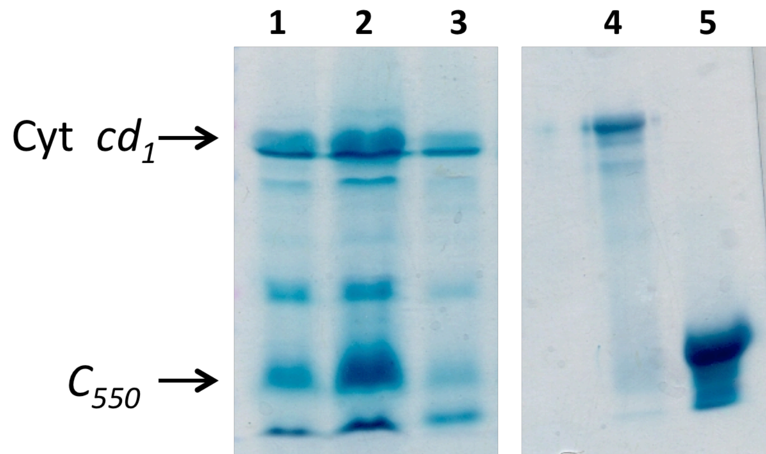

Figure 4.2-2 A haem stained SDS PAGE gel, loaded with periplasmic fractions (100 µg of protein is loaded per well) isolated from *P.denitrificans* grown in NO_3^- -sufficient succinate-limited medium with either 18 µM Cu (lane 1), 0.8 µM Cu (lane 3) or <0.5 µM Cu (lane 2). Purified cyt *cd_1*, 20 µg (lane 4) and 30 µg of *c_{550}* (lane 5) were loaded as a controls.

4.2.1.3 Nitrate Reductase

The membrane bound NO_3^- reductase, Nar, consists of three subunits, NarG is the larger subunit, approximately 140 kDa, Nar H is approximately 60 kDa and the third subunit NarI is 25 kDa. *P.denitrificans* membranes were isolated from steady state chemostat cultures, then run on an SDS-PAGE gel which was then blotted. Antibodies raised against *P.denitrificans* NarGH were used to see if a change in Cu concentrations would affect the expression of NarGH (Figure 4.2-3). It can be observed in the Western blotted SDS PAGE gel that *P.denitrificans* grown in 18 µM Cu expresses NarG, which is represented by the 100 kDa band. The intensity of the NarG band is marginally increased in the 0.8 µM Cu membrane fraction; and could not be detected in the <0.5 µM Cu periplasmic fraction (Figure 4.2-3). The NarH band was observed at approximately 60 kDa in the control western blotted SDS PAGE gel (Chapter 2 Figure 2.1), however it was not detected in the periplasmic fractions. NarH is co-expressed with NarG and therefore it must be transcribed with NarG, but the Abs were unable to detect the polypeptide.

The specific rate of NO_3^- reductase activity was monitored throughout the chemostat cultures. It was observed that the rate of NO_3^- reduction was lower during the aerobic phase, and once the cultures became anaerobic (24.5 h) the rate steadily increased between 24 – 40 h then entered steady state (Figure 4.2-4). The NO_3^- reductase activity was not significantly affected by Cu concentration in the whole cell assays. In the 18 µM Cu, 0.8 µM Cu and <0.5 µM Cu chemostat cultures at steady state the NO_3^- reductase activity was 36.5 ± 6.04 mol.min.mg drymass, 29 ± 4.2 mol.min.mg drymass and 38.9 ±8.89 nmol.min.mg drymass respectively (Figure 4.2-5 A). Therefore the Cu concentration has no significant affect on the rate of NO_3^- reduction. Thus even though Nar was unable to be detected using probing Western blotted SDS PAGE gels in the <0.5 µM Cu membrane fractions the Nar system was functional in all three of the Cu chemostat cultures. The rates observed from the membrane fractions (see Figure 4.2-5) also show no significant difference between the three cultures. The NO_3^- reductase rates observed in the membrane fractions in the 18 µM, 0.8 µM and <0.5 µM Cu cultures, were 69.4 ± 3.3, 72.8 ± 2 and 76 ± 0.1 nmol.min^{-1}mg protein^{-1}, respectively (Figure 4.2-5 B). The rates observed are due to the NarGHI as Nap is not expressed under anaerobic conditions in *P.denitrificans.* The results suggest that for Nar the MV+ dependent activity assay is a better probe for the enzyme than the Abs as cells lyse after freeze thaw and so Nar activity can be monitored in whole cell assays.

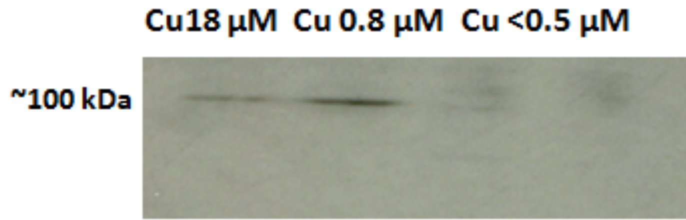

Figure 4.2-3 A Western-blotted SDS-PAGE gels probed with Abs raised against *P.denitrificans* NarGH from membrane fractions of 18 µM Cu, 0.8 µM Cu and <0.5 µM Cu chemostat cultures harvested at steady state. The 100 kDa band represents the NarG subunit (actual weight 140 kDa), and the 60 kDa band represent the NarH subunit. The control contains 0.2 µg of purified NarGH

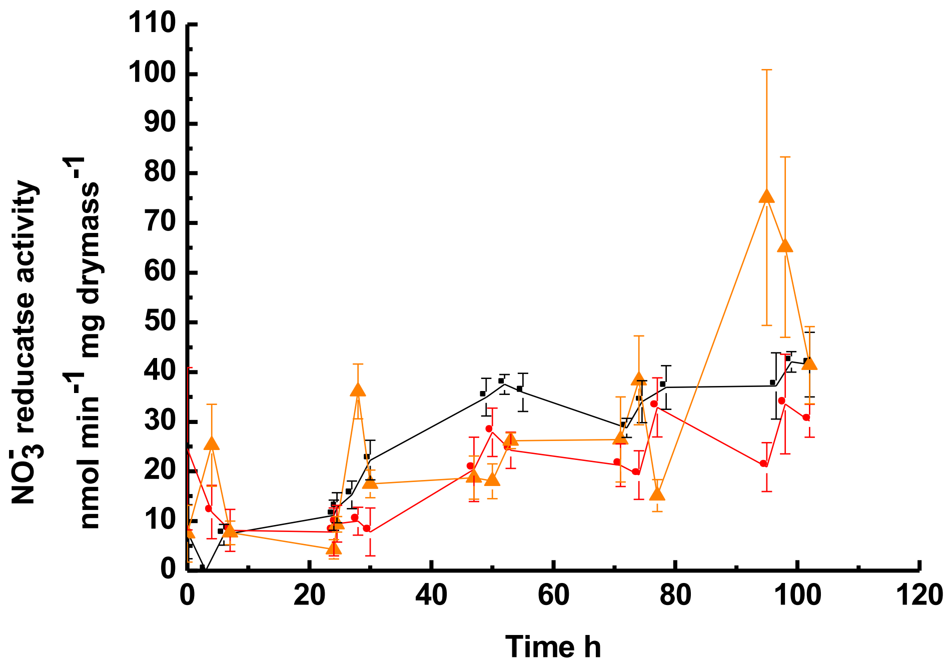

Figure 4.2-4 The activity of NO_3^- reductase monitored throughout the NO_3^- -sufficient succinate-limited chemostat cultures with either 18 µM Cu (■), 0.8 µM Cu (●) or <0.5 µM Cu (▲). Each sample was analysed twice from each chemostat culture, the error bars represent the standard error.

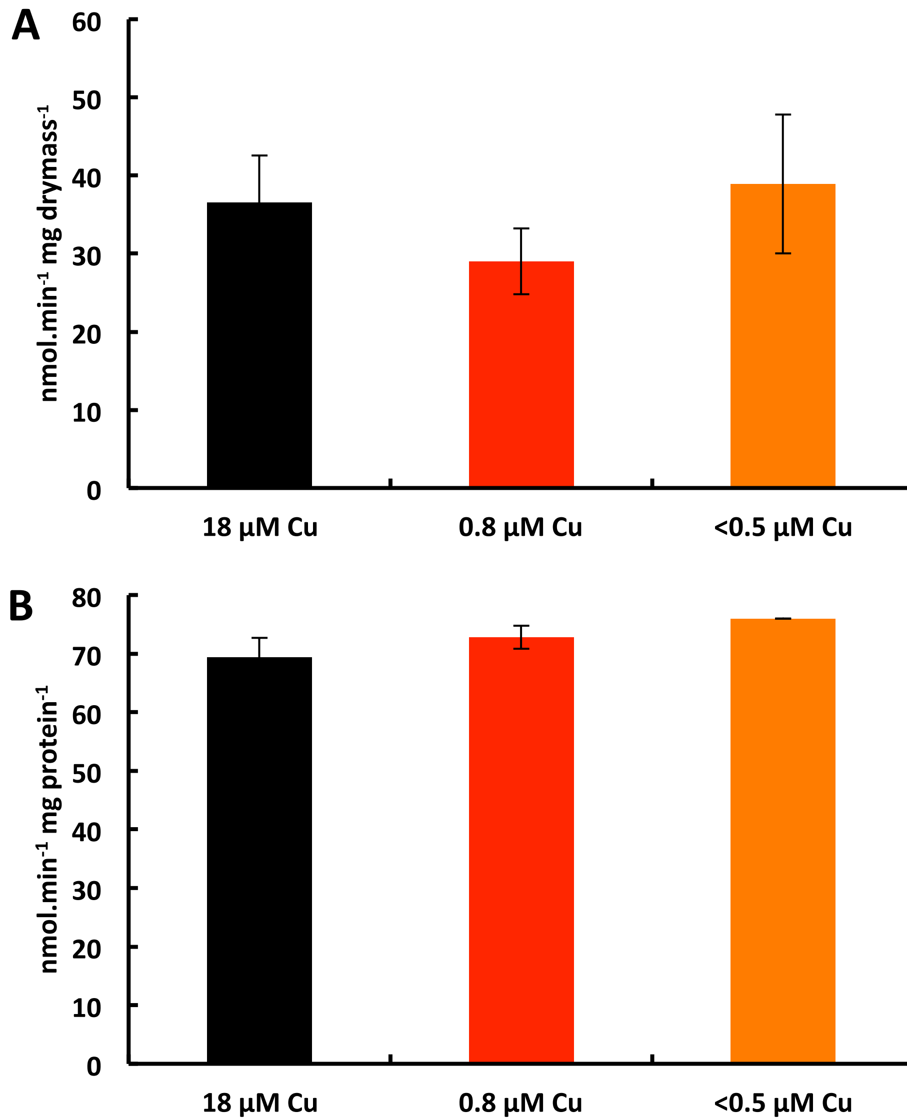

Figure 4.2-5 The specific activity of NO_3^- reductase measured in whole cell assays (A) and membrane fractions (B) isolated at steady state from chemostat cultures of *P.denitrificans* grown in NO_3^- sufficient *Paracoccus* media with either 18 µM Cu (black), 0.8 µM Cu (red) or <0.5 µM Cu (orange). Each sample was analysed twice from each chemostat culture, the error bars represent the standard error.

4.2.1.4 Nitrite reductase

Cytochrome *cd_1* is a periplasmic enzyme and is approximately 63 kDa. Western-blotted SDS PAGE gels probed with anti cyt *cd_1* Abs indicated that the cyt *cd_1* band was present in all 18 µM, 0.8 µM and <0.5 µM Cu chemostat periplasmic fractions. The intensity of the band marginally increased in the <0.5 µM, similar to that seen for the synthesis of c_{550} in the haem stain (Figure 4.2-2). In Figure 4.2-6 the <0.5 µM Cu periplasmic fraction shows a second band, at approximately 45-50 kDa, this band was also present in a lane containing the purified cyt *cd_1*. This could potentially be a contamination where the Abs cross react with another protein. However this 48 kDa band could potentially be the haem *d_1* binding domain of cyt *cd_1*. Previous research into cyt *cd_1* isolated from *Pseudomonas spp.* has observed that when cyt *cd_1* is incubated with a non specific protease it generates a 48 kDa protein, which represents the haem *d_1* domain (Horowitz, Muhoberac et al. 1982; Silvestrini, Cutruzzolà et al. 1996).

The specific activity of cyt *cd_1* was monitored throughout the chemostat cultures. After 24 h of aerobic growth the activity of the NO_2^- was at its lowest then when the cultures became anaerobic the activity increased. At steady state the activity in the 18 µM Cu chemostat cultures at steady state was 190 ± 47 nmol.min^{-1}mg drymass^{-1}; there was a slight decrease in activity in the 0.8 µM Cu culture, 94 ±25 nmol.min^{-1} mg drymass^{-1}. When the Cu concentration was reduced to <0.5 µM Cu the activity of the NO_2^- reductase was 204 ± 71 nmol.min^{-1}mg drymass^{-1}, showing no significant difference in the activity of cyt *cd_1* despite there being significantly more polypeptide in the <0.5 µM Cu periplasmic fraction. This suggests cyt *cd_1* is being synthesised but possibly the *d_1* haem is not being loaded correctly in all the cyt *cd_1* sites, rendering the enzyme inactive as it is the *d_1* haem that is responsible for reducing NO_2^- to NO.

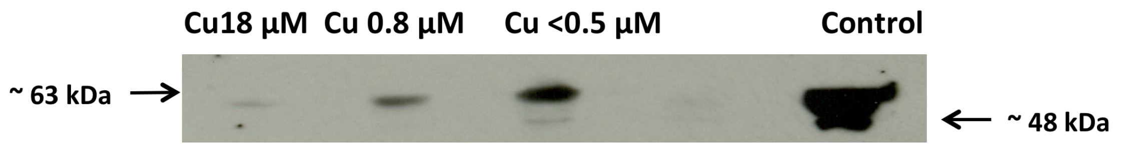

Figure 4.2-6 A Western-blotted SDS-PAGE gels probed with Abs raised against *P.denitrificans* cyt cd_1, 10 µg of protein from the periplasmic fractions was loaded into each well from the NO_3^- -sufficient succinate-limited 18 µM Cu, 0.8 µM Cu and <0.5 µM Cu chemostat cultures harvested at steady state. Cyt cd_1 was also loaded as a control (20 µg). For the full haem stained gel see Figure 4.2-2.

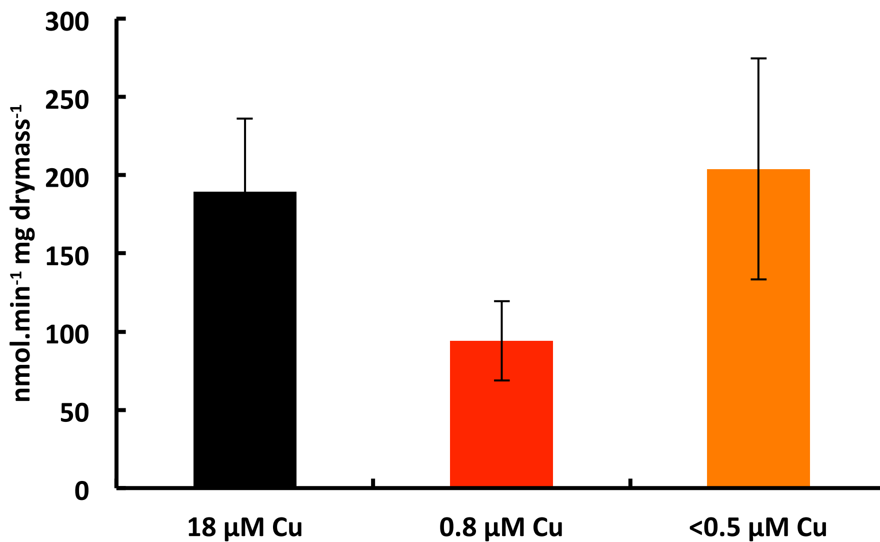

Figure 4.2-7 The specific activity of NO_2^- reductase measured in whole cell assays isolated at steady state from NO_3^- -sufficient succinate-limited chemostat cultures of *P.denitrificans* grown in NO_3^- sufficient *Paracoccus* media with either 18 µM Cu (black), 0.8 µM Cu (red) or <0.5 µM Cu (orange). Each sample was analysed twice from each chemostat culture, the error bars represent the standard error.

4.2.1.5 Nitric Oxide Reductase

The enzyme responsible for reducing the cytotoxic NO, NorCB, is a membrane bound enzyme. The Ab used was raised against NorB which is a 53 kDa protein containing a *b*-type haem (Butland, Spiro et al. 2001). A Western-blotted SDS PAGE gel probed with NorB Abs was carried out with the membrane fractions from *P.denitrificans* grown in 18 µM, 0.8 µM and <0.5 µM Cu chemostat cultures (Figure 4.2-8). NorB is present in all three 18 µM, 0.8 µM and <0.5 µM Cu, membrane fractions regardless of the Cu concentration. NO was not found to accumulate in the chemostat cultures, and therefore the activity and synthesis of the Nor system is unaffected by the concentration of Cu. As NO does not accumulate in the chemostat cultures, the rate of NO_2^- reduction must match the rate of NO reduction. Therefore as there is no significant difference in the rate of NO_2^- reduction, it would be expected that there would be no significant difference in the rate of NO reduction.

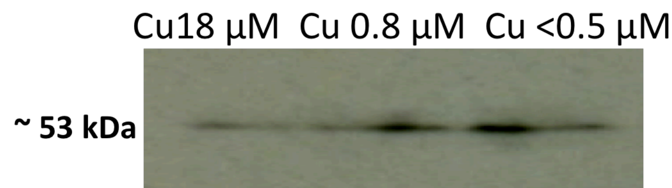

Figure 4.2-8 Western-blotted SDS-PAGE gels probed with Abs raised against *P.denitrificans* NorB. Each well has 10 µg of protein loaded per membrane fraction isolated from the NO_3^- sufficient succinate limted 18 µM Cu, 0.8 µM Cu and <0.5 µM Cu chemostat cultures harvested at steady state.

4.2.2 The transcription of *nosZ* in NO_3^- sufficient and limited chemostats cultures.

To identify whether *nosZ* was being transcribed at a reduced rate in the lower Cu concentration chemostat cultures Real Time PCR (RTPCR) was carried out. RNA was isolated after 4 h of the aerobic phase and the steady state anaerobic phase of the Pd1222 NO_3^- sufficient and NO_3^- limited chemostat cultures, and converted to cDNA by Reverse Transcriptase (Section 2.21). Primers were constructed by Primer Design Ltd for the *nosZ* and *RpoB* (RNA polymerase) genes from *P.denitrificans. RpoB* is a housekeeping gene and is used to normalise the RT PCR data for *nosZ*.

In the 18 µM, 0.8 µM and <0.5 µM Cu NO_3^- sufficient chemostat cultures *nosZ* is transcribed aerobically, 4 h sample, and anaerobically, 102 h sample (Figure 4.2-9). There is a significant effect on the transcription of *nosZ* and the Cu concentration, during aerobic growth as the Cu concentration decreases, so does the transcription of *nosZ* (Figure 4.2-9). When *P.denitrificans* is grown anaerobically in NO_3^- sufficient 18 µM Cu *Paracoccus* medium the transcription of *nosZ* is increased compared to the aerobic transcription. Under anaerobic conditions the transcription of *nosZ* is significantly lower when the Cu concentration is reduced. There is a 10 fold decrease in transcription in the <0.5 µM culture compared to the 18 µM Cu culture and a 40 fold decrease in the 0.8 µM Cu culture.

As *nosZ* is being transcribed in the low Cu chemostat cultures the NosZ polypeptide could be translated and exported to the periplasm. However if Cu is low and the Cu centres are not inserted then the enzyme would be inactive and therefore targeted for degradation. NosZ must be functional to a degree in the low Cu chemostats as N_2 is produced in all three of the NO_3^- sufficient chemostat cultures (Chapter 3). The detection of NosZ is explored in the following section.

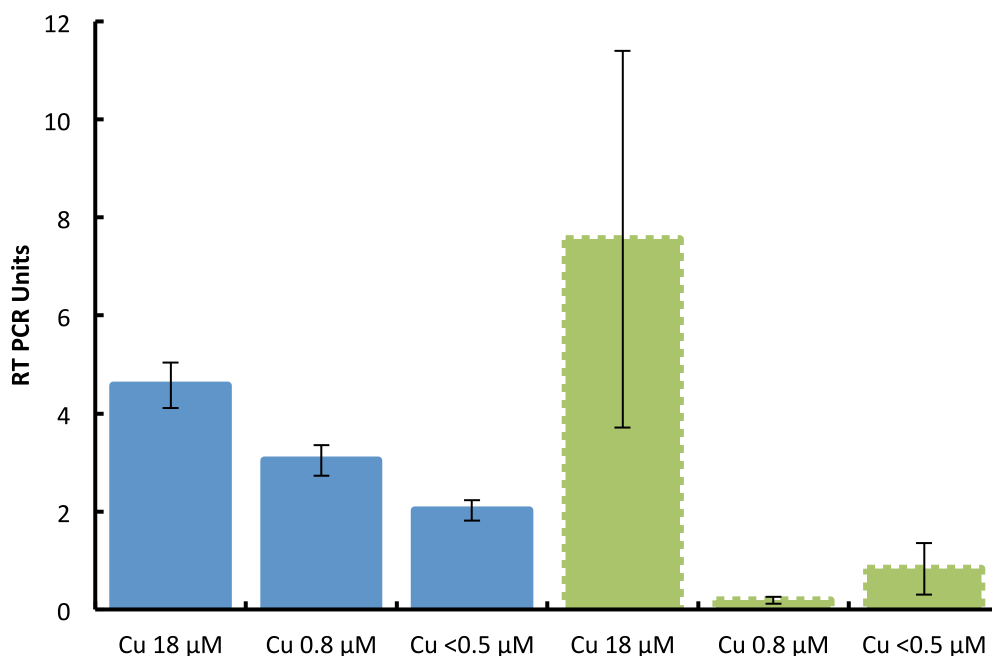

Figure 4.2-9 Real Time PCR showing the gene expression of *nosZ* in *P.denitrificans* chemostat cultures grown in NO_3^- -sufficient succinate-limited *Paracoccus* media with either 18 µM, 0.8 µM or <0.5 µM Cu. RNA samples were taken after 3 h, aerobic (blue) and at 102 h, anaerobic steady state (green, dotted outline). The data was normalised with *Rpo* as the housekeeping gene. Each RNA sample isolated was analysed twice from each chemostat culture, the error bars represent the standard error.

4.2.2.1 The detection of NosZ in NO_3^- sufficient chemostat cultures of *P.denitrificans*

NosZ is currently the only enzyme known to reduce N_2O to N_2, it is a Cu containing periplasmic enzyme that occurs as a dimer. The monomeric form of NosZ contains 6 Cu ions and is 66 kDa. In the chemostat cultures as the Cu concentration was reduced, N_2O release increased, this is of great interest as it seems to be that as Cu becomes limiting NosZ activity is decreasing, possibly due to the Cu centres not being formed. The 18 µM Cu periplasmic fraction shows a distinctive 66 kDa band, indicative of the monomeric form of NosZ, there is also a 50 kDa band present in the periplasm which cross reacts with the NosZ Ab (Figure 4.2-10). This 50 kDa band could be either a contamination or a degraded form of NosZ. As the Cu concentration

decreased so did the intensity of the 66 kDa band. NosZ was able to be detected in the 0.8 µM Cu periplasmic fractions however it is a very faint band, in the <0.5 µM Cu periplasmic fraction NosZ was unable to be detected (Figure 4.2-10). However in all three fractions the 50 kDa band is present. This band is also seen in the purified NosZ, which suggests that the 50 kDa band is a degraded form of NosZ rather than a cross reaction with the anti-NosZ Abs.

The 50 kDa band is of interest as it maybe a degraded form of NosZ, therefore a truncated NosZ polypeptide could be synthesised, exported to the periplasm and is possibly active even under limited Cu concentrations (Section 3.2.2.4). At first the 50 kDa band was thought to be a cross reaction of the anti NosZ Ab and lysozyme, which is used to lyse the cells (typically used at 10 mg/ml). A SDS PAGE gel loaded with lysozyme was Western-blotted and probed with anti NosZ Ab no bands were observed and so there was no cross reaction with lysozyme and the anti NosZ Abs. The question of whether this 50 kDa band was a degraded form of NosZ therefore needed to be resolved. If the Cu_A centre of NosZ is not formed the C terminal could potentially become fragile and degrade, possibly resulting in the N terminus being left exposed to further degradation (Bothe, Ferguson et al. 2007). Using ExPASy Compute pI/Mw the amino acid sequence of *P.denitrificans* N terminus NosZ was entered (protein sequence used for molecular mass determination was attained from Brown, Djinovic-Carugo et al. 2000). The theoretical molecular weight of the N terminus was 46 kDa, not far from 50 kDa, however this is dependent on where the protein is cleaved. The C terminal was predicted to be 15 kDa using the same program, giving a total mass of 61 kDa. The N terminal predicted molecular weight is similar to that of the 50 kDa band which observed (Figure 4.2-10), the 50 kDa band could therefore potentially be the β-propeller domain which contains the catalytic Cu_Z site.

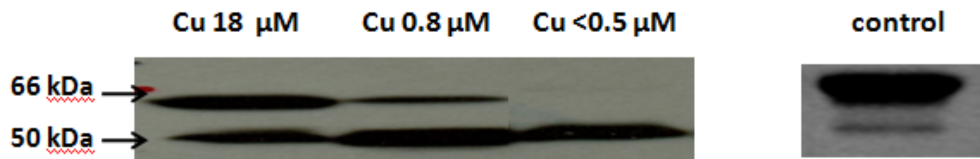

Figure 4.2-10 Western-blotted SDS-PAGE gels probed with Abs raised against NosZ isolated from *P.denitrificans* periplasmic fractions of the NO_3^- -sufficient succinate-limited 18 µM, 0.8 µM and >0.5 µM Cu chemostat cultures at steady state. Control contained 1 µg of purified *P.denitrificans* NosZ.

4.2.2.2 Does the degradation of NosZ generate a truncated form of NosZ?

To establish if the 50 kDa polypeptide is a degraded form of NosZ some purified NosZ was incubated with a non specific protease, proteinaseK, and then loaded onto an SDS PAGE gel as to see how the protein is degraded. Proteinase K has a broad specificity and cleaves many sites, therefore by incubating proteinase K and NosZ together the theory that the sites most susceptible to cleavage would be cleaved first by proteinase K. The samples were incubated at 37 ^{o}C for 0-64 minutes, the samples were then heat shocked at 100 ^{o}C to deactivate the proteinase K, stopping any further degradation at intervals of 1, 2, 4, 8, 16, 32 and 64 mins. NosZ proteinase K mix was then loaded on an 12.5 % SDS PAGE gel and stained using coomassie (Figure 4.2-11). Due to the concentration of purified protein loaded into each well, approximately 10 µg of NosZ, the staining is non quantifiable as the stain is saturated.

In Figure 4.2-11 the SDS PAGE gel shows that NosZ is present in all the lanes, approximately 66 kDa, and from 2-64 mins a 50 kDa band and a 16 kDa band appear. The linker region in the *nosZ* gene separates the C and N terminus. The smaller 16 kDa band could potentially be the C terminal of NosZ, the molecular weight of the 16 kDa band also decreases over time which could potentially be further degradation by protease (Figure 4.2-11). The 50 kDa band is therefore presumed to be the N terminal of NosZ, which contains the Cu_Z site.

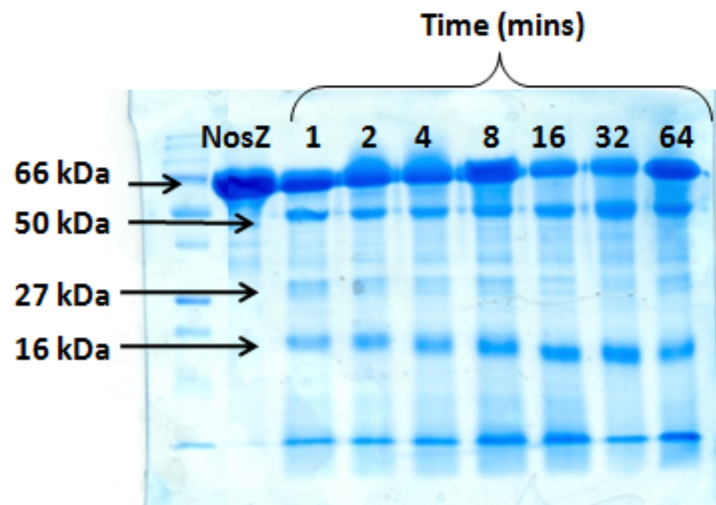

Figure 4.2-11 An SDS PAGE gel loaded with purified NosZ (NosZ) and NosZ incubated with proteinase k at 37 °C for 1, 2, 4, 8, 16, 32 and 64 minute interval. The 66 kDa band corresponds to the full NosZ polypeptide, a truncated form of NosZ appears at 50 kDa and 16 kDa. The 27 kDa band corresponds to proteinase k.

4.2.2.3 Isolation of the truncated NosZ - Affinity Chromatography

P.denitrificans was grown in NO_3^- -sufficient succinate-limited <0.5 µM Cu *Paracoccus* minimal media anaerobically at 37 ^oC for 24 h, as the 50 kDa NosZ band was observed in the periplasmic fractions isolated from the 18 µM Cu chemostat cultures (Figure 4.2-10). The cells were harvested and centrifuged at 3743 x g and lysed using the cell fractionation method (Section 2.3). The periplasmic fraction was collected and volume reduced using a 10 kDa cut off membrane (YM10) to ~ 4 ml. To immobilise the anti NosZ Abs a gravity column was packed with nProtein A Sepharose 4 Fast Flow matrix (GE healthcare) and 2 bed volumes of binding buffer were added (20 mM Sodium Phosphate buffer, pH 7.0). The anti NosZ Abs was diluted in binding buffer and 3 bed volumes were added to the gravity column which immobilised the anti NosZ Abs to the matrix, one more bed volume of binding buffer was added to the column as a wash. The *P.denitrificans* periplasmic fraction containing the 50 kDa band (confirmed by a Western-blotted SDS PAGE gel probed with anti NosZ Abs), was diluted in binding buffer and passed through the column. To elute the anti NosZ Abs and the proteins bound to the Abs, 0.1 M glycine buffer at pH 3, was added to the column and the fractions collected, the elution fractions were neutralised using 1 M Tris-HCl, pH 9.

Theoretically the 50 kDa NosZ protein and NosZ would bind to the immobilised anti NosZ Abs, and so the eluted fractions would contain the NosZ proteins bound to the Abs. The Ab-protein complex was denatured by the protein loading buffer and heating to ~100 ^oC, the samples were then run on a 12.5 % SDS PAGE gel and stained with coomassie blue. Figure 4.2-12 shows that there are 3 bands present in the first elution fraction. The 150 kDa band represents the anti NosZ Ab, and is therefore present in the elution fraction, as well as a 66 kDa band and a 50 kDa band (Figure 4.2-12). The 66 kDa band is indicative of NosZ and the 50 kDa is the proposed N terminal of NosZ. There were no other bands observed in the elution fractions.

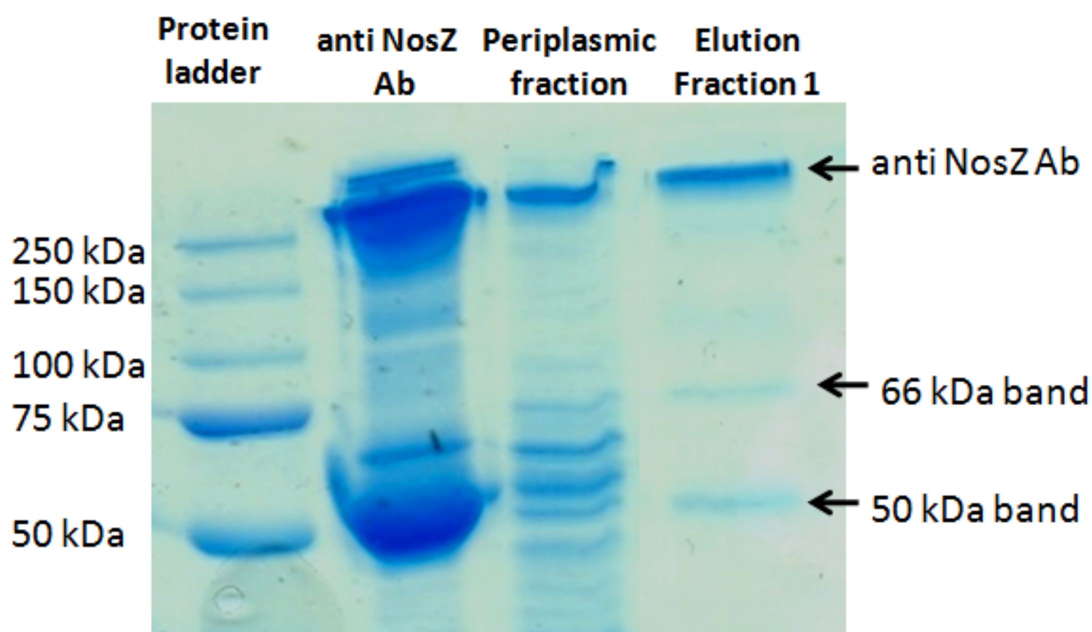

Figure 4.2-12 An SDS PAGE gel loaded with the anti NosZ Ab, (lane 1), the periplasmic fraction of *P.denitrificans* grown in <0.5 µM Cu *Paracoccus* media and the 2nd elution of the periplasmic fraction from the nProtein A Sepharose 4 Fast Flow

The elution fractions were pressure purified at a 10 kDa cut off, the fractions were then loaded onto an SDS PAGE gel. The 50 kDa band was extracted from the gel were sent for MALDI Mass fingerprinting at the John Innes Centre, along with a purified samples of NosZ.

The MALDI-Mass fingerprint trace was analysed using the Mascot database, the first 3 hits of the 50 kDa band isolated from the Cu depleted batch culture and the complete NosZ protein were compared to the database of denitrification genes. The MALDI-Mass fingerprint trace from the purified NosZ protein when compared to the Mascot database had the top hit as the full Nitrous oxide reductase from *P.denitrificans*, giving a protein score of 69 (Table 4-1). The second hit was a nitrous oxide reductase from an uncultured bacterium, with a protein score of 66. The trace attained from the MALDI-Mass Fingerprint of the 50 kDa band showed the top hit to be nitrous oxide reductase from *Rhizoobiales bacterium*, which is an α proteobacterium, protein score 56. The second hit was nitrous oxide reductase from an uncultured bacteria, protein score 39, and the third was nitrous oxide reductase

from *P.denitrificans*, protein score 31 (Table 4-2). The top three hits for the 50 kDa bands were NosZ proteins, even though the top hit was not the NosZ isolated from *P.denitrificans* this data strongly suggests that the 50 kDa band is a degraded form of NosZ.

The peptide sequences attained from the 50 kDa band MALDI Mass Fingerprinting were mapped on to the complete NosZ peptide sequences (Figure 4.2-13). The peptide sequences (Table 4-2) were all located on the N terminal domain of the NosZ sequence, suggesting that the 50 kDa band is not only a truncated form of NosZ but it is potentially the N terminal domain. This also suggests that the C terminal domain is cleaved off, potentially rendering the enzyme inactive. The chemostat cultures show that NosZ is active in the <0.5 μM Cu culture, and therefore this truncated form of NosZ maybe active, unless the full NosZ is present in the periplasmic fractions, but the concentration is below the detection limit of the Western blotted SDS PAGE gels. To synthesis NosZ under low Cu concentration and then degrade the polypeptide, seems energetically inefficient, however the RT PCR also confirms that *nosZ* is transcribed and so it suggests that Cu is not soley involved in the regulation of *nosZ*. It is not known whether the 50 kDa truncated form of NosZ is active, and can also bind Cu. Therefore further enzyme activity assays will have to be carried out. The synthesis of NosZ under low Cu concentrations indicates that if the Cu concentration was to increase then it could be inserted into NosZ, rendering an active NosZ.

Table 4-1 The top three hits from the MALDI-Mass Fingerprint of the purified NosZ identified using MASCOT

Hit	Accession number	Mass ppm	Protein Score	Description	Peptides Matched
1	**gi|2833444|sp|Q51705.1|NOSZ_PARDE**	71938	69	Full Nitrous-oxide reductase from *Paracoccus denitrificans*(immature polypeptide)	NDPMWAETER; NDPMWAETR +M; LSPTATVLDVTR; LDVHYQPGHLK; GNAYTSLFDSQVVK; YIPIANNPHGCNMAPDK +M; VYMSSVAPSFSIESFTVK; SAVVAEPELGLGPLHTAFDGR
2	**gi|76058410|emb|CAG34680.1|**	42722	66	Nitrous-oxide reductase [uncultured bacterium](mature poly peptide)	WNIEEAIR; LSPTVTVLDVTK; FDALFYNNAEPR; GNAYTSLFLDSQVVK; SEMDHVVVFNIVEIEK; SAVVAEPELGLGPLHTAFDGR
3	**gi|15988211|pdb|1FWX|A**	66850	56	Chain A, Crystal Structure Of Nitrous Oxide Reductase from *Paracoccus denitrificans* (mature polypeptide)	NDPMWAETR; NDPMWAETR +M; LDVHYQPGHLK; GNAYTSLFLDSQVVK; YIPIANNPHGCNMAPDK +M; VYMSSVAPSFSIESFTVK; SAVVAEPELGLGPLHTAFDGR

+M = oxidation of Methionine

Protein score is -10*Log(P), where P is the probability that the observed match is a random event.
Protein scores greater than 33 are a significant match (p<0.05).

Mature polypeptide refers to the sequence without the signal peptide

Table 4-2 The top three hits from the MALDI-Mass Fingerprint of the truncated NosZ identified usng MASCOT

Hit	Accession number	Mass ppm	Protein Score	Description	Peptides Matched
1.	**gi|122890430|emb|CAM12795.1|**	38161	56	Nitrous-oxide reductase from *Rhizobiales* bacterium D5-25 (mature polypeptide)	YLFMNDK; WNIEEAIR*; LSPTVTVLDVTK*; GNAYTSLFLDSQVVK*; IHDNGDLHHVHMSFTEGK +M*; SAVVAEPELGLGPLHTAFDGR*
2.	**gi|76058410|emb|CAG34680.1|**	42722	39	Nitrous-oxide reductase [uncultured bacterium](mature polypeptide)	YLFMNDK; WNIEEAIR*; LSPTVTVLDVTK*; GNAYTSLFLDSQVVK*; SAVVAEPELGLGPLHTAFDGR*
3.	**gi|2833444|sp|Q51705.1|NOSZ_PARDE**	71938	31	Full Nitrous-oxide reductase from *Paracoccus denitrificans* (immature sequence)	ILGIPSMR*; FLFMNDK*; FLFMNDK +M, GNAYTSLFLDSQVVK +M*; IHDNGDLHHVHMSFTEGK*

+M = oxidation of Methionine

Protein score is -10*Log(P), where P is the probability that the observed match is a random event. Protein scores greater than 33 are a significant match ($p<0.05$).

Mature polypeptide refers to the sequence without the signal peptide

*** polypeptide sequences observed in the Pd1222 NosZ sequence (Figure 4.2-13).**

MESKQEKGLSRRALLGATAGGAAVAGAFGGRLALGPAALGLGTAGVATVAGS

Signal peptide

GAALAASGDGSVAPGQLDDYYGFWSSGQSGEMRILGIPSMRELMRVPVFNRC

N terminal domain

SATGWGQTNESLRIHERTMSERTKKFLAANGKRIHDNGDLHHVHMSFTEGKY

N terminal domain

DGRFLFMNDKANTRVARVRCDVMKCDAILEIPNAKGIHGLRPQKWPRSNYVFC

N terminal domain

NGEDETPLVNDGTNMEDVANYVNVFTAVDADKWEVAWQVLVSGNLDNCDA

N terminal domain

DYEGKWAFSTSYNSEKGMTLPEMTAAEMDHIVVFNIAEIEKAIAAGDYQELNG

N terminal domain

VKVVDGRKEASSLFTRYIPIANNPHGCNMAPDKKHLCVAGKLSPTVTVLDVTRF

N terminal domain

DAVFYENADPRSAVVAEPELGLGPLHTAFDGRGNAYTSLFLDSQVVKWNIEDAI

N terminal domain

RAYAGEKVDPIKDKLDVHYQPGHLKTVMGETLDATNDWLVCLSKFSKDRFLNV

N terminal domain

GPLKPENDQLIDISGDKMVLVHDGPTFAEPHDAIAVHPSILSDIKSVWDRNDPM

N terminal domain

WAETRAQAEADGVDIDNWTEEVIRDGNKVRVYMSSVAPSFSIESFTVKEGDEV

C terminal domain

TVIVTNLDEIDDLTHGFTMGNYGVAMEIGPQMTSSVTFVAANPGVYWYYCQ

C terminal domain

WFCHALHMEMRGRMLVEPKEA

C terminal domain

Figure 4.2-13 Polypeptide sequence of *P.denitrificans* (Pd1222) NosZ indicating the N and C terminal domains. Polypeptide sequences attained from the 50 kDa MALDI Mass Fingerprint (Table 4-2) which correspond to *P.denitrificans* sequences are highlighted in red, and from the *Rhizobiales* bacterium and uncultured sequences in blue.

4.3 **Discussion**

This chapter has established that even under low Cu concentrations *nosZ* is transcribed and translated. *P.denitrificans* is capable of reducing N_2O to N_2 when grown in NO_3^- sufficient <0.5 µM Cu *Paracoccus* media, even though full length NosZ was not detected in the periplasmic fraction. Thus the complete NosZ polypeptide maybe below the detection level of the Western-blotted SDS PAGE gel probed with anti NosZ Abs, or the truncated NosZ can reduce N_2O to N_2.

It has been determined that the 50 kDa band isolated represents a degraded/truncated form of NosZ, more specifically the N terminal which contains the tetranuclear site Cu_Z. Whether this degradation occurs in the periplasm or cytoplasm is unclear, but it is able to be transported to the periplasm due to the twin argenine Tat signal sequence, which is located on the N terminus. The RT PCR data also supports this as *nosZ* is transcribed despite Cu concentration. Currently it is thought that the Cu centres of NosZ are inserted in the periplasm, and therefore the degradation would also occur in the periplasm since if the Cu_A site is not inserted then the C terminus of NosZ maybe degraded (Bothe, Ferguson et al. 2007).

The Cu concentration also had an effect on other denitrifying enzymes, directly or indirectly. Western-blotted SDS PAGE gels showed that there is a hierarchy to what protein Cu is inserted into. In the NO_3^--sufficient succinate-limited <0.5µM Cu periplasmic fractions a Paz was not detected and neither was a complete NosZ, but the N terminal was expressed which was expressed as a 50 kDa protein. *P.denitrificans* was capable of denitrifying without Paz present as the synthesis of c_{550} was increased. Therefore on a molecular level *P.denitrificans* can identify and regulate the expression of proteins in response to low Cu concentrations. However it is not known whether this is a direct response to Cu concentration or due to the redox potential of other enzymes or regulators in the respiratory pathway. It is also not known if the reduced concentration of Paz in the <0.5 µM Cu culture is due to the polypeptide being

degraded or the transcription is reduced. If the Cu centres are not inserted into Paz then the polypeptide could be targeted for degradation.

Previous work in *Pseudomonas pudita, nosA* is up regulated during low Cu anaerobic, and was thought to be involved in the maturation and insertion of the Cu centres in NosZ. However mutants in *nosA* showed no change in NosZ activity (Wunsch, Herb et al. 2003). It was therefore thought that the Sco proteins may be involved with Cu insertion, however Sco mutant strains also showed no significant change in NosZ activity. Therefore potentially both NosA and Sco are involved in Cu insertion into NosZ, and potentially can compensate for each other if one is absent.

The isolation of the truncated NosZ showed that even under Cu limitation *nosZ* is transcribed and translated. This may seem to be inefficient of the bacteria to synthesise NosZ if it is going to be degraded, however it is still capable of reducing N_2O. The possible degradation of the C terminus of NosZ could be to release, if any, Cu that may be bound, therefore releasing Cu for other proteins and possibly the Cu_z centre. It is also plausible that the complete NosZ is synthesised, however it was at an undetectable level. It may also be that the truncated NosZ is catalytically active. The Cu_A binuclear site is essential for e^- transport to the Cu_Z in the NosZ dimer. In *Wollinella succinogens* the *nosZ* gene also encodes a *c*-type cytochrome (Simon, Einsle et al. 2004), this brought about the question, is a *c*-tye cytochrome capable of direct e^- transfer to the Cu_Z site that resides in the β-propeller? In *P.denitrificans* is it therefore possible that c_{550} can transfer e^- to the Cu_z centre. Further work must now focus on purifying the “truncated NosZ” from the Cu limited cultures on a large scale and characterising the properties of the polypeptide. The polypeptide may be loaded with Cu and therefore the Cu_z site may have been inserted correctly, and therefore capable of reducing N_2O with a direct e^- donor, such as c_{550}.

Chapter 5: Characterisation of a *P.denitrificans nosZ* mutant

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5.3 **Discussion** **150**

5.1 **Introduction**

This current research has shown that *nosZ* is transcribed during aerobic respiration. However it was previously thought that the *nos* genes were regulated by FNR, therefore when O_2 is present FNR would be inactive and so transcription of the *nos* operon would not occur. The expression of NosZ has been found at low levels in *Pseudomonas stutzeri* in aerobic cultures, and so it is therefore thought not be completely dependent on FNR regulation (Cuypers, Viebrock-Sambale et al. 1992). It was also observed in Chapter 4 that NosZ is transcribed in aerobic cultures of *P.denitrificans*, the expression was increased under NO_3^- and Cu sufficient anoxic conditions (Figure 4.2-9). NosR is also thought to play a role in NosZ synthesis. In the majority of cases *nosR* is located upstream from *nosZ* in the *nos* operon. NosR in *P.stutzeri* contains 5 transmembrane helices. The C terminal contains cysteine residues thought to bind [4Fe-4S] clusters and is present on the cytoplasmic side of the membrane. NosR shows homology to the NirI proteins which are involved in the transcription activation of the *nir* operon and also NosR from *P.denitrificans* (Cuypers, Viebrock-Sambale et al. 1992; Saunders, Houben et al. 1999; Saunders, Hornberg et al. 2000; Wunsch and Zumft 2005). However NosR from *P.denitrificans* is predicted to contain 6 transmembrane helices and the C terminus contains 10 cysteine residues, 8 of which are predicted to bind 2[4Fe-4S] clusters (Saunders, Hornberg et al. 2000). The complete transcription of NosR is vital for NosZ activity, in NosR knock out mutant strains of *P.stutzeri* the NosZ polypeptide is synthesised but the activity is completely inhibited (Wunsch and Zumft 2005). NosR may therefore play an important role in the maturation of NosZ, NosA is thought to be involved in the insertion of the Cu centres, however NosA knockouts still synthesise active NosZ polypeptide in *Pseudomonas pudita* (Wunsch, Herb et al. 2003). This Chapter will investigate how knocking out *nosZ* and *nosR* genes affects aerobic and anaerobic respiration in *P.denitrificans.*

5.2 Results: The growth of *P.denitrificans nosZ* knockout mutant.

NosZ is responsible for the reduction of N_2O to N_2, which occurs in the periplasm of *P.denitrificans.* The previous section discusses how Cu concentration affects the denitrification pathway. Chemostat cultures of *P.denitrificans* grown in <0.5 µM Cu accumulated 2 mM of N_2O when in steady state and the specific rate of production was 5000 fold higher than the 18 µM Cu cultures. However, despite the culture being Cu limited, NosZ activity still occurred, but at a reduced level. Therefore to investigate how the synthesis of the denitrification proteins is affected by the absence of NosZ, chemostat cultures of a *nosZ* knockout mutant were carried out in 18 µM Cu. Therefore it can be determined whether the Cu concentration affects the synthesis of denitrification proteins, or if the absence of a functional NosZ.

Pd10221 is a *P.denitrificans nosZ* knockout mutant, chemostat cultures of Pd10221 can therefore be compared to the <0.5 µM Cu Pd1222 chemostat cultures to observe if N_2O accumulates in a similar manor. N_2O reduction in the <0.5 µM Cu cultures is reduced compared to the 18 µM Cu cultures, and so it was therefore hypothesised that the Pd10221 chemostat cultures would act similarly. The *nosZ* mutant was obtained from Rob van Spanning, University of Amsterdam. The mutant was constructed by inserting a kanamycin resistance marker (Km^R) into the *nosZ* gene, rendering the NosZ protein inactive.

5.2.1 The growth of *P.denitrificans nosZ* mutant in batch cultures

To ensure that the strain grown was the mutant, LB agar plates containing 25 µg/ml rifampacin (Rif) and 40 µg/ml kanamycin (Km), were used to subculture the mutant. As a control and to check for contamination Pd10221 was streaked onto LB plates containing 40 µg/ml ampacillin (Amp) or 40 µg/ml chloramphenicol (Cm), no growth was seen suggesting the Pd10221 culture was indeed correct and there were no contaminants, which were Cm resistant.

Pd10221 was grown in NO_3^- sufficient (20 mM), succinate limited (5 mM) *Paracoccus* minimal media with 18 µM Cu. The Pd10221 culture had a lower final OD_{600} compared to the Pd1222 culture.

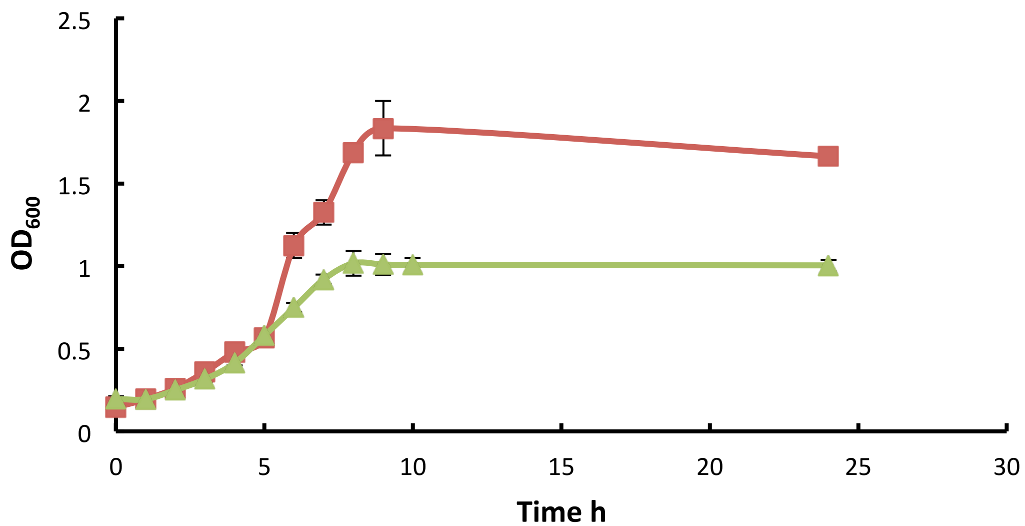


Figure 5.2-1 Anaerobic batch culture of wild type *P.denitrificans*, Pd1222 (■) and NosZ knockout (▲) grown in NO_3^- -sufficient succinate-limited 18 µM Cu *Paracoccus* medium. The batch cultures were repeated three times for each conditions, the error bars represent the standard error.

5.2.2 Chemostat cultures of *P.denitrificans* NosZ mutant grown in NO_3^- -sufficient and succinate limited medium.

The previous <0.5 µM Cu chemostats show that the synthesis of NosZ is partially inhibited as N_2O accumulates but some N_2O is reduced to N_2. Chemostat cultures of Pd10221 were run at the same parameters as the previous *P.denitrificans* cultures in NO_3^- -sufficient succinate-limited *Paracoccus* minimal media with 18 µM Cu. It was hypothesised that for Pd10221 the chemostat culture would act similarly to <0.5 µM Cu Pd1222 culture, but due to NosZ being completely knocked out, the N_2O would account for 100 % of the total consumed NO_3^-.

5.2.2.1 Drymass generated in chemostat cultures of Pd10221

The Pd10221 chemostat was run at the same D as determined in Chapter 3 (D = 0.06 h^{-1}). The first 24 h of growth was an aerobic batch culture, there was a slight difference in growth between Pd10221 culture and the Pd1222 <0.5 µM Cu culture (Figure 5.2-2). After 24 h of aerobic growth the Pd10221 culture reached 0.21 ± 0.03 mg/ml, which is 20 % lower than the growth seen at 24 h of the <0.5 µM Cu culture. After 24 h the air supply was removed and both cultures became anaerobic within 30 min. At 24 h the feed was also started and a drop in drymass, which was observed in both cultures, between 24-70 h. The cultures entered steady state at approximately 70 h, where 3 vessel volumes of feed have passed through the vessel. The Pd10221 culture showed no significant difference in the final drymass compared to the <0.5 µM Cu culture and the 18 µM Cu *P.denitrificans* cultures, this is also seen in the growth yield (Table 5-1). This therefore confirms that NosZ is not essential for the *P.denitrificans* denitrification pathway. There are bacteria that are denitrifiers that do not possess NosZ such as *Agrobacterium tumefaciens*, however they are still classed as denitrifiers as they posses nitrate, nitrite and nitric oxide reductases.

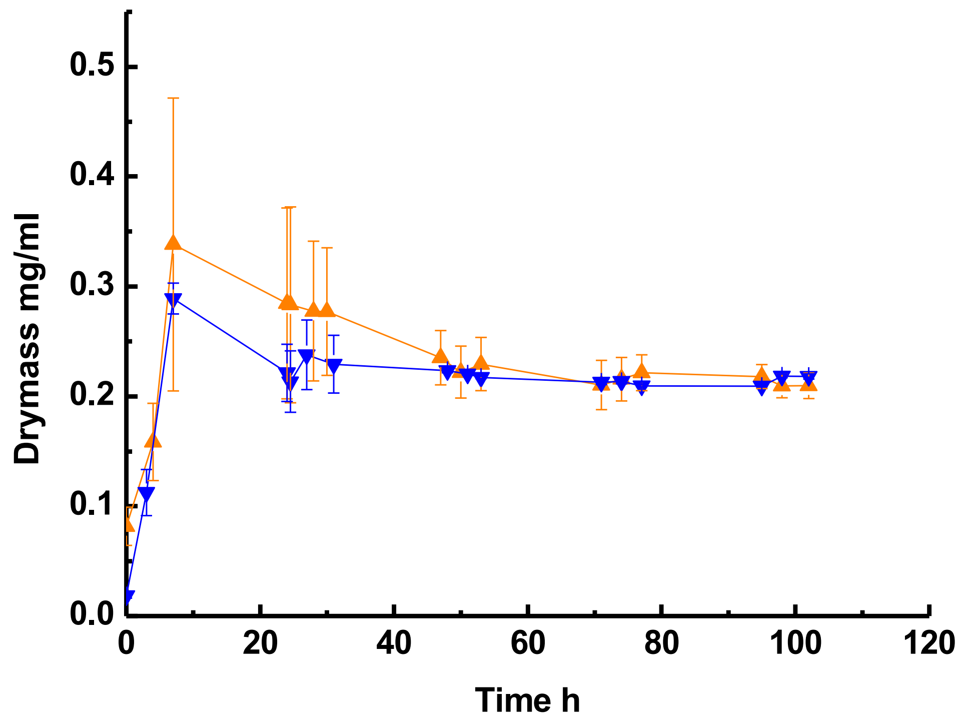


Figure 5.2-2 The drymass of Pd10221 (▼) grown in NO_3^- -sufficient succinate-limited 18 µM Cu *Paracoccus* medium and the WT *P.denitrificans*, Pd1222, grown in NO_3^- -sufficient succinate-limited <0.5 µM Cu Paracoccus minimal medium (▲). At 24 h the air supply was removed and the feed was added (D = 0.06 h^{-1}).

5.2.2.2 Nitrate consumption in chemostat cultures of Pd10221

The first 24 h of growth in chemostat cultures is aerobic, there was little to no NO_3^- consumption during the aerobic phase, consumption began in both cultures as soon as the cultures had turned anaerobic at approximately 24.5 h (Figure 5.2-3). The NO_3^- concentration continually decreases throughout the chemostat run in both the Pd10221 culture and the <0.5 µM Cu culture. At steady state the total net of NO_3^- consumed was 12.7 ± 0.5 mM, which is similar to that consumed in the Pd1222 <0.5 µM Cu culture. The total net of NO_3^- consumed in the Pd10221 and <0.5 µM Cu cultures is significantly more than that consumed in the Pd1222 18 µM Cu culture, which was 7.5 ± 0.3 mM. This is increase in NO_3^- consumption is potentially due to the decreased capacity to use the electron acceptor N_2O. Therefore as to increase the amount of e^- acceptor available an increase in NO_3^- consumption is required. The reduction of NO_3^- to N_2O requires 4 e^- per NO_3^-, compared to 5 e^- in the reduction of NO_3^- to N_2, therefore a 30 % increase in NO_3^- consumption compensates for the 20 % decrease in e^- available in the Pd10221 and <0.5 µM Cu cultures.

The specific rate of NO_3^- consumption, $qcNO_3^-$, in the Pd10221 culture and the wild type (WT) <0.5 µM Cu culture shows no significant difference (Table 5-1). The total net consumption of NO_3^- also shows no significant difference, with 12.07 ± 0.5 mM of NO_3^- being consumed in the Pd10221 culture and 14.3 ± 2.2 mM in the <0.5 µM Cu. The NO_3^- consumed in the 18 µM Cu cultures (Chapter 3) is approximately 30 % less than in the <0.5 µM Cu and Pd10221 cultures.

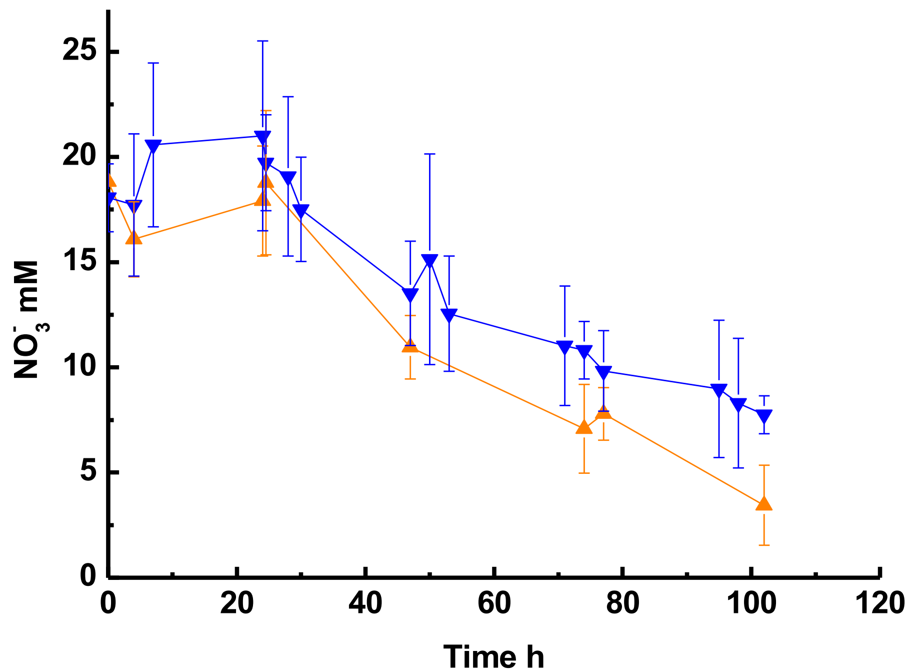


Figure 5.2-3 The consumption of NO_3^- in chemostat cultures of Pd10221 (▼) grown in 18 µM Cu *Paracoccus* minimal media (NO_3^- -sufficient succinate-limited) and the WT *P.denitrificans,* Pd1222 grown in <0.5 µM Cu *Paracoccus* minimal media (NO_3^- -sufficient succinate-limited) (▲).At 24 h the air supply was removed and the feed was added (D= 0.06 h^{-1}). Each sample isolated from the chemostat cultures was analysed twice, the error bars represent the standard error.

5.2.2.3 Nitrite production kinetics in chemostat cultures of Pd10221

In Chapter 3 it was observed that in Pd1222 cultures NO_2^- did not accumulate regardless of the Cu concentration. During the first 24 h there was little to no production or consumption of NO_2^-, ruling out aerobic denitrification. After the vessel became anaerobic at 24.5 h the Pd10221 culture produced NO_2^-, this peak could correspond to a lag phase seen in the synthesis of cyt cd_1, once the culture has turned anaerobic Reaction 1 is faster than Reaction 2, causing an accumulation of NO_2^-, between 30 -70 h Reactions 1 and 2 are matched:

$$2NO_3^- + 4e^- + 4H^+ \rightarrow 2NO_2^- + 2H_2O \quad \text{(Reaction 1)}$$

$$2NO_2^- + 2e^- + 4H^+ \rightarrow 2NO + 2H_2O \quad \text{(Reaction 2)}$$

From 72 h onwards the Pd10221 culture has entered steady state and reaction 1 becomes faster than reaction 2, therefore NO_3^- reduction is faster than NO_2^- reduction. In the Pd1222 <0.5 µM Cu chemostat the total net production of NO_2^- was 0.07 ± 0.003 mM at steady state (Figure 3.2-9). However the NO_2^- detected in the bioreactor for the Pd10221 culture reached 10.08 ± 0.06 mM at steady state (Figure 5.2-4), over 1000 times more than previously seen in any *P.denitrificans* chemostats, and it accounts for 77 ± 4.7 % of the total NO_3^- consumed.

The specific rate of NO_2^- production ($qpNO_2^-$) shows a significant increase in the Pd10221 culture, compared to the 18 µM Cu and < 0.5 µM Cu Pd1222 cultures. This increase in $qpNO_2^-$ could be due to a decrease in the synthesis of cyt cd_1 which would result in a lower NO_2^- reductase activity (See section 5.2.3.4). The net consumption of NO_2^- ($qcNO_2^-$, Table 5-1) is 60 % lower in the Pd10221 culture compared to the Pd1222 <0.5 µM Cu culture. Therefore the flux of N through the Pd10221 culture is lower.

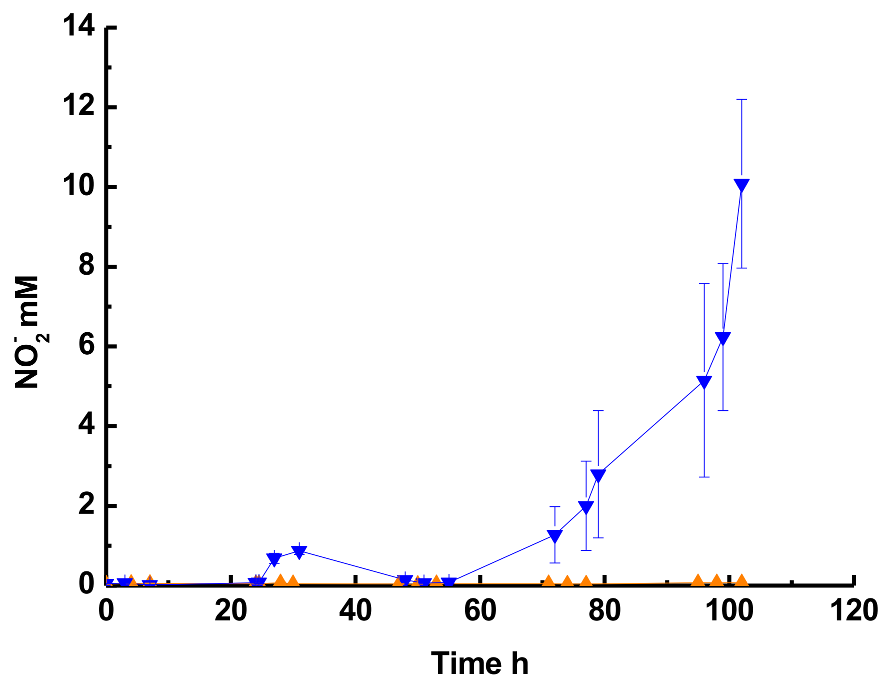
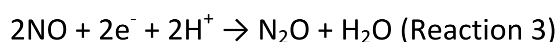


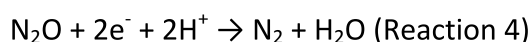
Figure 5.2-4 The accumulation of NO_2^- in chemostat cultures of Pd10221 (▼) grown in 18 µM Cu *Paracoccus* minimal media (NO_3^- -sufficient succinate-limited) and the WT *P.denitrificans,* Pd1222 grown in <0.5 µM Cu *Paracoccus* minimal media (NO_3^- -sufficient succinate-limited) (▲).At 24 h the air supply was removed and the feed was added (D= 0.06 h^{-1}). Each sample isolated from the chemostat cultures was analysed twice, the error bars represent the standard error.

5.2.2.4 Nitrous Oxide production in chemostat cultures of Pd10221

In Chapter 3 it was observed that as Cu became limited the production of N_2O increased, and this was due to a reduced amount of the full NosZ polypeptide being synthesised. The Pd10221 culture has a Km^R marker inserted into *nosZ*, therefore the gene is terminated early, which renders the polypeptide inactive. In the Pd10221 chemostat culture there was little to no production or consumption of N_2O during the aerobic phase (Figure 5.2-5). The accumulation of N_2O occurs differently in the Pd10221 culture and the Pd1222 <0.5 µM Cu culture. In the Pd10221 culture N_2O production occurred immediately after the culture became anaerobic, this is due to the lack of active NosZ. N_2O production began in the Pd1222 <0.5 µM Cu after 50 h. Therefore up until 50 h in the <0.5 µM Cu culture reaction 3 and 4 are matched, and therefore there is little to no N_2O produced, as reaction 4 becomes slower, N_2O begins to accumulate, this is observed after 50 h (Figure 5.2-5):



$2NO + 2e^- + 2H^+ \rightarrow N_2O + H_2O$ (Reaction 3)



$N_2O + 2e^- + 2H^+ \rightarrow N_2 + H_2O$ (Reaction 4)

The lag in N_2O accumulation in the Pd1222 <0.5 µM Cu culture suggests that there is active NosZ present. In the Pd10221 culture reaction 3 is the final step of denitrification, as reaction 4 is absent, and so once NO_3^- consumption begins N_2O is produced, this also occurs in the Pd1222 18 µM Cu culture, but to a lesser extent (Figure 5.2-5 and Figure 3.2-10 B). NO_3^- consumption began at 24.5 h, by which time the N_2O concentration in the bioreactor had doubled to 0.35 ± 0.02 µM, and increased to 7.8 ± 3.5 µM by 28 h. The total net of N_2O produced in the bioreactor was 1.7 ± 0.2 mM, which is approximately 20 % less than that observed in the <0.5 µM Cu culture.

The specific rate of N_2O production, qpN_2O, in the Pd10221 culture is approximately 30 % lower in the Pd10221 culture than the Pd1222 <0.5 µM Cu culture (Table 5-1). Both the Pd10221 culture and the Pd1222 <0.5 µM Cu culture have a significantly higher qpN_2O compared to the Pd1222 18 µM Cu culture. Due to the ‘loss’ of ability to use the electron acceptor N_2O, there is an increase in the $qcNO_3^-$ which therefore increases the amount of electron acceptors available.

Even though the total net N_2O produced was 20 % less in the Pd10221 culture, the N_2O concentration in the bioreactor accounts for 31 ± 7 % of the total net consumption of NO_3^-. The total NO_3^- accounted for therefore equates to approximately 100 %, with 31 % converted to N_2O, and 77 % converted to NO_2^-. This is also illustrated by the flux of N through the bioreactor, $qcNO_2^-$, is nearly matched by the qpN_2O, suggesting that all NO_2^- consumed is reduced to N_2O (Table 5-1). The qpN_2O does not exactly match the $qcNO_2^-$ due to standard error in the measurements of NO_2^- and N_2O. Due to 100 % of the converted NO_3^- being reduced to NO_2^- and N_2O, N_2 was not monitored. The absence of a functional NosZ polypeptide has affected the synthesis of other denitrifying proteins as a means of maximising the amount of electron acceptor available. The following section investigates the synthesis and expression of the proteins involved in denitrification.

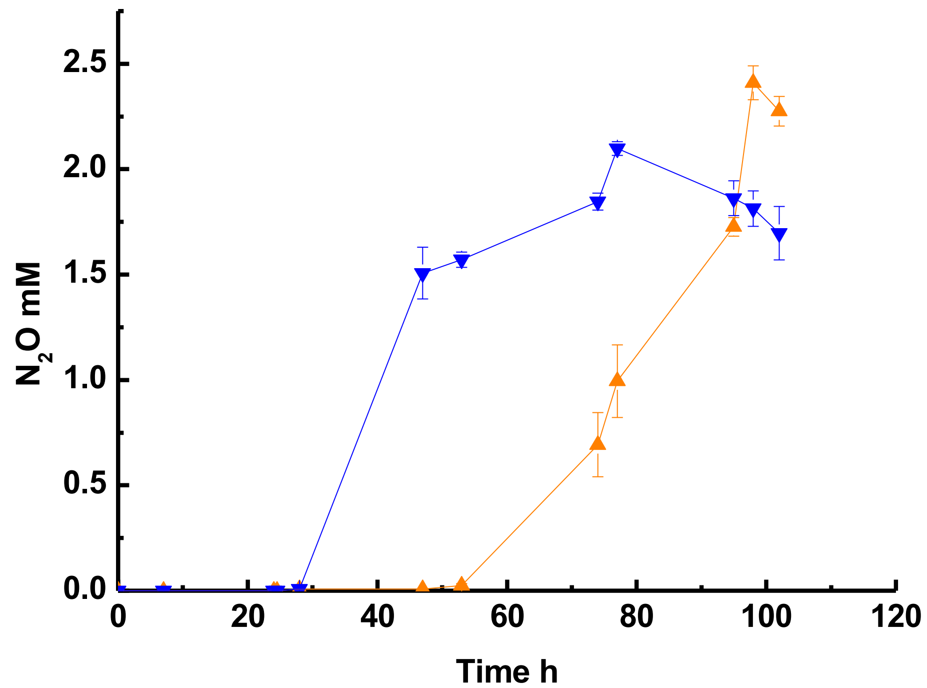


Figure 5.2-5 The accumulation of N_2O in chemostat cultures of Pd10221 (▼) grown in 18 µM Cu *Paracoccus* minimal media (NO_3^- -sufficient succinate-limited) and the WT *P.denitrificans,* Pd1222 grown in <0.5 µM Cu *Paracoccus* minimal media (NO_3^- -sufficient succinate-limited) (▲).At 24 h the air supply was removed and the feed was added (D= 0.06 h^{-1}). Each gas sample taken from the chemostat cultures was analysed twice, the error bars represent the standard error.

Table 5-1 Productivity rates for NO_3^- consumptions and NO_2^-, N_2O and N_2 accumulation of *P.denitrificans* wild types and *nosZ* knockout (Pd10221) chemostat cultures at steady state (102h). NO_3^- and NO_2^- reductase assays were carried out in steady state.

	Growth Yield	qcNO$_3^-$	qpNO$_2^-$	qcNO$_2^-$	qpN$_2$O
	mg.ml.h^{-1}	mmol.g drymass^{-1} h^{-1}			
Cu 18 µM	0.011 ±0.000	2.010 ±0.098	-0.006 ±0.001	2.016	0.0001 ±0.000
Cu <0.5 µM	0.011 ±0.000	3.166 ±.790	0.009 ±0.005	3.275	0.527 ±0.007
Pd10221	0.013 ±0.000	2.83 ±0.153	1.50 ±0.746	1.33	0.377 ±0.041

Growth Yield -biomass production = Dx; where x = the biomass at steady-state.

NO_3^- consumption $(qcNO_3^-) = ([NO_3^-]_R - [NO_3^-]_r) D / x$; where [$NO_3^-$]R is the NO_3^- concentration in the reservoir, [NO_3^-]r is the residual NO_3^- concentration in the reactor vessel, D is the dilution rate and x is the drymass concentration in the reactor vessel.

NO_2^- production $(qp\ NO_2^-) = [NO_2^-]\, r\, D / x$ where [NO2-]r is the NO_2^- concentration in the reactor vessel – the initial/background NO_2^- concentration

NO_2^- consumption $(qc\ NO_2^-) = qcNO_3^- - qp\ NO_2^-$

N_2O production $(qp\ \ N_2O) = [N_2O]\, r\, D / x$; where [$N_2O$]r is the N_2O concentration in the reactor vessel – the initial/background N_2O concentration

' - 'indicates net consumption

5.2.3 Detection of Denitrification enzymes in the NosZ mutant of *P.denitrificans* (Pd10221) chemostat cultures.

5.2.3.1 Pseudoazurin

The *nos* operon is arranged in *P.denitrificans* as *paz nosCRZDFYL,* the *paz* gene is transcribed in the opposite direction to the *nos* gene cluster. Even though *paz* is close to the *nos* operon it was expected that the transcription of *paz* as well as *nosC* and *nosR* would not be affected by the Km^R inserted into *nosZ*. Therefore the presence of Paz in the periplasm of Pd10221 should be similar to Pd1222 18 µM Cu chemostat culture. A Western-blotted SDS PAGE gel probed with anti Paz Abs shows that there was indeed no significant difference in the band intensity of Paz between the 18 µM Cu and Pd10221 periplasmic fractions (Figure 5.2-6). Therefore transcription of *paz* continued as wild type despite *nosZ* being knocked out. This confirms that the synthesis of Paz is directly influenced by the Cu concentration, however it is not known whether this is at a transcriptional or protein level.

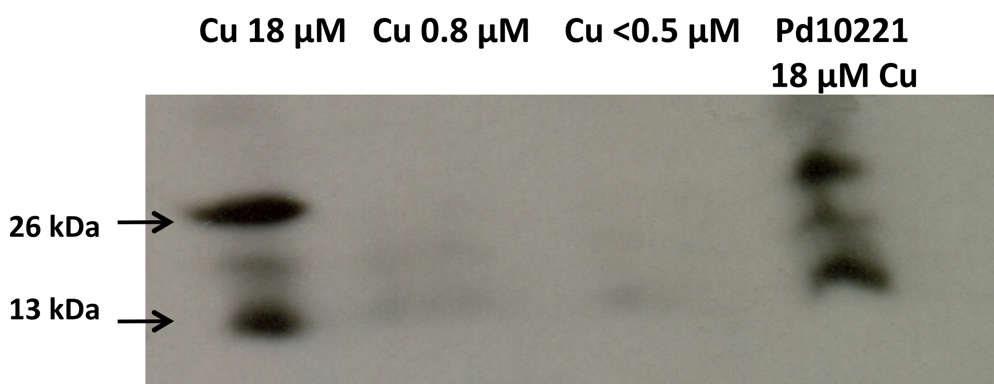


Figure 5.2-6 Western-blotted SDS-PAGE gels probed with Abs raised against P.denitrificans Paz from periplasmic fractions (100 µg of protein loaded per well) of NO_3^- -sufficient succinate-limited Pd1222 18 µM Cu, 0.8 µM Cu or <0.5 µM Cu and Pd10221 chemostat cultures harvested at steady state. The two bands at 26 kDa and13 kDa represent the dimeric and monomeric form of Paz, respectively.

5.2.3.2 Cytochrome c_{550}

The transcriptional regulator for c_{550} is currently unknown, however in low Cu and NO_3^- -sufficient succinate-limited the expression of c_{550} is significantly increased, whether this is a direct response of the Cu concentration is also unknown (Figure 4.2-2). In Pd10221 chemostat culture the haem stained SDS gel indicates that c_{550} is expressed at a similar concentration to that of the Pd1222 18 µM Cu chemostat culture. When the NO_3^- was limited the expression was generally lower and the expression of c_{550} was not significantly elevated under <0.5 µM Cu. Therefore the expression of c_{550} is more complex than originally thought; the regulation of c_{550} is dependent on the NO_3^- concentration as well as Cu concentration, direct or indirectly.

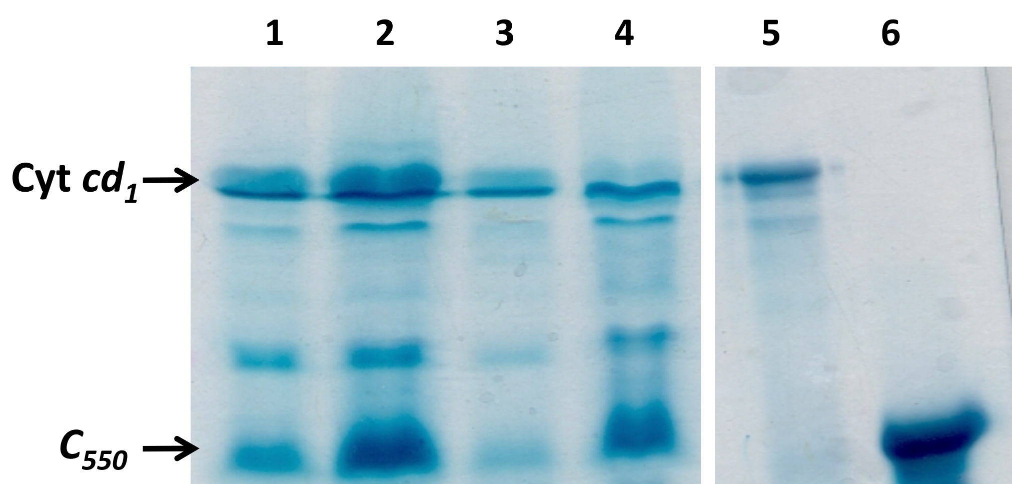


Figure 5.2-7 A haem stained SDS gel loaded with periplasmic fractions (100 µg of protein per well) isolated from *P.denitrificans* chemostat cultures grown in NO_3^- -sufficient succinate-limited media with either 18 µM Cu (lane 1), 0.8 µM Cu (lane 2), <0.5 µM Cu (lane 3). Pd10221 was grown in NO_3^- -sufficient succinate-limited media with 18 µM Cu (lane 4). Purified cyt cd_1 , 20 µg (lane 5) and 30 µg of c_{550} (lane 6) were loaded as controls.

5.2.3.3 Nitrate reductase

The membrane bound NO_3^- reductase, NarGHI, was identified using Western-blotted SDS-PAGE gels probed with Abs raised against NarGH , two bands were observed at ~100 kDa and 60 kDa which represent NarG and NarH, respectively, the previous Western blotted SDS PAGE gel against NarGH in the Pd1222 chemostat culture, only the NarG polypeptide was detected (Figure 4.3-3). The synthesis of the NarGH in Pd10221 is similar to that of the Pd1222 18µM Cu culture. The <0.5 µM Cu membrane fraction only the NarG band is present, however NarH must be present, but at a lower level due to NarGHI dependent NO_3^- reduction still occurs in the purified membrane fraction.

The specific rate of NO_3^- reductase activity was monitored at steady state in the Pd10221 culture. The specific cell NO_3^- activity reached 24 ± 3 $nmol.min^{-1}mg\ drymass^{-1}$, which shows no significant difference compared to the activity in the Pd1222 18 µM and <0.5 µM Cu chemostat cultures (Figure 5.2-9 A), however the membrane fraction showed a 2 fold increase in the Pd10221 culture to 124 ± 9 $nmol.min^{-1}mg\ drymass^{-1}$ (Figure 5.2-9 B). This increase in activity in the membrane fraction could potentially be due to the Nar system being upregulated in the Pd10221 culture, NarR is involved in regulating the transcription of the *nar* genes, the presence of NO_3^- is required to activate transcription. Another possibility could potentially be the amount of NO_3^- imported into the cell, via the NarK1/K2 systems, being limited.

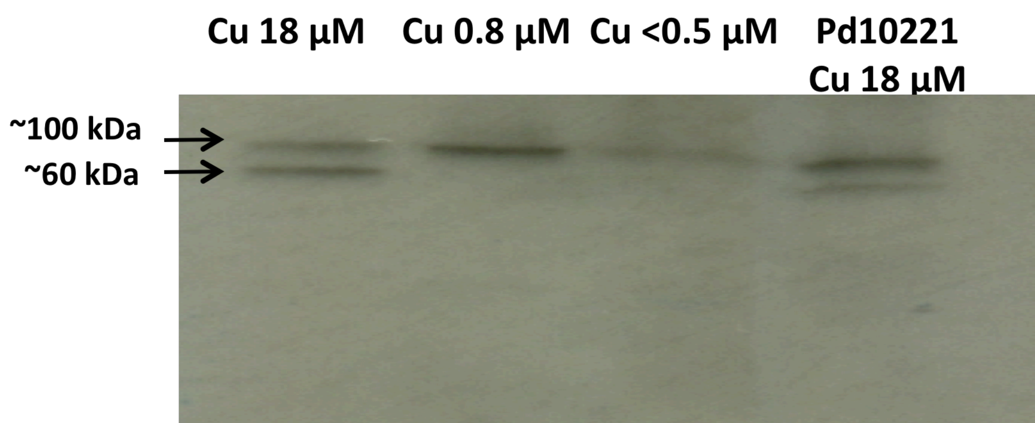


Figure 5.2-8 Western-blotted SDS-PAGE gels probed with Abs raised against *P.denitrificans* NarGH from membrane fractions (100 μg protein loaded per well) isolated from the Pd1222 18 μM Cu, 0.8 μM Cu, <0.5 μM Cu and Pd10221 18 μM Cu chemostat cultures harvested at steady state. The 100 kDa band represents the NarG subunit (actual weight 140 kDa), and the 60 kDa band represent the NarH subunit. The membrane fractions were isolated at steady state. The control contains approximately 0.2 μg of purified NarGH.

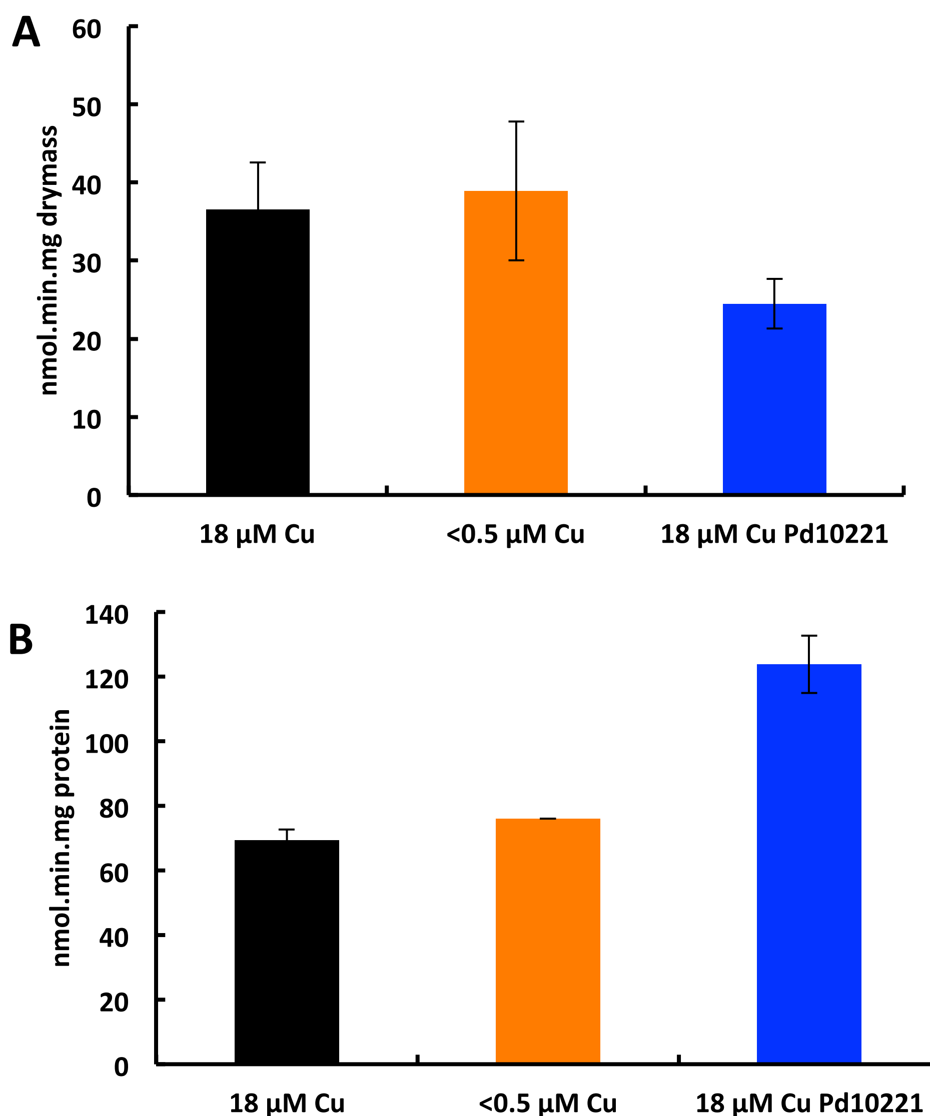


Figure 5.2-9 The specific activity of NO_3^- reductase measured in whole cell assays (A) and membrane fractions (B) isolated at steady state from chemostat cultures of *P.denitrificans* grown in NO_3^- -sufficient succinate-limited *Paracoccus* media with either 18 µM Cu (black) or <0.5 µM Cu (orange) and Pd10221 18 µM Cu culture (blue). Each sample isolated from the chemostat cultures was analysed twice, the error bars represent the standard error.

5.2.3.4 Nitrite reductase

In the Pd10221 chemostat culture, NO_2^- was found to accumulate to 10.2 ± 0.1 mM, 100 times more than observed in previous Pd1222 chemostat cultures. A Western-blotted SDS-PAGE gels probed with Abs raised against cyt *cd_1* showed that cyt *cd_1* was present in the periplasmic fractions of the Pd1222 18 µM and <0.5 µM Cu cultures and the Pd10221 cultures. The expression of cyt *cd_1* was elevated in the <0.5 µM Cu cultures, compared to the Pd1222 18 µM Cu culture (Figure 5.2-10). In the Western-blotted SDS-PAGE gel the presence in cyt *cd_1* in the Pd10221 periplasmic fraction is comparable to the 18 µM Cu chemostat culture, the haem stained SDS PAGE gel also indicates that the concentration of cyt *cd_1* in the periplasm is comparable in the Pd10221 and Pd1222 <0.5 µM Cu cultures. The fact that NO_2^- accumulates in the Pd10221 implies that the activity of cyt *cd_1* is decreased. Figure 5.2-10 also shows that a 48 kDa band in present in the Pd10221 periplasmic fraction. In previous Pd1222 cultures the 48 kDa band is only present in the NO_3^- sufficient succinate limited <0.5 µM Cu culture, this band is most likely to be the haem *d_1* binding domain, produced after the enzyme is proteolysed (Horowitz, Muhoberac et al. 1982). This also suggests that in the Pd10221 culture the d_1 haem is not properly being inserted, hence the degradation of the protein in the low Cu cultures.

The specific rate of cyt *cd_1* activity in the Pd10221 culture at steady state is 38.5 ± 9.5 $nmol.min^{-1}mg\ drymass^{-1}$, which is marginally lower compared to the Pd1222 18 µM and <0.5 µM Cu cultures. At steady state the NO_3^- reductase activity is 4 times higher in the Pd10221 culture compared to the <0.5 µM Cu culture, however the NO_2^- reductase activity fell slightly in the Pd10221 culture. The difference in the activity of cyt *cd_1* between the Pd10221 culture and the Pd1222 18 µM Cu culture suggests that there is a regulatory system involved in the synthesis of cyt *cd_1*, such as NNR. It also suggests that the activity of cyt *cd_1* polypeptide in the <0.5 µM Cu culture is lower than the Pd10221 culture and 18 µM Cu culture, as the expression of cyt *cd_1* Is significantly increased (Figure 5.2-10).

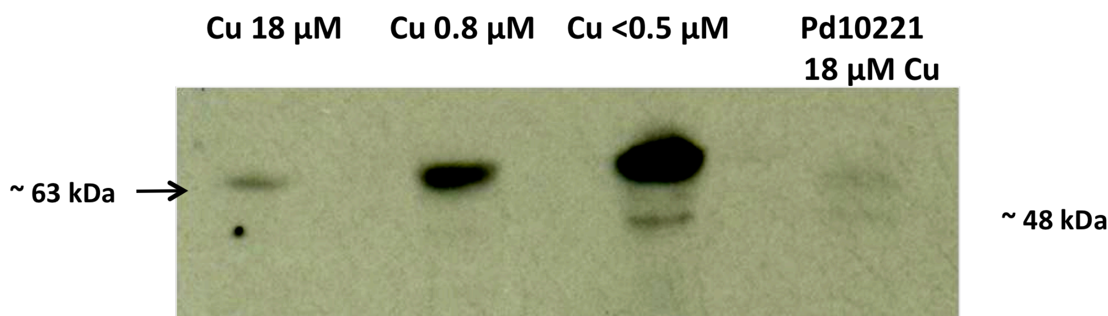


Figure 5.2-10 Western-blotted SDS-PAGE gels probed with Abs raised against *P.denitrificans* cyt *cd_1* containing periplasmic fractions (100 µg of protein loaded per well) isolated from the Pd1222 NO_3^- -sufficient succinate-limited 18 µM Cu, 0.8 µM Cu, <0.5 µM Cu and Pd10221 18 µM Cu chemostat cultures at steady state. The bands present at 63 kDa are indicative of cyt *cd_1*. For the complete haem stained SDS PAGE gel see Figure 5.2-7.

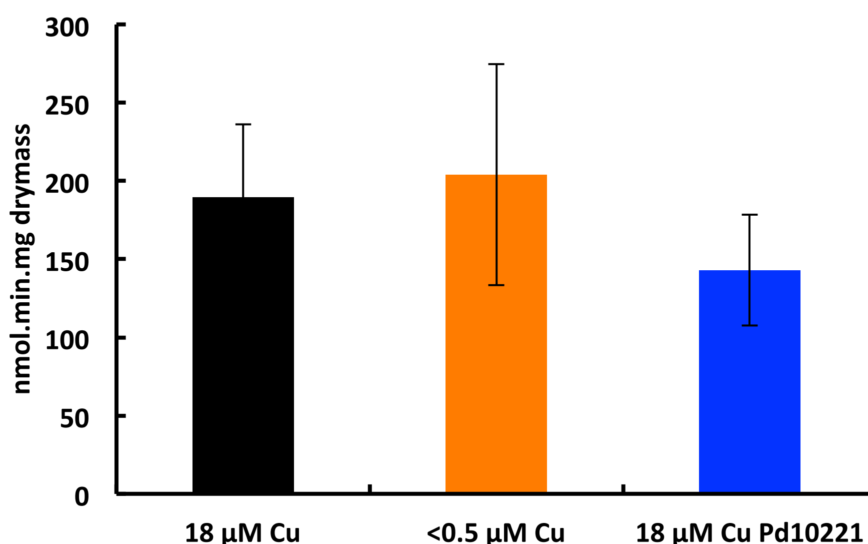


Figure 5.2-11 The specific activity of NO_2^- reductase measured in whole cell assays isolated at steady state from chemostat cultures of *P.denitrificans* grown in NO_3^- sufficient *Paracoccus* media with either Pd1222 18 µM Cu (black) or <0.5 µM Cu (orange) and Pd10221 18 µM Cu culture (blue). Each sample isolated from the chemostat cultures was analysed twice, the error bars represent the standard error.

5.2.3.5 Nitric oxide reductase

Throughout the Pd10221 culture NO was unable to be detected, which was the case for all the Pd1222 chemostat cultures, suggesting that the Nor system was unaffected by Cu concentration and the absence of NosZ. A Western-blotted SDS-PAGE gels probed with Abs raised against NorB indicate that NorB is present in the membrane fractions of Pd1222 18 µM and <0.5 µM Cu and the Pd10221 cultures (Figure 5.2-12). The presence of NorB was marginally increased in the Pd1222 <0.5 µM Cu culture compared to the Pd1222 18 µM Cu and Pd10221 culture which show a similar band intensity, implying a similar concentration of the NorB (Figure 5.2-12).

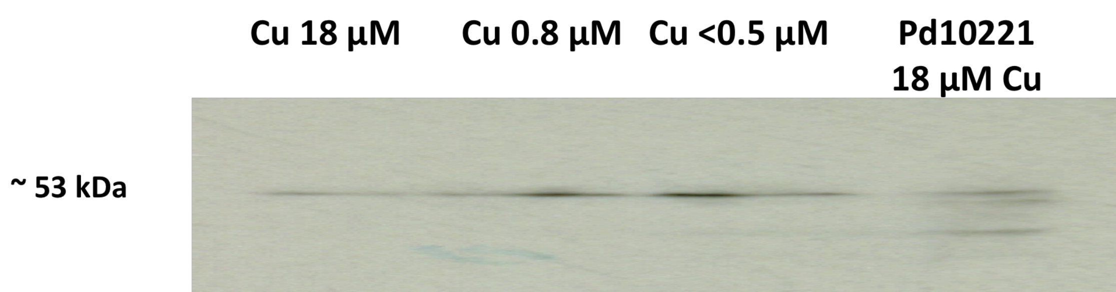


Figure 5.2-12 A Western-blotted SDS-PAGE gels probed with Abs raised against *P.denitrificans* NorB from membrane fractions (100 µg of protein loaded per well) isolated from the NO_3^- -sufficient succinate-limited Pd1222 18 µM Cu, 0.8 µM Cu, <0.5 µM Cu and Pd10221 18 µM Cu chemostat cultures harvested at steady state.

5.2.4 The synthesis of NosZ in chemostat cultures of *P.denitrificans* and Pd10221

5.2.4.1 Nitrous oxide reductase Pd10221

By inserting a Km^R marker into *nosZ* the transcription of the gene is interrupted by the Km^R cassette and transcription is terminated early due to stop codons at the end of the cassette. Therefore the enzyme is rendered inactive as the complete *nosZ* not being transcribed. A Western-blotted SDS-PAGE gel probed with Abs raised against NosZ indicated that there was no 66 kDa band present in the Pd10221 periplasmic fraction, indicating that no fully functional NosZ polypeptide is present. However the Pd10221 periplasmic fraction showed a band of similar mass to the band observed in the <0.5 µM Cu periplasmic fraction, 50 kDa, however the band observed in the Pd10221 culture did run slower, suggesting its mass was higher than 50 kDa (Figure 5.2-13). Primarily this band was thought to be a cross reaction with the anti NosZ Abs and another polypeptide, possibly lysozyme as it is used to lyse the cells, however this was proved wrong. It was therefore suggested that the 50 kDa band in the Pd10221 chemostat also represented a degraded from of NosZ and that the 50 kDa band, similar to that observed in the <0.5 µM Cu chemostat was also a degraded from of NosZ. The 18 µM Cu periplasmic fractions also shows a 50 kDa band, but the 66 kDa band, indicative of NosZ, is also present. If the 50 kDa present is a degraded form of NosZ, then it should not be present in the Pd10221 periplasmic fraction, unless a the Km^R cassette was inserted in the linker region of the gene, between the Cu_A and Cu_Z coding region.

The construction of Pd10221 involved the insertion of a Km^R cassette into the Sal I restriction sites, which occur at 1590 and 1926 bp into the gene (total gene length is 2417 bp). This section also so happened to be the linker region between the N and C terminal coding regions of the *nosZ* gene (See Chapter 4 section 4.5). Due to the Km^R cassette being inserted inbetween the two coding regions, potentially the Cu_z coding

region (5’ region) could be transcribed, rendering an estimated 50 kDa protein (predicted using ExPASy Peptide Mass – using mature protein sequence).

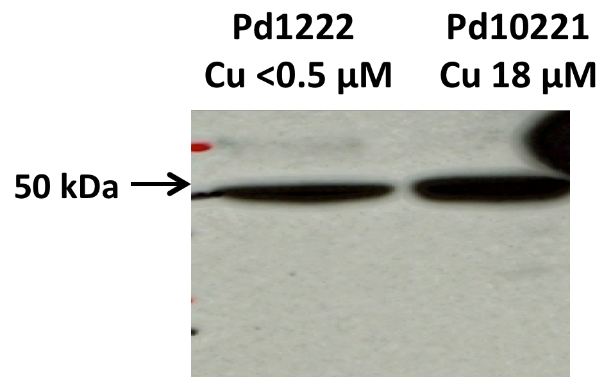


Figure 5.2-13 A Western-blotted SDS-PAGE gels probed with Abs raised against NosZ from *P.denitrificans* periplasmic fractions (100 µg of protein loaded per well) isolated from the NO_3^- -sufficient succinate-limited Pd1222 <0.5 µM Cu and Pd10221 18 µM Cu cultures.

5.2.5 Identification of the NosZ polypeptide expressed in *P.denitrificans* NosZ mutant

The 50 kDa band observed in the <0.5 µM Cu periplasmic fraction is potentially a degraded form of NosZ. The fact that there is a 50 kDa band present in the Pd10221 culture suggests that NosZ is synthesised, however due to the Km^R marker being inserted into the gene, the 50 kDa band in the Pd10221 culture is more likely to be a cross reaction between a similar polypeptide and the anti-NosZ Abs. However it is still plausible that the 50 kDa band in the <0.5 µM Cu culture is a degraded form of NosZ due to the MALDI Mass Fingerprinting data (See section 4.2.2.3).

The NosZ polypeptide was extracted from periplasmic fractions by immobolising the NosZ Ab on the gravity column packed with nProtein A Sepharose 4 Fast Flow matrix (GE healthcare) and the periplasmic fraction was passed through the column, then the Ab-protein complexes were eluted from the matrix (Section 4.2.2.3). It was observed in the Pd10221 periplasmic culture that, as observed in Figure 5.2-13, the 50 kDa band thought to be NosZ, was of a higher mass, and therefore the Pd10221 strain may not therefore produce NosZ. The bands were extracted from the gel and were sent for MALDI Mass fingerprinting at the John Innes Centre, along with a purified samples of NosZ. It was noted that the 50 kDa band from the Pd10221 periplasmic fraction ran slightly higher than the other 50 kDa fractions, but this could potentially be due to the Km^R marker being attached to the end of the NosZ polypeptide.

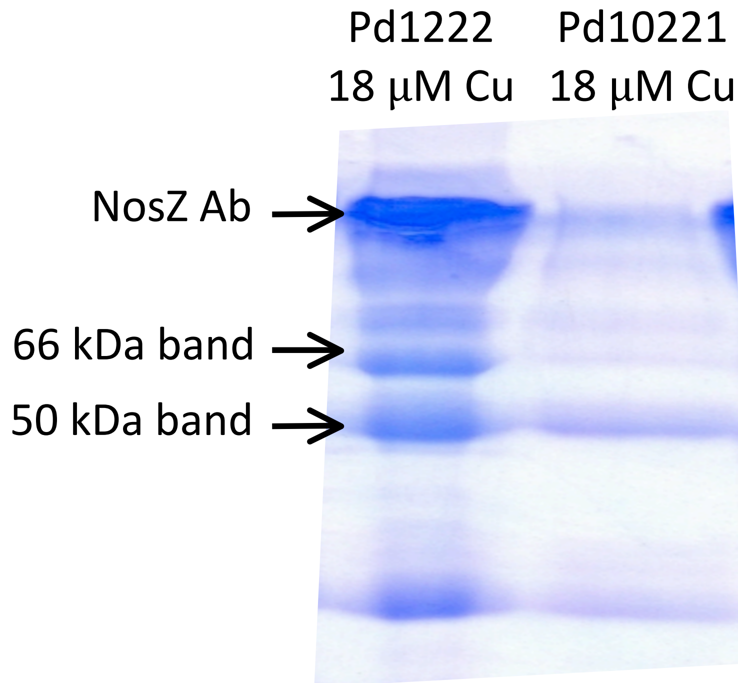


Figure 5.2-14 A SDS PAGE gel loaded with the Pd10221 and Pd10021 Ab-protein complex eluted from the nSepharose column. The gel was stained with comassie and the 50 kDa from the Pd10221 periplasmic fractions was isolated then sent for MALDI-Mass Fingerprinting.

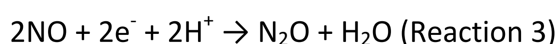
The MALDI-Mass finger print from the 50 kDa band isolated from the Pd10221 culture was analysed using Mascot. The 50 kDa band showed no significant match to nitrous oxide reductase, the top hit was a putative RNA methylase (accession number Q46XE4) and the second putative hydrolase (accession number Q0FAD5), with a protein score of 68 and 62. In Chapter 4 the 50 kDa band was identified as a truncated NosZ polypeptide. Therefore Pd10221 is a NosZ knockout mutant, and there is no NosZ activity, which is also confirmed by the total net consumption of NO_3^- in the chemostat cultures accumulating as NO_2^- and N_2O. Therefore the band observed at 50 kDa may not be NosZ but a polypeptide that interacts with the NosZ Ab. This may also be the reason to why less polypeptide was eluted from the nSepharose column (Figure 5.2-14).

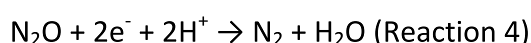
5.3 Discussion

This Chapter has attempted to characterise the *nosZ P.denitrificans* mutants. In chemostat cultures of Pd10221 the accumulation of denitrification intermediates varies greatly from the Pd1222 chemostat cultures (Chapter 3). Under NO_3^- -sufficient succinate-limited cultures NO_2^- and N_2O were not found to accumulate, therefore the rate of Reaction 1 is equal to that as Reactions 2, 3 and 4:

$2NO_3^- + 4e^- + 4H^+ \rightarrow 2NO_2^- + 2H_2O$ (Reaction 1)

$2NO_2^- + 2e^- + 4H^+ \rightarrow 2NO + 2H_2O$ (Reaction 2)


$2NO + 2e^- + 2H^+ \rightarrow N_2O + H_2O$ (Reaction 3)


$N_2O + 2e^- + 2H^+ \rightarrow N_2 + H_2O$ (Reaction 4)

However when NosZ activity is knocked out NO_2^- accumulated to nearly 10 mM, 77 % of the total net consumption of NO_3^-. As the N_2O electron acceptor is being 'lost' in the Pd10221 culture there is an increased rate of NO_3^- consumption compared the Pd1222 18 µM Cu culture ($qcNO_3^-$ Table 5-1). The $qcNO_3^-$ and qpN_2O in the Pd10221 culture and the Pd1222 <0.5 µM Cu culture are similar. The Pd1222 <0.5 µM Cu culture was capable of reducing N_2O to N_2, but at a lower rate than the 18 µM Cu cultures, therefore the final step of denitrification (reaction 4) was still functional. The Pd10221 culture is unable to carry out the reduction of N_2O (reaction 4) and therefore the denitrification system has adjusted and compensated for the bioenergetic consequences of losing reaction 4. Therefore reaction 1 is faster than NO_2^- reaction 2, causing an accumulation of NO_2^-. The presence of cyt cd_1 in the periplasmic fraction of the Pd10221 culture was similar to that as the Pd1222 18 µM Cu culture, however the activity of cyt cd_1 was nearly 40 % lower than the Pd1222 <0.5 µM Cu culture (Figure 5.2-11), therefore the NO_2^- reaction 2 is lower than reaction 1 and so NO_2^- begins to accumulate. Therefore the activity of cyt cd_1 in the Pd10221 culture shows a slight decrease, this could be due to the insertion of the d_1 haem being inserted incorrectly. N_2O may therefore affect the synthesis of cyt cd_1.

The Chapter has shown that in the Pd10221 culture the proteins involved in denitrification are synthesised as similar levels compared to the Pd1222 18 µM Cu culture. Therefore the increase in synthesis of cytochromes, such as cyt cd_1 and c_{550}, may be directly due to the Cu concentration in the bioreactor. However the mechanism involved in Cu sensing and the expression of cytochromes is unknown. The Sco proteins are involved in inserting Cu ions in to cytochromes and it maybe that there is a regulatory system involved. To understand this regulation and to see what genes are upregulated under Cu limitation future work will involve analysis gene expression by using Microarrays.

Chapter 6: The effect of copper on nitrous oxide production by *Paracoccus denitrificans* in nitrate-limited succinate-sufficient chemostat cultures

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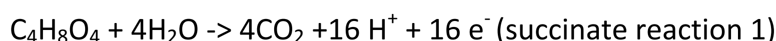
6.1 Introduction

The previous Chapters have discussed the effect that Cu concentration has on the denitrification pathway in *P.denitrificans* grown in NO_3^- -sufficient succinate-limited chemostat cultures. However there are many cases in the environment where NO_3^- is limited. There have been many soil studies looking at the effect of N concentration and the release of N_2O and N_2. The rate of denitrification is lower in low nitrate soils (32 - 96 µM) compared to higher NO_3^- (640 – 1400 µM), therefore the $N_2O:N_2$ ratios are lower in low NO_3^- soils (Weier, Doran et al. 1993). The production of N_2O and NO increase as water filled pore space (WTPS) percentage increases as more anaerobic micro environments form, promoting denitrification (Weier, Doran et al. 1993; Sánchez Martín, Vallejo Garcia et al. 2008; Allen, Kingston et al. 2009). Additions of carbon, such as glucose, also caused an increase in the production of N_2O (Weier, Doran et al. 1993; Sánchez Martín, Vallejo Garcia et al. 2008). As carbon source is oxidised electrons are released, electron acceptors, such as NO_3^-, can then be metabolised. N_2O emissions have been found to be lower in the autumn and winter seasons which coincides with lower temperatures and rainfall (Allen, Kingston et al. 2009). Although directed additions of NO_3^- to soils have been shown to lead to significant increase in N_2O release, the application of fertilisers to agricultural fields have been reported to increase N_2O emissions, however this is dependent on the type of fertiliser, the method of application and soil type, as well as the time of the year (Allen, Kingston et al. 2009).

In NO_3^- -sufficient succinate-limited chemostat cultures, as the Cu concentration decreased the production of N_2O increased (Chapter 3). There was also a significant effect on the synthesis of NosZ, as in low Cu, a truncated form of NosZ was expressed (Chapter 4). To explore the impact of NO_3^- limitation on N_2O production in different Cu concentrations this chapter aims to investigate the effect of Cu concentration on denitrification in *P.denitrificans* grown in NO_3^- -limited succinate-sufficient chemostat cultures.

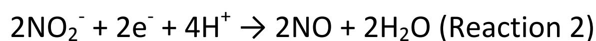
6.2 Results: *P.denitrificans* Chemostat Cultures operated under NO_3^- limited and succinate sufficiency

It was observed in chemostat cultures run under NO_3^- sufficient (20 mM) and succinate limited (5 mM) *Paracoccus* media that when the Cu concentration was reduced to ≤ 0.8 µM the N_2O produced increased dramatically. From batch culture data presented in Chapter 3 it was expected that the culture would be NO_3^- limited when the NO_3^- was reduced to 5 mM and the succinate would be sufficient when increased to 20 mM. Under these conditions the production and reduction of the denitrification intermediates as by increasing the succinate concentration the ratio of electron donor to acceptor has also shifted. For anaerobic respiration 20 mM succinate can yields a maximum of 320 mM electrons:

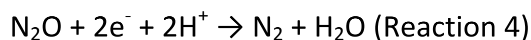

$C_4H_8O_4 + 4H_2O \rightarrow 4CO_2 + 16\ H^+ + 16\ e^-$ (succinate reaction 1)

The complete reduction of 2 molecules of NO_3^- to 1 molecule of N_2 consumes 10 electrons and so 5 mM of NO_3^- can consume a maximum of 25 mM of electrons

$2NO_3^- + 4e^- + 4H^+ \rightarrow 2NO_2^- + 2H_2O$ (Reaction 1)


$2NO_2^- + 2e^- + 4H^+ \rightarrow 2NO + 2H_2O$ (Reaction 2)

$2NO + 2e^- + 2H^+ \rightarrow N_2O + H_2O$ (Reaction 3)


$N_2O + 2e^- + 2H^+ \rightarrow N_2 + H_2O$ (Reaction 4)

This chapter investigates how the Cu concentration affects chemostat cultures of *P.denitrificans* grown in NO_3^- -limited succinate-sufficient medium.

6.2.1.1 Drymass generated in chemostat cultures of *P.denitrificans.*

The first 24 h of growth is an aerobic batch culture, after 24 h the air supply is removed, and the feed was started; the culture became anaerobic by 24.5 h. Aerobic growth was not affected by Cu concentration, as both cultures grew to a high 0.5-0.7 mg/ml after 24 h (Figure 6.2-1). Due to the media containing 20 mM succinate the growth yield during the aerobic phase is much higher than seen previously in the succinate-limited medium (Chapter 3). Steady state was entered at about 80 h in both the 18 µM and <0.5 µM Cu cultures, after approximately 3 vessel volumes. When the cultures entered steady state there is no significant difference between the drymass of two cultures (Figure 6.2-1) and therefore Cu concentration has no significant affect on growth, this is also illustrated by the biomass yields (Table 6-1). At steady state the growth yield in the NO_3^- -limited succinate-sufficient cultures compared to the NO_3^- -sufficient succinate-limited cultures show no significant difference, despite the succinate concentration being 4 times higher. Therefore the reduced growth is potentially due to the limited availability of the electron acceptor, NO_3^-.

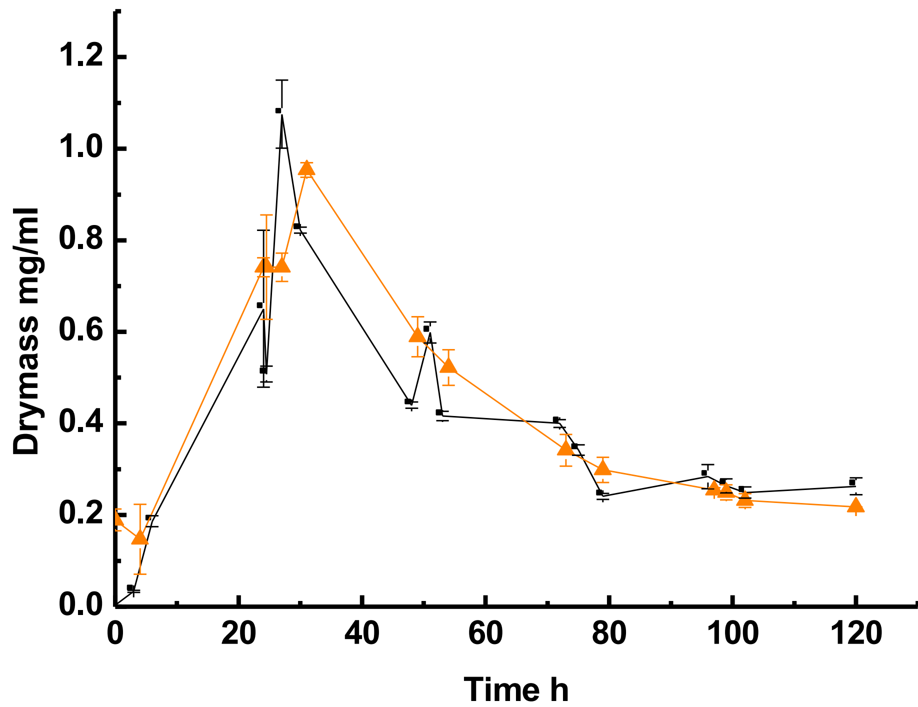


Figure 6.2-1 The drymass of *P.denitrificans* chemostat cultures grown on NO_3^- -limited succinate-sufficient *Paracoccus* minimal media, with either 18 µM Cu (■) or <0.5 µM Cu (▲). At 24 h the air supply was removed and the feed was attached, with the flow at D = 0.06 h^{-1}.

6.2.1.2 Nitrate consumption in NO_3^- -limited succinate-sufficient chemostat cultures of *P.denitrificans*

The first 24 h of growth was aerobic and little NO_3^- was consumed, suggesting that NO_3^- assimilation and aerobic NO_3^- denitrification was not significant. NO_3^- consumption began as soon as the culture became anaerobic at approximately 24.5 h. In both the 18 µM and the <0.5 µM Cu cultures the NO_3^- feeding into the chemostat had been completely consumed after 48 h of growth (24 h anaerobic growth). The NO_3^- concentration at steady state in the reactor vessel was then below the detection limit (Figure 6.2-1). This confirms that NO_3^- is limiting and contrasts to the > 5 mM residual NO_3^- observed in the NO_3^- -sufficient succinate-limited chemostat cultures (Chapter 3 Figure 3.2.7).

The specific rate of NO_3^- consumption, $qcNO_3^-$, showed no significant difference between the 18 µM and <0.5 µM Cu NO_3^- limited cultures. The $qcNO_3^-$ observed in the NO_3^- limited chemostat cultures are significantly lower than that observed in the NO_3^- sufficient cultures. The $qcNO_3^-$ in the NO_3^- sufficient cultures was 2.01 ± 0.1 mmol.g drymass^{-1}h^{-1}, and as the Cu concentration became limited the $qcNO_3^-$ increased to 3.3 ± 0.4 mmol.g drymass^{-1}h^{-1} (Table 3.2-3). Compared to the NO_3^- limited cultures, the $qcNO_3^-$ is on average 4 times lower than the rates observed in the NO_3^- sufficient cultures.

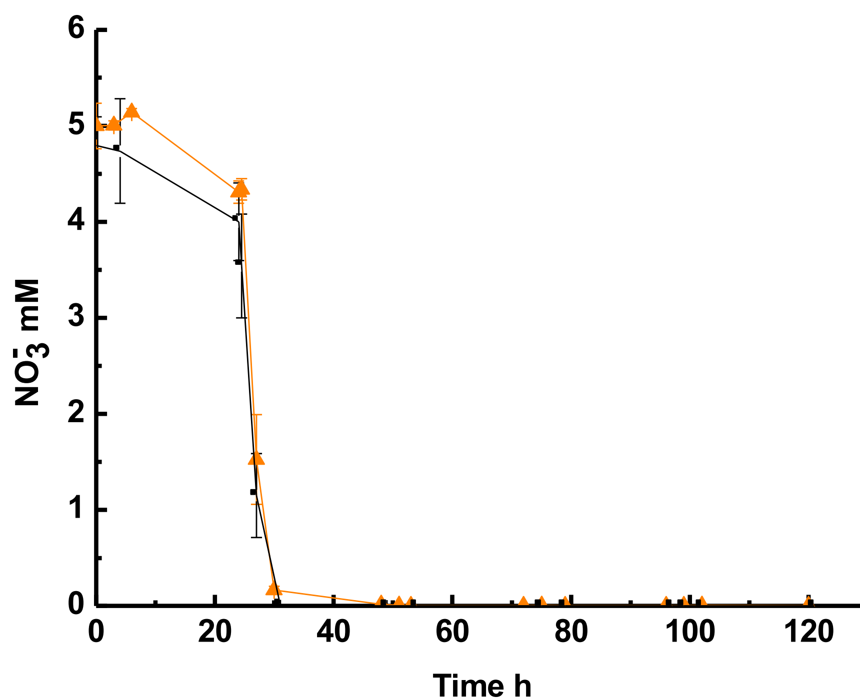


Figure 6.2-2 The consumption of NO_3^- in *P.denitrificans* chemostat cultures grown on NO_3^- sufficient succinate limited *Paracoccus* minimal media, with either 18 µM Cu (■) or <0.5 µM Cu (▲). At 24 h the air supply was removed and the feed was added (D=0.06 h^{-1}). Each sample isolated from the chemostat cultures was analysed twice, the error bars represent the standard error.

6.2.1.3 Nitrite production kinetics in NO_3^- -limited succinate-sufficient chemostat cultures of *P.denitrificans.*

The first 24 h of growth was aerobic and no NO_2^- was found to accumulate. The production of NO_2^- was only detected in the first 3 h of anaerobic growth in both cultures, more so in the <0.5 µM Cu culture (Figure 6.2-3). Therefore Reaction 1 is faster than Reaction 2. The net concentration of NO_2^- in the bioreactor then decreased after 3 h to approximately 20 – 30 µM in the <0.5 µM Cu culture and between 10 – 18 µM in the 18 µM Cu culture, throughout steady state, suggesting that NO_3^- reaction 1 and 2 are now at similar rates. The initial production of NO_2^- could potentially be due to a lag in synthesising the cyt cd_1 so as NO_3^- is reduced the NO_2^- accumulates. After 27 h the levels of NO_2^- are similar to that observed in NO_3^- -sufficient succinate-limited cultures (Chapter 3) and the NO_2^- does not accumulate above 18 µM in the 18 µM Cu culture and 35 µM in the <0.5 Cu culture.

The specific rate of NO_2^- production ($qpNO_2^-$) in both the 18 µM and <0.5 µM Cu cultures shows a negative value (Table 6-1). Therefore more NO_2^- is consumed than produced in the NO_3^- limited cultures, the NO_2^- present at the beginning of the culture is contamination in the medium in the reservoir. The $qpNO_2^-$ between the two cultures shows no significant difference, however compared to the NO_3^- sufficient chemostat cultures the $qpNO_2^-$ in the NO_3^- limited cultures are significantly lower, -0.006 ± 0.001 mmol.g drymass^{-1}h^{-1} and 0.009 ± 0.005 mmol.g drymass^{-1}h^{-1} in the 18 µM and <0.5 µM Cu cultures respectively (Table 3.2.3).

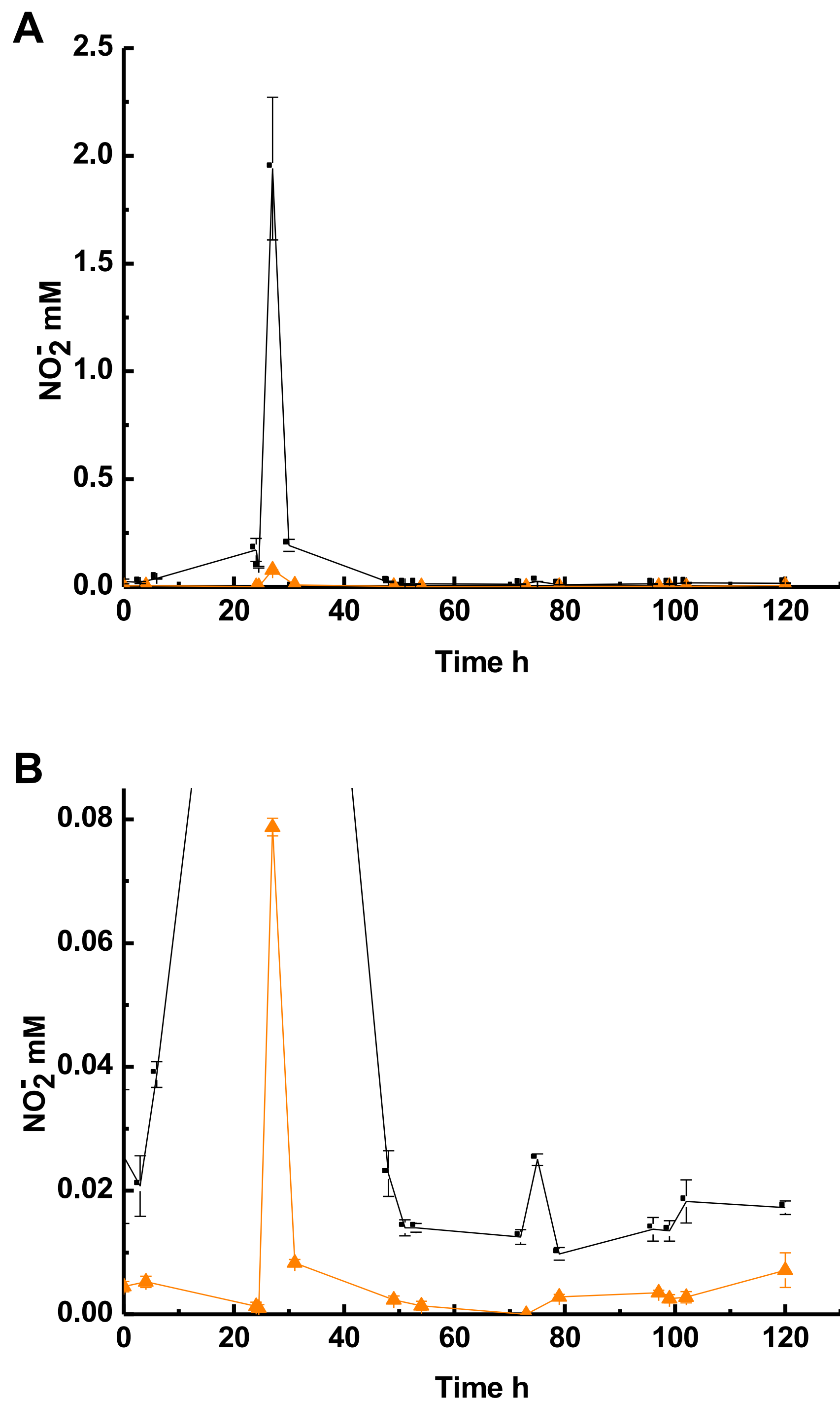


Figure 6.2-3 The production of NO_2^- in *P.denitrificans* chemostat cultures grown NO_3^- -limited succinate-sufficient *Paracoccus* minimal media (A and B), with either 18 µM Cu (■) or <0.5 µM Cu (▲). The second graph (B) show the first graph on an enlarged scale. At 24 h the air supply was removed and the feed was added, by 24.5 h the vessel became anaerobic. Each sample isolated from the chemostat cultures was analysed twice, the error bars represent the standard error.

6.2.1.4 Nitrous oxide production in NO_3^- -limited succinate-sufficient chemostat cultures of *P.denitrificans*

Throughout the chemostat experiment no extracellular NO was detected and therefore NO_2^- was being reduced to NO and then N_2O. There was a small amount of N_2O detected in the first 24 h of growth, however this was not due to denitrification as NO_3^- was not being consumed and so the N_2O detected was atmospheric. Typically the atmospheric N_2O detected in the gas phase is < 1 µM, which corresponds to the atmospheric N_2O concentration. In the 18 µM Cu chemostat culture the N_2O detected in the headspace is less than atmospheric. Therefore more N_2O is consumed than produced, therefore the specific productivity rate of N_2O, qpN_2O, is represented as a negative rate (Table 6-1). In the <0.5 µM NO_3^- -sufficient succinate-limited chemostat culture the production of N_2O was significantly elevated, especially in the first 3 h of anaerobic growth. By 27 h the N_2O concentration reached 483 ± 154 µM, this implies that the initial activity of NosZ is reduced in low Cu cultures. However by 48 h the concentration of N_2O in the vessel had decreased to 35 ± 17 µM, therefore N_2O was being reduced to N_2 by NosZ (Figure 6.2-4). This therefore implies that cellular NosZ is active but at a reduced rate in the <0.5 µM Cu culture compared to the 18 µM Cu culture, suggesting that the synthesis of NosZ is affected by low Cu concentrations, but the activity is not completely knocked out. At steady state in the <0.5 µM Cu culture the N_2O accumulation is 4 times higher than in the 18 µM Cu culture, 4.59 ± 1.7 µM (Figure 6.2-4). However the production of N_2O in the NO_3^- -limited succinate-sufficient chemostat cultures is significantly lower than that observed in the NO_3^- -sufficient succinate-limited chemostat cultures (Chapter 3 Figure 3.2-10).

The specific rate of N_2O production, qpN_2O, for the <0.5 µM Cu culture is significantly increased compared to the 18 µM Cu culture (Table 6-1). The <0.5 µM Cu culture accumulates N_2O, whereas in the 18 µM Cu culture no N_2O accumulates. Therefore even in NO_3^- limited cultures the Cu concentration plays a major role in the release of N_2O and the activity of NosZ, but to a lesser extent compared to the NO_3^- sufficient chemostats. The qpN_2O observed in the <0.5 µM Cu NO_3^- -sufficient

succinate-limited culture was 0.527 ± 0.007 mmol.g $drymass^{-1}h^{-1}$, 1000 fold increased compared to the NO_3^- -limited succinate-sufficient culture (Table 3.2-3).

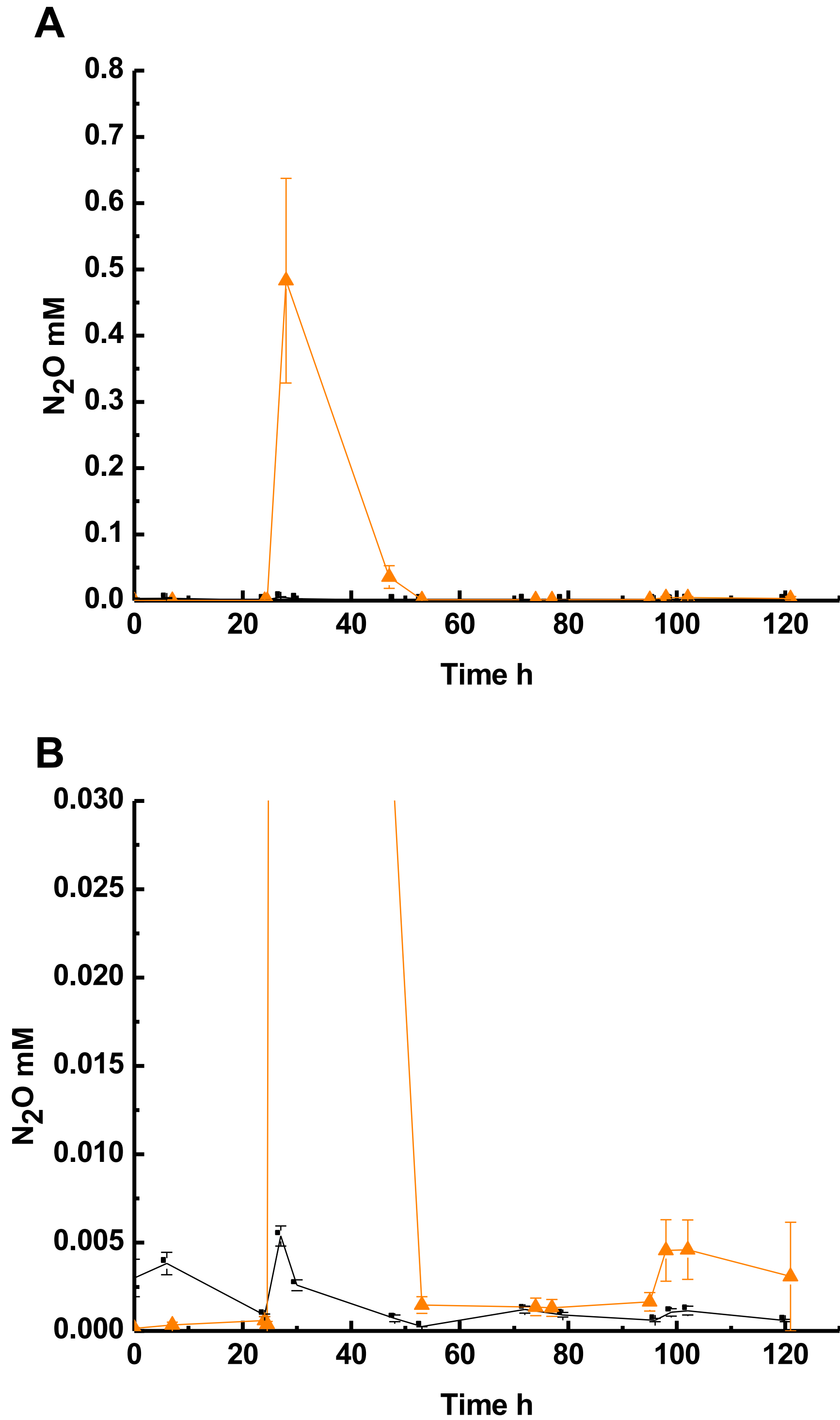


Figure 6.2-4 The accumulation of N_2O in *P.denitrificans* chemostat cultures grown on NO_3^- -limited succinate-sufficient *Paracoccus* minimal media (A and B), with either 18 µM Cu (■) or <0.5 µM Cu (▲). The second graph (B) show the first graph on an enlarged scale. At 24 h the air supply was removed and the feed was added, by 24.5 h the vessel became anaerobic. Each gas sample from the chemostat cultures was analysed twice, the error bars represent the standard error.

6.2.1.5 Dinitrogen production in nitrate-limited succinate-sufficient chemostat cultures of *P.denitrificans*

The production of N_2 in NO_3^- sufficent chemostat cultures indicated that NosZ was still active under low Cu concentrations as N_2O was being reduced to N_2. It was therefore hypothesised that NosZ would also be active in the NO_3^- limited chemostat cultures. At steady state in the 18 µM Cu chemostat culture a total of 589 ± 93.6 µM of N_2 was produced, accounting for 23.5 ± 3.7 % of the total NO_3^- consumed. The amount of N_2 produced in the <0.5 µM Cu chemostat culture was nearly half that observed in the 18 µM Cu chemostat culture. A total of 240.3 ± 73.9 µM of N_2 was produced at 102 h in the <0.5 µM Cu chemostat culture, which accounts for 12.5 ± 2.7 % of the total NO_3^- consumed. The fact that the N_2 produced does not account for 100 % of the total consumed NO_3^- is concerning, as the chemostat cultures are an "open" system, gases are able to diffuse, there is also the possibility of error in the measurements of NO_3^-, NO_2^- and N_2O, or due to some NO_3^- being consumed in NO_3^- assimilation, therefore further work will be carried out to address this matter. Methods to increase the accuracy of ^{15}N measurements involve using a closed system that is connected to a Gas Chromatorgaphy Mass Spectrometer, as many systems that use ^{15}N have problems with accounting for 100 % of the ^{15}N put into the system (Isobe, Suwa et al. 2009).

The specific rate of N_2 production (qpN_2) is higher in the 18 µM Cu chemostat culture compared to the <0.5 µM Cu culture (Table 6-1). Compared to the NO_3^- -sufficient succinate-limited chemostat cultures, the qpN_2 rates are significantly lower, which is due to a lower initial NO_3^- concentration. The qpN_2 in the NO_3^- -sufficient succinate-limited cultures were 0.9 ± 0.2 mmol.g drymass^{-1}h^{-1} in the 18 µM Cu culture and 0.2 ± 0.04 mmol.g drymass^{-1}h^{-1} in the <0.5 µM Cu cultures (Table 3.2-3), on average the rates observed are 18 times higher in the 18 µM Cu culture and 10 times higher in the <0.5 µM Cu culture compared to that observed in the NO_3^- -limited succinate-sufficient cultures. This therefore comfirms that under NO_3^- limitation

P.denitrificans is still capable of reducing N_2O to N_2 under low Cu concentrations, but the activity of cellular NosZ is reduced under low Cu concentrations. This could potentially be due to not all the Cu centres being inserted in NosZ, or the Cu centres are not being inserted correctly, or even due to the transcription of *nosZ* being lower which will be investigated later in this Chapter.

Table 6-1 The rate for NO_3^- consumption and NO_2^-, N_2O and N_2 production in *P.denitrificans* chemostat cultures at steady state (102h). *P.denitrificans* was grown in NO_3^- -limited succinate-sufficient *Paracoccus* minimal media with either 18 µM or <0.5 µM Cu.

	Growth Yield		$qcNO_3^-$		$qpNO_2^-$		$qcNO_2^-$	qpN_2O		qpN_2	
	mg.ml.h^{-1}		mmol.g drymass^{-1}h^{-1}								
Cu 18 µM	0.010	±0.0005	0.533	±0.012	$-6.3x10^{-4}$	$±2.5x10^{-3}$	0.533	$-1.9x10^{-4}$	$±9.6x10^{-5}$	0.050	±0.006
Cu <0.5 µM	0.009	±0.0006	0.557	±0.095	-0.002	$±2.7x10^{-4}$	0.557	$7.9x10^{-4}$	$±3.1x10^{-4}$	0.035	±0.007

Growth Yield -biomass production = Dx; where x = the biomass at steady-state.

NO_3^- consumption $(qpNO_3^-) = ([NO_3^-]_R - [NO_3^-]_r) D / x$; where $[NO_3^-]_R$ is the NO_3^- concentration in the reservoir, $[NO_3^-]_r$ is the residual NO_3^- concentration in the reactor vessel, D is the dilution rate and x is the drymass concentration in the reactor vessel.

NO_2^- production $(qp\ NO_2^-) = [NO_2^-]\ r\ D / x$ where $[NO_2^-]_r$ is the NO_2^- concentration in the reactor vessel – the initial/background NO_2^- concentration

N_2O production $(qp\ N_2O) = [N_2O]\ r\ D / x$; where $[N_2O]_r$ is the N_2O concentration in the reactor vessel – the initial/background N_2O concentration

N_2 production $(qp\ N_2) = [^{15}N - N_2]\ D/x$

Negative (-) numbers represents the consumption of the intermediate

6.2.2 Detection of Paz, *c_{550}*, Nar, Nir and Nor in *P.denitrificans* grown in nitrate limited chemostat cultures.

The release of denitrification intermediates in *P.denitrificans* NO_3^- -limited succinate-sufficient chemostat cultures were significantly lower than that observed in the NO_3^- -sufficient succinate-limited chemostat cultures. Due to the concentration of NO_3^- being limited the NO_3^- was being reduced to N_2 in order to gain as much energy as possible, and therefore intermediates did not accumulate. Therefore the synthesis of denitrification proteins and enzymes were investigated in order to identify how NO_3^- limitation and Cu concentrations affects the denitrification pathway.

6.2.2.1 Pseudoazurin

In *P.denitrificans* grown in NO_3^- -sufficient succinate-limited conditions with either 0.8 µM or <0.5 µM Cu Paz was not present in the periplasm (Figure 4.3-1). It was therefore hypothesised that Paz would also not be present in the NO_3^- limited <0.5 µM Cu culture due to any Cu present in the cell being inserted into NosZ for reducing N_2O to N_2. A Western-blotted SDS PAGE gel probed with anti-Paz Abs, shows that Paz is present in the periplasmic fraction of the 18µM Cu culture, seen in the monomeric form which is 13 kDa (Figure 6.2-5). Paz was undetectable in the <0.5 µM Cu culture, this was also observed in the NO_3^- -sufficient succinate-limited culture (Figure 4.3-1). The reduced levels of Paz in <0.5 µM Cu cultures could potentially be due to Paz being targeted for degradation if the Cu centres are not inserted or that the transcription of Paz is affected due to low Cu levels, however the mechanism involved is unknown.

In the NO_3^- -limited succinate-sufficient chemostat cultures in 18 µM and <0.5 µM Cu culture no denitrification intermediates were found to accumulate as the culture is e^- acceptor limited and e^- donor sufficient. As previously discussed the c_{550} is capable of transferring e^- to NirS, NorCB and NosZ, therefore if Paz is not present c_{550} can sustain e^- transport.

6.2.2.2 Cytochrome c_{550}

The synthesis of Paz is reduced in the <0.5 µM Cu NO_3^- -limited succinate-sufficient chemostat culture, which is also observed in the 0.8 µM and <0.5 µM Cu NO_3^- sufficient cultures, it was therefore hypothesised that the expression of c_{550} would be elevated in the <0.5 µM Cu cultures so that e^- transport can be sustained. The expression of c_{550} is elevated in the <0.5 µM Cu chemostat culture compared to the 18 µM Cu culture (Figure 6.2-6). The levels of c_{550} in the NO_3^- -limited succinate-sufficient chemostat culture are significantly lower than the levels observed in the NO_3^- sufficient chemostat cultures. NO_3^- respiration could therefore potentially play a role in a regulatory factor which senses NO_3^-, Cu or the redox state of other enzymes in the denitrification pathway.

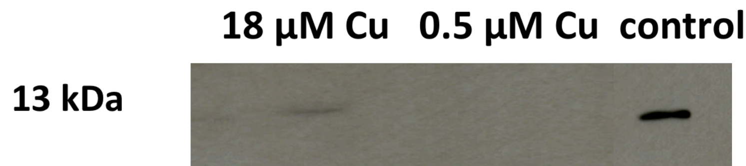


Figure 6.2-5 A Western-blotted SDS-PAGE gels probed with Abs raised against Pseudoazurin from *P.denitrificans* periplasmic fractions grown NO_3^- -limited succinate-sufficient *Paracoccus* media with either 18 µM or <0.5 µM Cu. The periplasmic fractions were isolated at steady state. The control contained 2 µg of purified *P.denitrificans* Paz and 100 µg of protein were loaded for each periplasmic fraction.

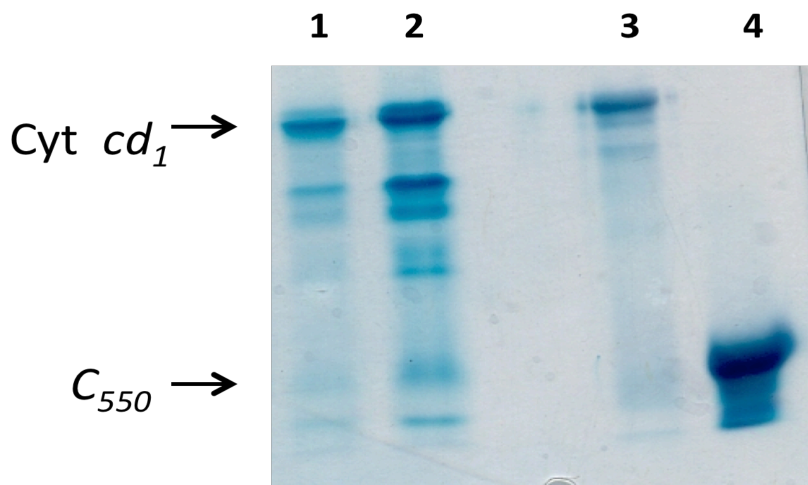


Figure 6.2-6 A haem stained SDS PAGE gel loaded with periplasmic fractions (100 µg of protein per well) isolated from *P.denitrificans* grown in NO_3^- -limited succinate-sufficient media either 18 µM Cu (lane 1) or <0.5 µM Cu (lane 2). Purified cyt cd_1 20 µg (lane 3) and 30 µg of c_{550} (lane 4) were loaded as controls. The band seen at approximately 20 kDa represents cyt c_{550}, the 66 kDa represents cyt cd_1.

6.2.2.3 Nitrate reductase

In chemostat cultures of *P.denitrificans* grown in NO_3^- -sufficient succinate-limited medium the level of NarGH polypeptide appeared to be decreased in the <0.5 µM Cu membrane fractions compared to the 18 µM membrane fraction (Figure 4.3-3). However the activity of Nar showed no significant difference between the three Cu concentrations. Under NO_3^- limitation and succinate sufficiency the effect of the Cu concentration on NarGH is similar to that as observed in the NO_3^- sufficient chemostats. A Western-blotted SDS PAGE gel probed with anti NarGH Abs, shows that the NarGH subunits are both present in the 18 µM Cu culture (Figure 6.2-7). This therefore implies that the Nar system is being synthesised correctly and is active. The <0.5 µM Cu membrane fraction shows that only the NarG band is present and the NarH band is absent, however the enzyme is still active. The immunochemistry does not therefore appear to reflect the activity of the Nar system, and so monitoring the activity of NarGHI will give a better understanding into the effect that Cu concentration has on NO_3^- reduction.

The activity of NO_3^- reductase was monitored at steady state using MV+ assays. The rates observed in the 18 µM and <0.5 µM Cu chemostat cultures showed no significant difference in the whole cell assays, with the activity detected in the 18 µM Cu culture being 36 ± 11 nmol.min^{-1}mg drymass^{-1}, and 35 ± 2 nmol.min^{-1}mg drymass^{-1} in the and <0.5 µM Cu culture (Figure 6.2-8A). The activity of the NarGHI system in the membrane fractions isolated from the NO_3^- -limited succinate-sufficient 18 µM Cu and the <0.5 µM Cu membrane fractions also showed no significant difference in the rates (Figure 6.2-8 B). The rates observed from the whole cell assays in the NO_3^- limited cultures are similar to the rates observed in the NO_3^- -sufficient succinate-limited whole cells assays, at steady state (Figure 4.2-4 A). However, interestingly the rates derived from the membrane fractions are higher in the NO_3^- -sufficient succinate-limited culture than the NO_3^- -limited succinate-sufficient chemostat culture, perphaps due to more NO_3^- being present in the cultures.

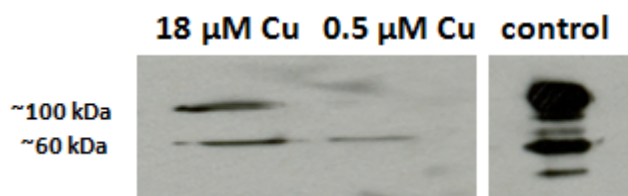


Figure 6.2-7 A Western-blotted SDS-PAGE gels probed with Abs raised against NarGH from *P.denitrificans* membrane fractions isolated from NO_3^- -limited succinate-sufficient chemostat cultures, with either 18 µM Cu or <0.5 µM Cu. The 100 kDa band represents the NarG subunit (actual weight 140 kDa) and the 60 kDa band represents the NarH subunit. The membrane fractions were isolated at steady state. The control contains approximately 0.2 µg of purified NarGH.

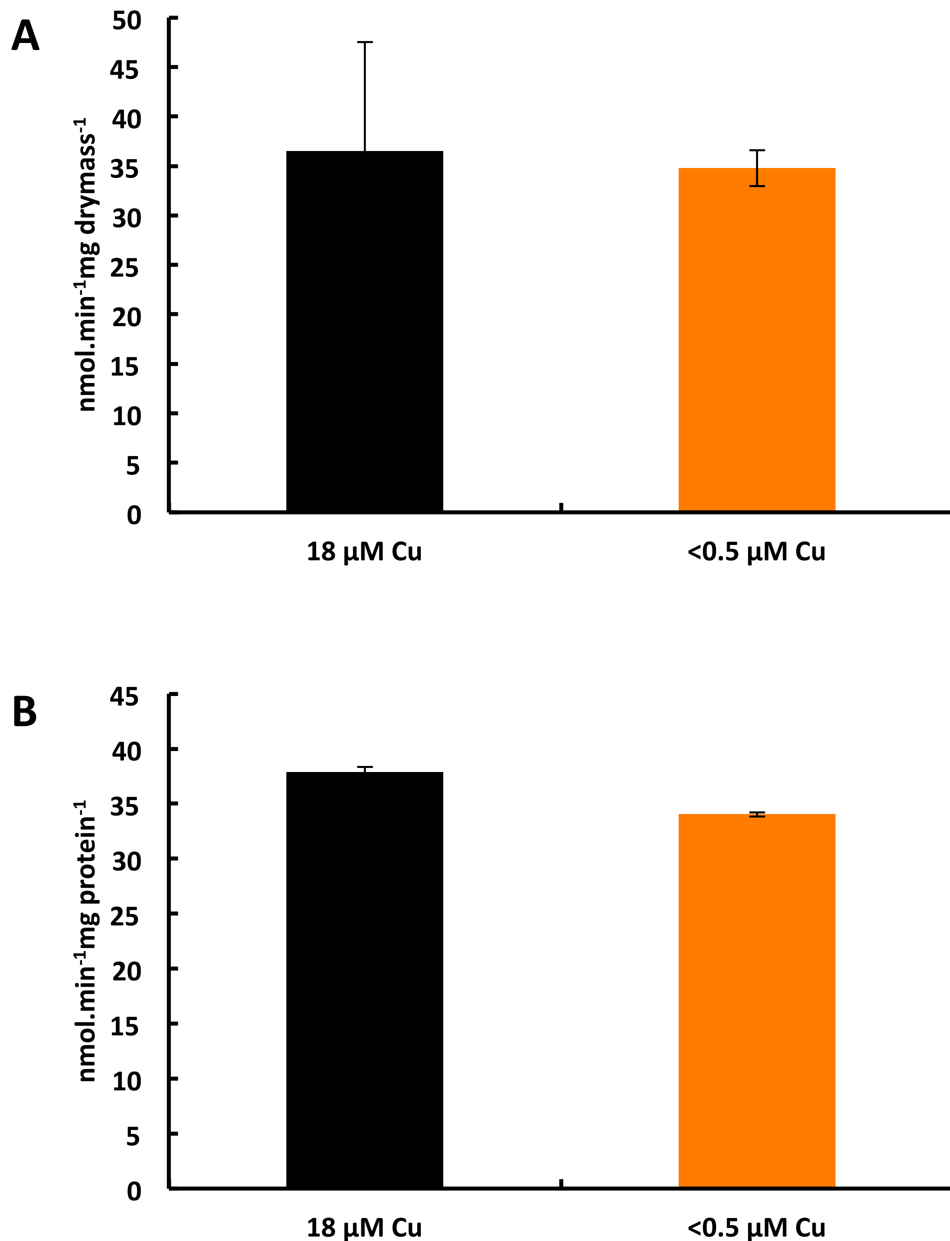


Figure 6.2-8 The specific activity of NO_3^- reductase measured in whole cell assays (A) and membrane fractions (B) isolated at steady state from chemostat cultures of *P.denitrificans* grown in NO_3^- -limited succinate-sufficient *Paracoccus* media with either 18 µM Cu (black) or <0.5 µM Cu (orange). ach sample isolated from the chemostat cultures was analysed twice, the error bars represent the standard error.

6.2.2.4 Nitrite reductase

When *P.denitrificans* was grown in NO_3^- -sufficient succinate-limited media the Cu concentration directly affected the synthesis of cyt cd_1, which was increased under low Cu concentrations (Figure 4.2-6). A Western-blotted SDS PAGE gel probed with anti cyt cd_1 Abs shows that in the NO_3^- -limited succinate-sufficient periplasmic fraction there is no significant difference in cyt cd_1 concentration between the two 18 µM and <0.5 µM Cu periplasmic fractions (Figure 6.2-9). Therefore the Cu concentration has no significant affect on the presence of cyt cd_1 in NO_3^- limited conditions. It can be observed in a haem stained SDS PAGE gel that there is a marginal increase in the level of cyt cd_1 polypeptide present in the <0.5 µM Cu periplasmic fraction (Figure 6.2-6).

The specific activity of cyt cd_1 was determined using MV+ assays at steady state, and it was observed that activity in the <0.5 µM Cu culture was 48 ± 20 nmol.min^{-1}mg drymass^{-1}, which is approximately 10 times lower than the activity observed in the 18 µM Cu culture (Figure 6.2-10). In the NO_3^- -sufficient succinate-limited <0.5 µM Cu chemostat culture the activity of cyt cd_1 showed no significant difference to the 18 µM Cu culture, however there was a significant increase in the amount of polypeptide. In the NO_3^- limited <0.5 µM Cu culture the amount of polypeptide present in the periplasm is similar to that as the 18 µM Cu culture, and so the polypeptide in the <0.5 µM Cu culture is less active. Therefore the Cu concentration affects the activity of cyt cd_1 possibly due to the d_1 haem not being inserted correctly. The activity of cyt cd_1 in the NO_3^- -limited succinate-sufficient 18 µM Cu culture was 145 ± 23 nmol.min^{-1}mg drymass^{-1} (Figure 6.2-10), which is three times as much as the activity observed in the NO_3^- sufficient 18 µM Cu chemostat. Therefore despite a decrease in the cyt cd_1 polypeptide being present in the NO_3^- -limited succinate-sufficient cultures, the activity of cyt cd_1 is elevated.

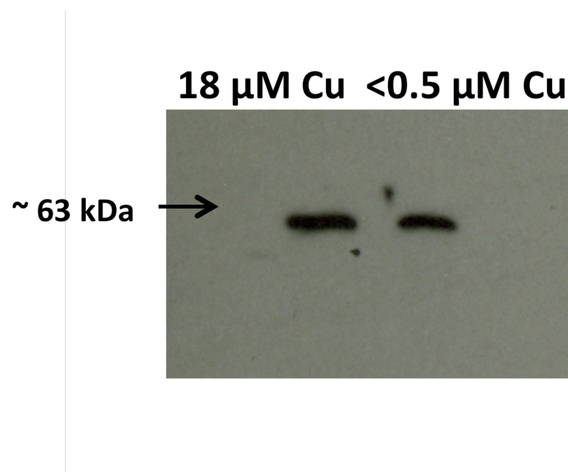


Figure 6.2-9 A Western-blotted SDS-PAGE gels probed with Abs raised against *P.denitrificans* cyt *cd₁* from periplasmic fractions of the NO_3^- -limited succinate-sufficient 18 µM Cu and <0.5 µM Cu chemostat cultures harvested at steady state. 100 µg of protein was loaded into each well.

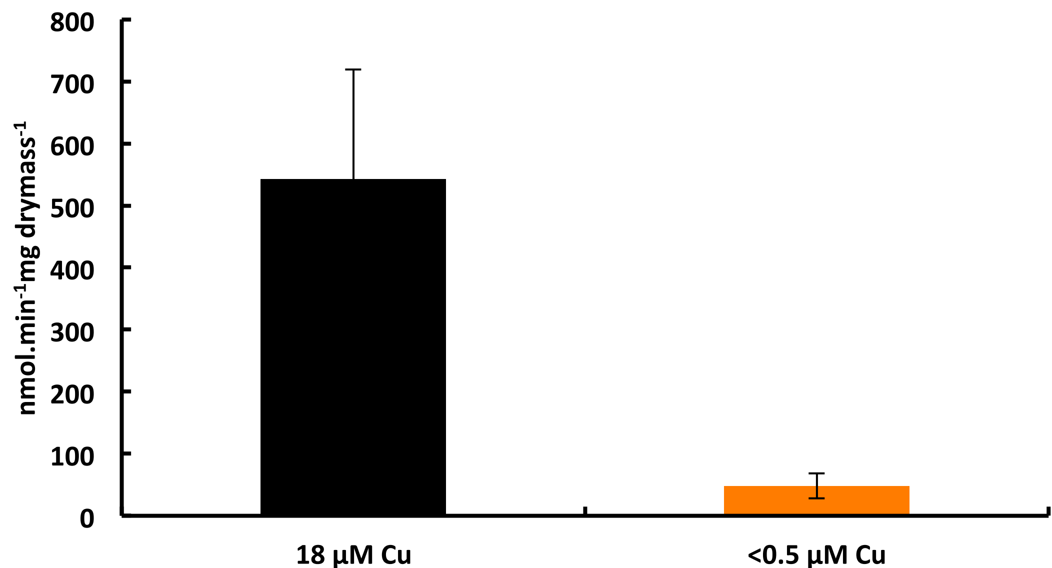


Figure 6.2-10 The specific activity of NO_2^- reductase measured in whole cell assays isolated at steady state from chemostat cultures of *P.denitrificans* grown in NO_3^- -limited succinate-sufficient *Paracoccus* media with either 18 µM Cu (black) or <0.5 µM Cu (orange). ach sample isolated from the chemostat cultures was analysed twice, the error bars represent the standard error.

6.2.2.5 Nitric oxide reductase

Throughout the chemostat cultures extracellular NO was unable to be detected. This therefore indicates that the activity of NorCB is unaffected by NO_3^- concentration or Cu concentration. On Western-blotted SDS PAGE gels probed with anti NorCB Abs it was not possible to detect NorCB in the membrane fractions. NorCB must be present in the membrane fractions as NorCB knockouts in *P.denitrificans* are unable to grow under denitrifying conditions (Butland, Spiro et al. 2001). However the level of polypeptide was unable to be detected.

6.2.3 The synthesis of nitrous oxide reductase in NO_3^- -limited succinate-sufficient chemostat cultures

The production of N_2O in the NO_3^--limited succinate-sufficient chemostat cultures was significantly lower than that observed in the NO_3^--sufficient succinate-limited culture in Cu limitation. However, N_2 production still continues under low Cu and NO_3^- concentations and therefore the activity of NosZ seems unaffected. The transcription of *nosZ* in the NO_3^- limited chemostats is generally lower than the transcription observed in the NO_3^- sufficient chemostat cultures. During the aerobic phase *nosZ* transcription is lower in the <0.5 μM Cu culture compared to the 18 μM Cu culture (Figure 6.2-11). Compared to the NO_3^- sufficient chemostat cultures, the transcription of *nosZ* aerobically in the NO_3^- limited 18 μM and <0.5 μM Cu cultures is decreased by 68 % and 93 %, respectively (Figure 4.3-10). During the NO_3^- limited anaerobic phase the transcription of *nosZ* is up regulated, more so in the <0.5 μM Cu culture. Compared to the transcription in the NO_3^- sufficient culture the 18 μM Cu culture shows a 78 % decrease, however the <0.5 μM Cu culture shows an increase by

325 % in *nosZ* transcription. This suggests that the transcription of *nosZ* is affected by the Cu concentration as well as the NO_3^- concentration.

The RT PCR confirms that *nosZ* is being transcribed in the 18 µM and <0.5 µM Cu NO_3^- -limited succinate-sufficient chemostat cultures. To identify whether the NosZ polypeptide is present in the periplasmic fractions isolated from the chemostat cultures a Western-blotted SDS PAGE gel was probed with anti NosZ Abs. The periplasmic fractions isolated from the 18 µM and <0.5 µM Cu NO_3^- limited chemostat cultures both contained the complete NosZ polypeptide, represented by the 66 kDa band. The intensity of the NosZ band is increased in the 18 µM Cu culture, and there is also a 50 kDa present which is predicted to be a degraded from of NosZ, this 50 kDa band was also present in all the periplasmic fractions in the NO_3^- sufficient chemostats. The 50 kDa band is absent in the NO_3^- limited <0.5 µM Cu periplasmic fraction, contrary to the NO_3^- sufficient <0.5 µM Cu chemostat culture which only contained a 50 kDa band and no 66 kDa band, which is indicative of NosZ. Therefore Cu limitation only has a significant effect on the presence of the NosZ polypeptide when the NO_3^- is sufficient, because when the NO_3^- was limited the complete NosZ polypeptide is synthesised correctly.

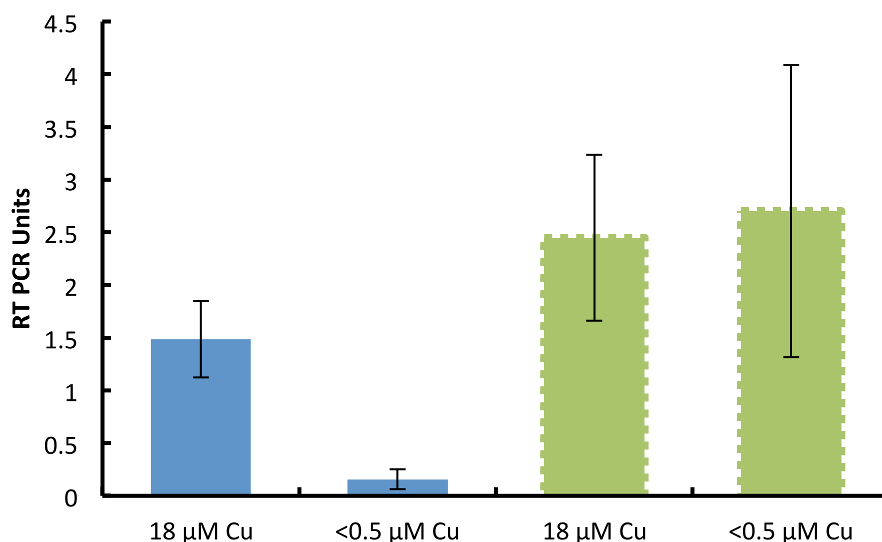


Figure 6.2-11 Real Time PCR showing the gene expression of *nosZ* in *P.denitrificans* chemostat cultures grown in NO_3^- -limited succinate-sufficient *Paracoccus* media with either 18 µM or <0.5 µM Cu. RNA samples were taken after 3 h, aerobic (blue) and at 102 h, anaerobic steady state (green, dotted outline). The data was normalised with *Rpo* as the housekeeping gene. Each RNA sample isolated was analysed twice from each chemostat culture, the error bars represent the standard error.

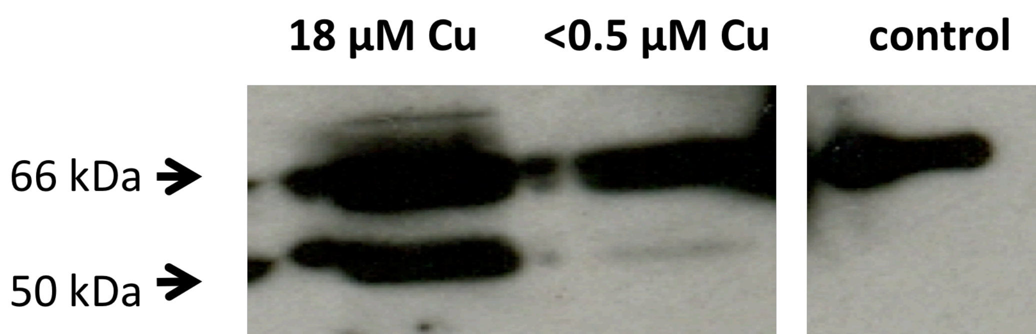


Figure 6.2-12 Western-blotted SDS-PAGE gels probed with Abs raised against NozZ from *P.denitrificans* periplasmic fractions grown on NO_3^- -limited succinate-sufficient *Paracoccus* minimal media with either 18 µM Cu or <0.5 µM Cu.

6.3 Discussion

To investigate the influence of carbon-versus NO_3^--limitation on N_2O production under different Cu concentrations, continuous culture experiments were run with the reservoir medium feed containing 5 mM nitrate and 20 mM succinate to achieve an electron donor-sufficient (succinate) / electron acceptor-limited (NO_3^-). The results can be compared to those of Chapter 3 collected under NO_3^- -sufficient succinate-limited conditions. In the NO_3^- limited chemostat cultures the NO_3^- levels in the bioreactor remained approximately constant at ~5 mM during the aerobic batch phase and no significant net accumulation of NO_2^- or N_2O was observed. However, the growth density reached at 24 h of aerobic growth was over twice as high during this phase compared to the succinate-limited culture, which reflects the higher level of electron donor available in cultures that are not limited for electron acceptor (dissolved oxygen) in this first batch phase. When the cultures turned to anaerobic continuous cultures there were transient spikes of NO_2^- and N_2O release in Cu<0.5 culture, but in the steady-state no net release of these intermediates was observed. The entire reservoir NO_3^- was consumed by the steady-state cultures, confirming they the NO_3^- was limiting.

Complete catabolism of 20 mM succinate to CO_2 maximally yields 320 mM electrons available for anaerobic respiration. The complete reduction of 5 mM of NO_3^- to 2.5 mM N_2 consumes 25 mM electrons. Thus the cultures are extremely electron acceptor limited. The rate of NO_3^- consumption was ~0.5 mmol g drymass^{-1} h^{-1} regardless of the Cu-status (Table 6-1), which is ~4 fold lower than observed in the NO_3^--sufficient cultures (Table 3.2-3). Since there is no accumulation of NO_2^- or N_2O then all reactions (Reactions 1-4) are matched but the rate is lower than the rate in the NO_3^- -sufficient succinate-limited cultures. By growing *P.denitrificans* in NO_3^- -limited succinate-sufficient conditions the qpN_2O is considerably lower compared to the NO_3^- -sufficient succinate-limited culture. Therefore by reducing the e^- acceptor availability *P.denitrificans* is capable of driving the reduction of NO_3^- through to N_2, despite Cu being limited, utilising the maximum amount of available e^- acceptor. Therefore when relating this research to the environment, in agricultural fields with excessive NO_3^-, due to excessive fertiliser

application, could result in higher N_2O emissions compared to fields that have lower NO_3^-. Therefore better management of fertiliser application could result in lower N_2O emissions from agricultural soils.

Changes in the NO_3^- and Cu concentrations significantly affected the expression of proteins and enzymes involved in denitrification. The Paz polypeptide was absent from the <0.5 µM Cu periplasmic fractions, therefore for e^- transport to continue the synthesis of c_{550} was increased. Most significantly though the 66 kDa intact NosZ band, rather than the 50 kDa truncated NosZ band was assembled in the periplasm in the <0.5 µM Cu culture. It is clear that under electron-acceptor limited conditions there is a strong selective pressure to consume as much of the available electron acceptor as possible and thus it appears that to achieve this even under copper deplete conditions. The cultures must therefore prioritise the synthesis of intact holo-NosZ as to ensure that all N_2O is reduced, so that there is a sufficient amount of NosZ present as to accommodate the low flux. Under electron-acceptor sufficient conditions there is less need to do this and hence the partial shutdown of the reduction of N_2O to N_2, the synthesis of poorly active Cu_zNosZ and the release of millimolar levels of N_2O.

In earlier work, contextualised for oceanic denitrification, Granger and Ward (2003) studied two defined species of denitrifying bacteria, *P. denitrificans* and *Pseudomonas stutzeri*. The work was conducted in batch cultures, containing only 250 µM nitrate at the outset, which was quickly consumed and thus the nitrate cultures became nitrate-limited as there was not a continuous delivery of nitrate into the closed system. Nevertheless, Cu-limitation did lead to the release of N_2O, but the levels of production were in the low µM range not the thousands of µM range observed in the present study under nitrate-sufficient conditions (Chapter 3). These previous results are more consistent with the cultures being nitrate-limited where, as now established in the current Chapter, the rate of N_2O release is orders of magnitude lower than under nitrate-sufficiency. It was notable though that when electron-acceptor is limited and the culture needs to maximise utilisation of all available electron acceptor an intact NosZ is synthesised even under copper depleted conditions.

Chapter 7: The effect of copper on *Achromobacter xylosoxidans* in nitrate-limited and sufficient chemostat cultures

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7.1 Introduction

The previous Chapters have discussed the affect that Cu concentration has on the denitrification pathway in *P.denitrificans.* Although *P.denitrificans* is a model denitrifying bacterium, there are many soil isolates that denitrify that can use different proteins and enzymes than *P.denitrificans* to catalyse the reduction of nitrogenous compounds. In soil samples it has been estimated that up to 10^6 bacterial genomes are present per gram of soil (Wu, Chellemi et al. 2008). Many soil bacteria are unable to be cultured, *Pseudomonas spp., Actinobacteria spp.,* and *Achromobacter spp.* are commonly found in many soil samples and are capable of denitrification (Dandie, Burton et al. 2007; Inceoglu, Hoogwout et al. 2010). Some of these bacterial species have genomes that encode the copper-type nitrite reductase, NirK, rather than the haem dependent cyt cd_1. Thus Cu limitation might impact denitrification in these species differently to the way the Cu limitation impacts on denitrification in *P.denitrificans* cultures.

7.1.1 *Achromobacter xylosoxidans*

A.xylosoxidans is capable of utilising NO_3^- and/or NO_2^- as an e^- acceptor in anaerobic environments. The denitrification process differs slightly compared to *P.denitrificans* in many ways, the NO_2^- reductase is the blue Cu containing NirK and the NO reductase is a qNor. *A.xylosoxidans* also contains two distinctive azurins, Az-I and Az-II, these are e^- transfer proteins, both contain a single Cu ion and are the physiological e^- donors for CuNir (Inoue, Shibata et al. 1994; Murphy, Dodd et al. 2002; Hough, Ellis et al. 2005; Harris, Eady et al. 2006). *In vitro* it has been found that Az-I is marginally more effective at e^- transfer to the CuNir, than Az-II (Kataoka, Yamaguchi et al. 2004; Harris, Eady et al. 2006). CuNir contains two Cu centres Cu(I) and Cu(II), e^- are transferred to the Cu(I) site via the azurins and then to the Cu(II) site where NO_2^- reduction occurs (Suzuki, Horikoshi et al. 1999; Hough, Ellis et al. 2005). This reaction has a low driving

force, due to the redox potential difference of the two Cu sites being small, this is so that the Cu(II) site is not reduced prematurely, which results in loss of enzyme activity (Hough, Ellis et al. 2005). The binding of NO_2^- increases the potential of the catalytic site, which consequently increases the driving force of e^- transfer from Cu(I) to Cu(II) (Hough, Ellis et al. 2005). In *A. xylosoxidans* this reaction can become rate limited due to protein-protein interactions with the azurins and the CuNir, rather than the catalytic centres. (Hough, Ellis et al. 2005). The reduction of NO to N_2O is carried out by the qNor, this is a quniol oxidising protein and therefore unlike Nor, qNor receives its electrons from a quinol, as it lacks the *c*-type cytochrome subunit (Hendriks, Oubrie et al. 2000; Braker and Tiedje 2003; Dandie, Burton et al. 2007). The qNor consists primarily of NorB along with containing an N-terminal extension allowing quinol oxidation (Cramm, Pohlmann et al. 1999; Hendriks, Oubrie et al. 2000).

Currently this research has investigated the effect of Cu and NO_3^- concentration on denitrification in *P.denitrificans*. In this Chapter the impact of NO_3^- and Cu concentration on denitrification in chemostat cultures of *A.xylosoxidans* will be investigated.

7.2 Results: The effect of Cu concentration on the growth of *Achromobacter xylosoxidans*

7.2.1 Batch Culture

A.xylosoxidans was capable of growing in *Paracoccus* minimal media, with an addition of 0.1% Yeast Extract for vital amino acids which *A.xylosoxidans* is unable to synthesise, growth was severely inhibited without the addition of Yeast Extract. *A.xylosoxidans* could sustain growth in NO_3^- -sufficient succinate-limited 18 µM and <0.5 µM Cu medium under aerobic and anaerobic conditions. Aerobic growth was sustainable due to the presence of cytochrome *bd* a haem dependent oxidase, the other haem-Cu

oxidases may also be active as there is potentially a small amount of Cu available to the culture. Compared to the batch cultures of *P.denitrificans,* the extent of growth in *A.xylosoxidans* batch cultures is similar, as the final OD_{600} is between 1-1.3 OD_{600} units (Figure 7.2-1). The µmax was determined using the log/OD_{600} (Figure 7.2-1B) and both the 18 µM and <0.5 µM Cu cultures the µmax was approximately 0.2 h^{-1}, which is similar to the µmax observed in the *P.denitrificans* batch cultures (Chapter 3, section 3.2.1.2). There was no apparent affect of the Cu concentration on the growth rate (µ) in the *A.xylosoxidans* batch cultures (Figure 7.2-1). The effects of Cu concentration on denitrification in *A.xylosoxidans* can therefore make a good comparison against the effects of Cu concentration on denitrification in *P.denitrificans* cultures.

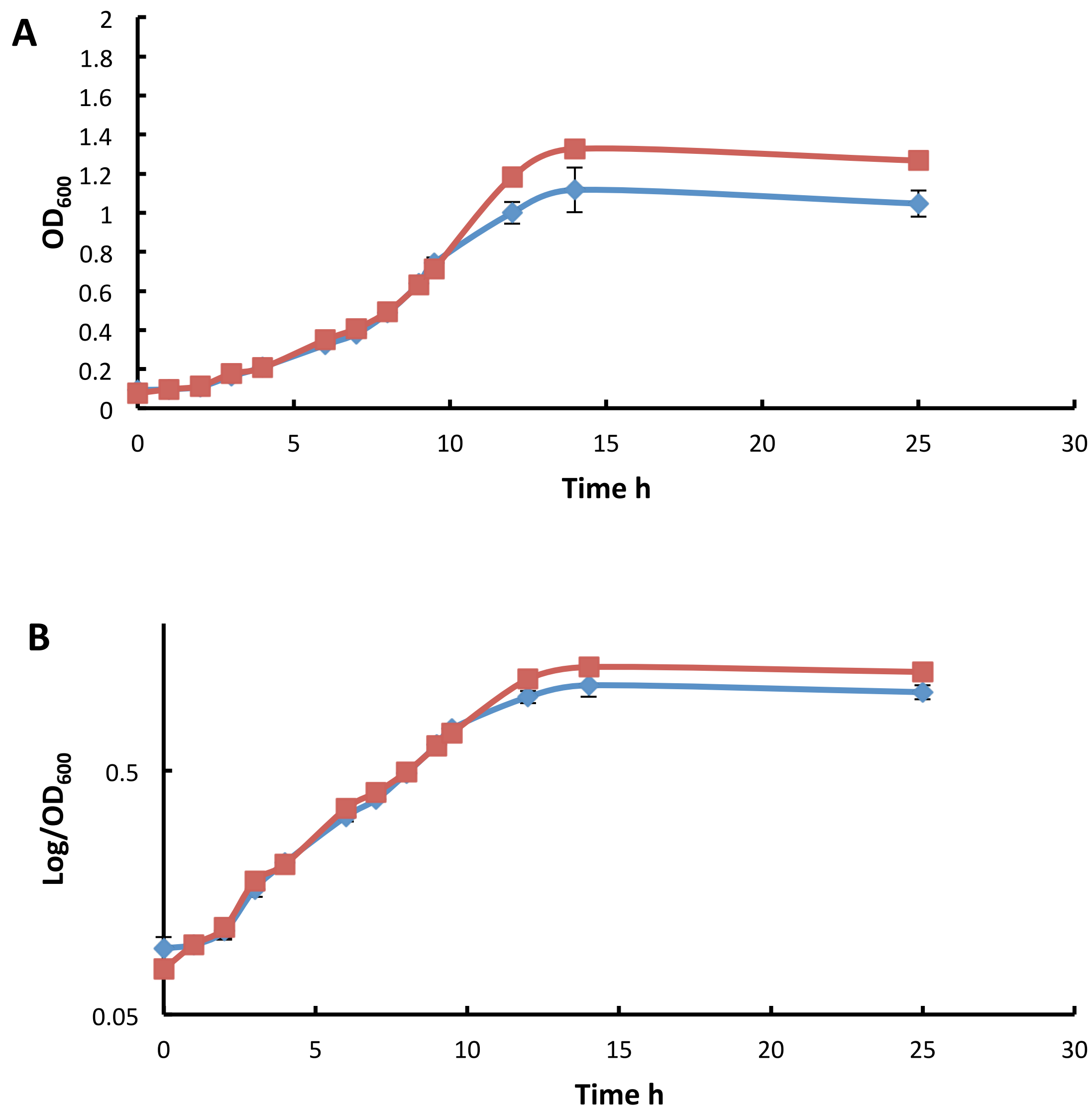


Figure 7.2-1 Batch culture experiments with *A.xylosoxidans,* with varying the Cu concentration, monitored over time by observing the OD_{600} (A) or Log/OD_{600} (B). The cultures were grown anaerobically in *Paracoccus* minimal medium with 0.1 % yeast extract, 20 mM NO_3^-, 5 mM succinate with either Cu 18 µM (◆) <0.5 µM Cu (■). The batch cultures were repeated three times for each condition, the error bars represent standard error.

7.2.2 The effect of Cu concentration on chemostat cultures of *A.xylosoxidans* grown in NO_3^- sufficient media.

A.xylosoxidans was grown in NO_3^- sufficient (20 mM) succinate limited (5 mM) *Paracoccus* media supplemented with 0.1% yeast extract, the Cu concentration was either at 50 µM, 18 µM or <0.5 µM and all parameters remained the same as *P.denitrificans* cultures. Due to the µmax being similar to that of the *P.denitrificans* batch cultures the D was set at 0.06 h^{-1}, the same as used for *P.denitrificans.*

7.2.2.1 The production of drymass in chemostat cultures of A.xylosoxidans

The first 24 h of growth was an aerobic batch culture, and therefore an increase in drymass was observed in all three cultures, however the Cu concentration had a significant effect on the aerobic growth (Figure 7.2-2). The 50 µM Cu culture showed approximately a 20 % decrease in drymass after 24 h of aerobic growth compared to the 18 µM Cu culture. At 24 h the air supply was removed and the feed was added at a D of 0.06 h^{-1}, the vessel became anaerobic 30 minutes after the air was turned off. The drymass began to decrease after the feed was added and after approximately 3 vessel volumes of feed were passed through the vessel, the 50 µM, 18 µM and <0.5 µM Cu cultures entered steady state at approximately 70 h. At steady state over 90 % of the original aerobic culture had been diluted out, theoretically the cultures could be maintained indefinitely but due to the risk of mutations and contamination the cultures were stopped at 120 h. At steady state there is a significant difference in the final drymass between the three cultures, as the Cu concentration increases the drymass decreases, suggesting that Cu has a toxic affect on *A.xylosoxidans* anaerobic cultures. At steady state the drymass in the 18 µM Cu cultures is approximately 0.11 mg/ml, which is approximately 30 % less growth than observed in the <0.5 µM Cu culture (Figure 7.2-2).

The effect of Cu concentration on anaerobically grown *A.xylosoxidans* cultures is illustrated by the growth yields (Table 7-1). The highest growth yield was in the <0.5 μM Cu cultures, the growth yield dropped by 30 % when the Cu was increased to 18 μM, and then a further 15 % when the Cu was increased to 50 μM. It was hypothesised that due to *A.xylosoxidans* encoding azurins and NosZ the demand for Cu would be higher, however as the Cu concentration is increased the growth yield is reduced which indicates that Cu is more toxic to *A.xylosoxidans* than in *P.denitrificans* continuous cultures, growth yields in the 18 μM and <0.5 μM Cu showed no significant difference.

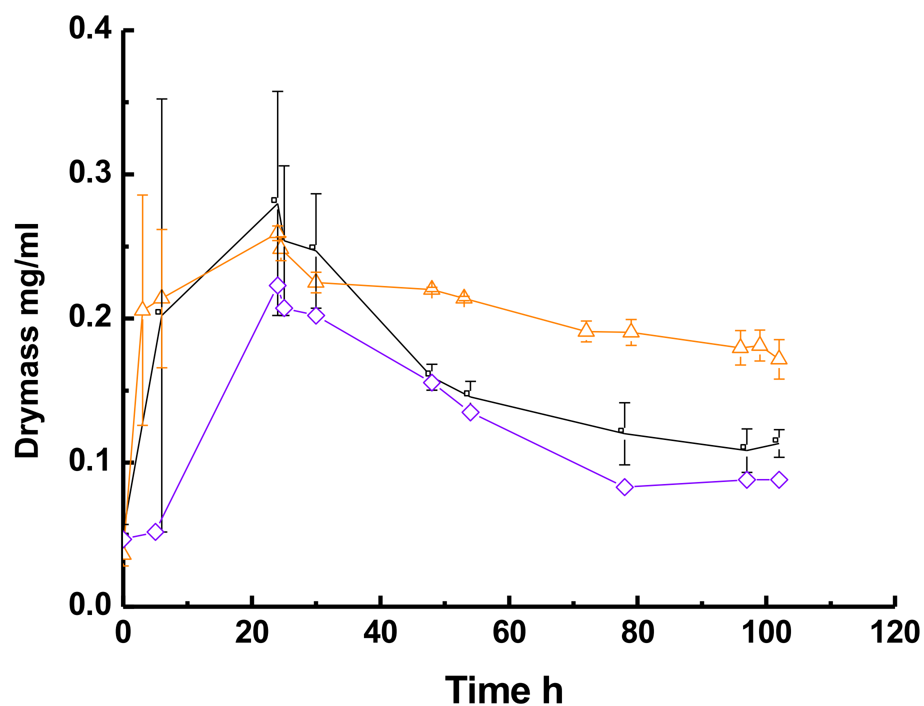


Figure 7.2-2 The drymass production of *A.xylosoxidans* chemostat cultures grown in NO_3^- -sufficient succinate-limited *Paracoccus* minimal media with either 50 μM (◇), 18 μM (□) or <0.5 μM Cu (△). At 24 h the air supply was removed and the feed was added (D=0.06 h^{-1}). Each chemostat culture was repeated three times for each conditions, the error bars represent the standard error.

7.2.2.2 The consumption of nitrate in chemostat cultures of A.xylosoxidans

In the first 24 h of growth there was little to no NO_3^- consumed and so aerobic NO_3^- reduction and NO_3^- assimilation were not occurring. The air supply was removed at 24 h and the consumption of NO_3^- began once the culture became anaerobic at approximately 24.5 h. When the cultures entered steady state the NO_3^- consumed was highest in the <0.5 µM Cu culture with a total of 20.3 ± 1.4 mM of NO_3^- being consumed (Figure 7.2-3). This was reduced by 30 % in the 18 µM and 50 µM Cu cultures, at steady state there is a net consumption of 15.6 ± 0.5 mM and 15.7 ± 0.05 mM of NO_3^-.

The specific rates of NO_3^- consumption, $qcNO_3^-$, reveal that the 18 µM and <0.5 µM Cu culture are similar (Table 7-1). The $qcNO_3^-$ is approximate 20 % lower in the 50 µM Cu culture than in the 18 µM and <0.5 µM Cu cultures. The highest $qcNO_3^-$ was observed in the 18 µM Cu culture. This could be due to the rate of the Nar system being higher in the 18 µM Cu culture, however a decrease in drymass was observed suggesting that the culture is under stress. The specific NO_3^- reductase activity measured by MV+ assays on whole cells isolated at steady state was significantly higher in the 18 µM Cu chemostat culture, 27 ± 10 $nmol^{-1}min^{-1}$mg drymass, compared to the <0.5 µM Cu culture which showed a 65 % drop in activity, 3 ± 1.4 $nmol^{-1}min^{-1}$mg $drymass^{-1}$ (Figure 7.2-4). NO_3^- reductase activity was highest in the 50 µM Cu culture, reaching 34 ± 15 $nmol^{-1}min^{-1}$mg $drymass^{-1}$ at steady state (Figure 7.2-4). The increase in NO_3^- reductase activity correlates with the increase in the $qcNO_3^-$. Therefore as Cu increases the activity of NO_3^- reductase increases. This could be potentially due to the maturation of NarGHI involving Cu in the Mo binding chaperone (Blasco, Guigliarelli et al. 2001). If the Mo is not inserted correctly then the activity of NarGHI would be profoundly reduced.

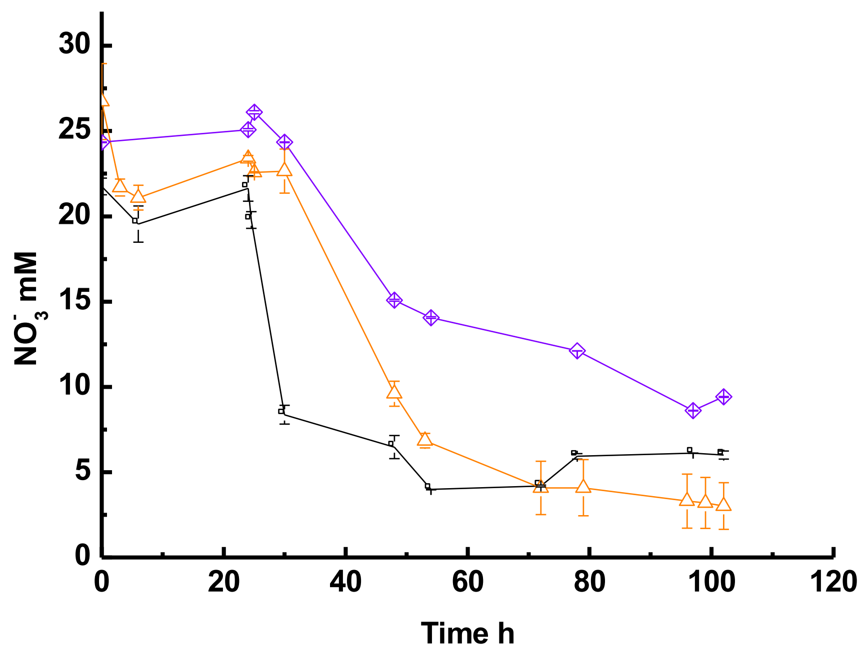


Figure 7.2-3 The concentration of NO_3^- in *A.xylosoxidans* chemostat cultures grown in NO_3^- sufficient *Paracoccus* minimal media with either 50 µM (◇), 18 µM (□) or <0.5 µM Cu (△).At 24 h the air supply was removed and the feed was added (D=0.06 h^{-1}). Each sample from the chemostat cultures was analysed twice, the error bars represent the standard error.

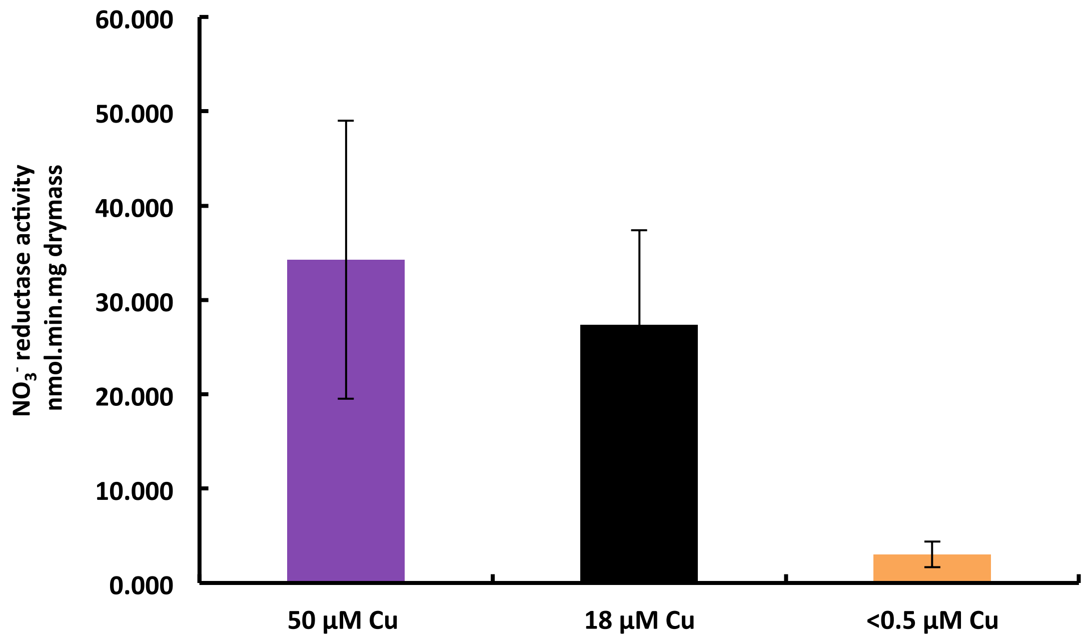


Figure 7.2-4 The activity of NO_3^-reductase at a 102 h in chemostat cultures of *A.xylosoxidans* grown in NO_3^- -sufficient succinate-limited *Paracoccus* media with either 50 µM (purple), 18 µM (black) or <0.5 µM Cu (orange). Each sample isolated from the chemostat cultures was analysed twice, the error bars represent the standard error.

7.2.2.3 Nitrite production kinetics in chemostat cultures of *A.xylosoxidans*

In chemostat cultures of *A.xylosoxidans* the NO_2^- concentration in the reactor vessel was found to accumulate to concentrations 1000 times more than observed in any *P.denitrificans* chemostat culture. In the 18 µM Cu chemostat at steady state the NO_2^- produced reached 4.0 ± 0.6 mM, which accounts for 30.2 % of the net NO_3^- consumed (Figure 7.2-5). The production of NO_2^- increased by 60 % in the <0.5 µM Cu culture, reaching a total of 10.1 ± 3 mM at steady state, accounting for 50.2 % of the total NO_3^- consumed (Figure 7.2-5). When the Cu concentration was increased to 50 µM the NO_2^- produced at steady state reached 6 ± 1.3 mM.

The specific rate of NO_2^- production ($qpNO_2^-$) shows no significant difference (Table 7-1). When the Cu concentration was increased to 50 µM the $qpNO_2^-$ is nearly 50 % higher than the rates observed in the 18 µM and <0.5 µM cultures. The fact that the 18 µM and <0.5 µM Cu cultures have a similar $qcNO_2^-$ indicates that the NO_2^- reductase is active in both cultures and therefore there must be a small amount of Cu available in the bioreactor and it is incorporated into NirK.

The specific NO_2^- reductase activity of NirK was measured using MV+ assays. The activity observed in the 18 µM culture reached 129 ± 23 $nmol^{-1}min^{-1}mg\ drymass^{-1}$ at steady state, the activity in the <0.5 µM Cu chemostat culture decreased by approximately 95 % to 6.5 ± 1.4 $nmol^{-1}min^{-1}mg\ drymass^{-1}$ (Figure 7.2-6). The activity observed in the 50 µM Cu culture increased 10 fold, compared to the 18 µM Cu culture (Figure 7.2-5). This suggests that the activity of NirK is directly dependent on the Cu concentration in NO_3^- sufficient cultures. This could potentially be due to an increase in NirK synthesis or more Cu centres being inserted into the NirK polypeptide in high Cu conditions. Under low Cu concentrations NirK is still active therefore there must be Cu available to the cells which is then incorporated into NirK. If the Cu available is incorporated into NirK, then it maybe that there is not sufficient Cu to form a functional NosZ, this will be discussed in the following section.

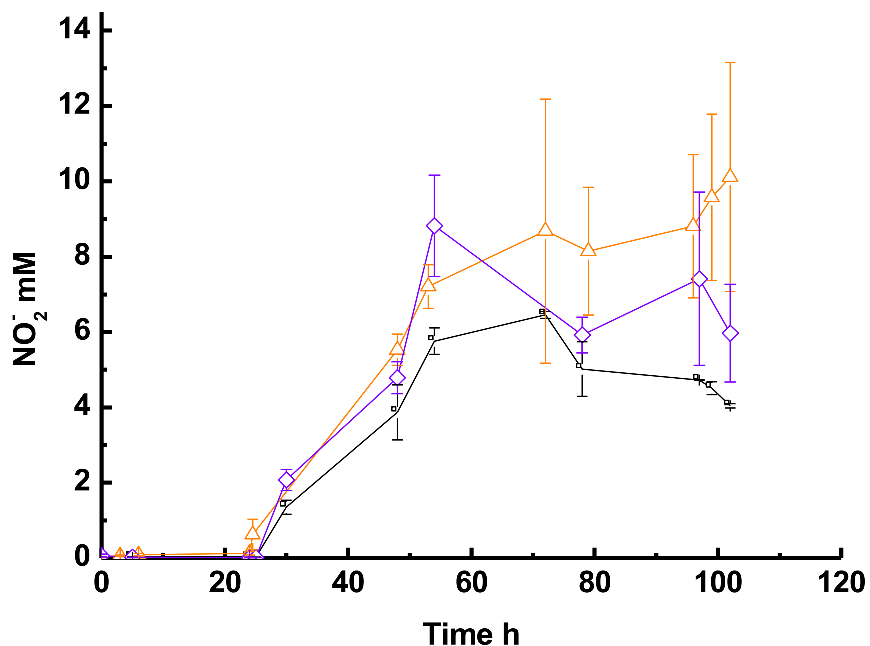


Figure 7.2-5 The accumulation of NO_2^- in *A.xylosoxidans* chemostat cultures grown in NO_3^- -sufficient succinate-limited *Paracoccus* minimal media with either 50 µM (◇), 18 µM (☐) or <0.5 µM Cu (△). At 24 h the air supply was removed and the feed was added (D=0.06 h^{-1}). Each sample from the chemostat cultures was analysed twice, the error bars represent the standard error.

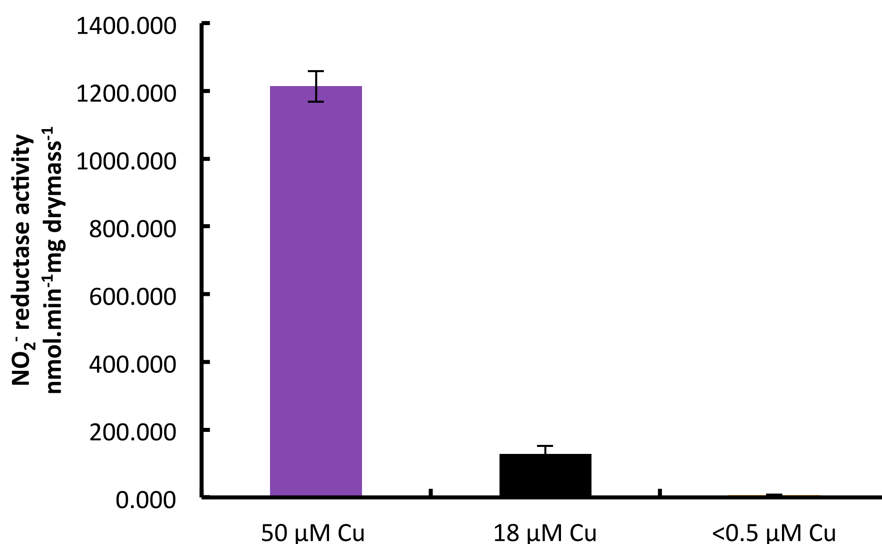
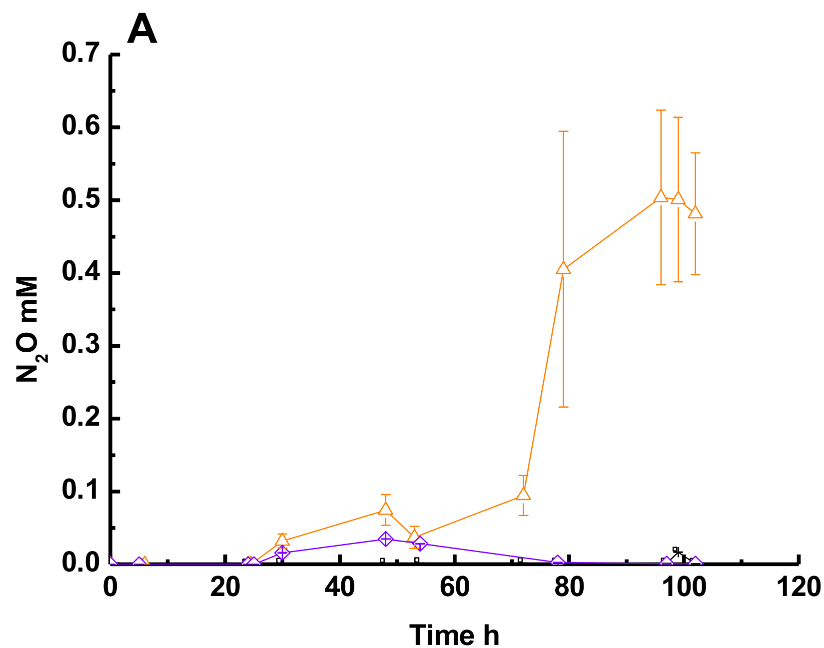


Figure 7.2-6 The specific activity of NO_2^- reductase at a 102 h in chemostat cultures of *A.xylosoxidans* grown in NO_3^- -sufficient succinate-limited *Paracoccus* media with either 50 µM (purple), 18 µM (black) or <0.5 µM Cu (orange). Each sample was analysed twice, the error bars represent standard error.

7.2.2.4 Nitrous oxide production kinetics in chemostat cultures of *A.xylosoxidans*

Throughout the time course of the *A.xylosoxidans c*hemostat cultures no NO was detected, and therefore NO_2^- was being reduced to NO and subsequently to N_2O. The N_2O released in the 18 µM Cu chemostat culture reached a maximum of 17 ± 0.2 µM, at steady state (Figure 7.2-7). The N_2O released from the <0.5 µM Cu chemostat culture increased by nearly 30 fold, totalling 500 ± 113 µM at 99 h (Figure 7.2-7). The N_2O produced in the 50 µM Cu chemostat culture shows a peak between 30-80 h. The production then decreases and at steady state the net accumulation of N_2O reaches 0.9 µM at 102 h. Therefore even in low Cu concentrations NirK is capable of reducing NO_2^- to NO and Nor is active and therefore N_2O is being produced. The activity of NosZ is reduced at low Cu concentration as N_2O production increases when Cu concentration is low.

The specific rate of N_2O production, qpN_2O, also indicates that as NosZ activity is dependent on the Cu concentration (Table 7-1). The qpN_2O observed in the 18 µM Cu culture is similar to that observed in the *P.denitrificans* 18 µM Cu NO_3^- sufficient culture, 0.001 ± 0.000 mmol.g drymass^{-1}h^{-1}. As the Cu concentration increased the qpN_2O decreased, suggesting that the activity of NosZ is increased. When the Cu concentration was decreased to <0.5 µM the qpN_2O increases by 250 fold to 0.03 ± 0.01 mmol.g drymass^{-1}h^{-1} (Table 7-1). However compared to the *P.denitrificans* <0.5 µM Cu NO_3^- sufficient culture the rate is approximately 18 fold less (Table 3.2-3). As NirK is active when Cu is limited, then it is plausible that the NosZ is also active, which is the case in the *P.denitrificans* cultures (Chapters 3, 4 and 6), this is discussed in the following section.



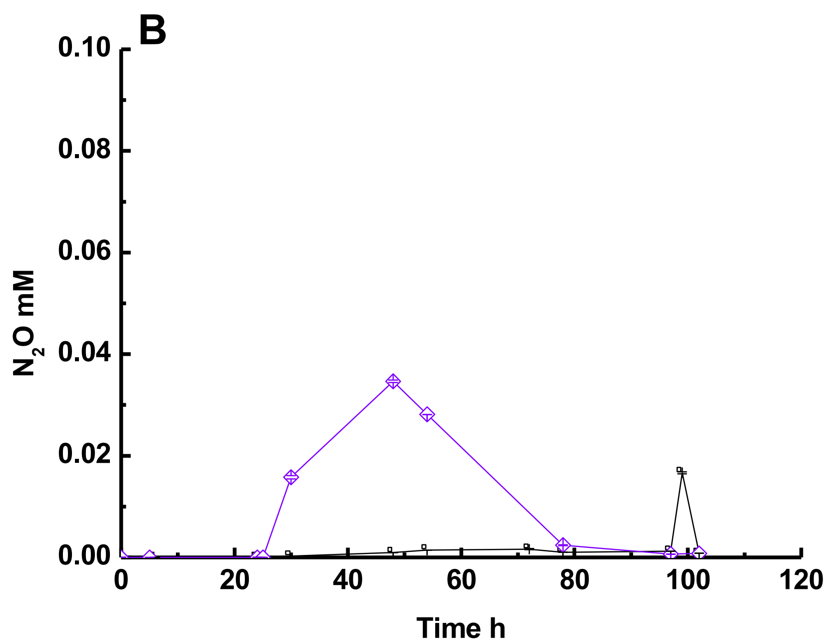


Figure 7.2-7 The production of N_2O in *A.xylosoxidans* chemostat cultures grown in NO_3^- sufficient *Paracoccus* minimal media with either 50 µM (◇), 18 µM (□) or <0.5 µM Cu (△).The second graph (B) shows the an enlarged view of the 50 µM (◇) and 18 µM (□) cultures. At 24 h the air supply was removed and the feed was added (D=0.06 h^{-1}). Each gas sample from the chemostat cultures was analysed twice, the error bars represent the standard error.

7.2.2.5 The production of dinitrogen in chemostat cultures of *A.xylosoxidans*

Under low Cu concentrations the activity of NirK and NosZ is reduced, as both NO_2^- and N_2O accumulate. At steady state the 18 µM Cu chemostat culture produced a total of 2.6 ± 0.9 mM of N_2. The amount of N_2 produced in the <0.5 µM Cu chemostat culture was 67 % lower than that observed in the 18 µM Cu culture, 0.87 ± 0.28 mM of N_2 accumulated at steady state. This therefore suggests that under low Cu concentrations NosZ is still active, however at a reduced rate.

The specific rate of N_2 production, qpN_2, also indicates that there is an increased production in the 18 µM Cu culture. In the <0.5 µM Cu culture the qpN_2 is decreased by approximately 60 % (Table 7-1). This could potentially be due to a reduced amount of polypeptide being synthesised or that due to a low Cu concentration that the Cu centres in NosZ are not being inserted correctly, consequently reducing the activity.

7.2.2.6 The flux of NO_3^- to N_2

The rates for the consumption of NO_3^- and the production of NO_2^-, N_2O and N_2 can illustrate the flux of NO_3^- through to N_2 in the chemostat cultures during steady state. The specific rate of NO_2^- consumption, $qcNO_2^-$, indicates the N flux. The $qcNO_2^-$ in the 18 µM Cu chemostat culture matches the qN_2, therefore suggesting that the rate at which the NO_2^- consumed is the same as the rate of N_2 production (Table 7-1). The flux of N through the <0.5 µM Cu culture is lower than that observed in the 18 µM Cu culture, and as observed in the 18 µM Cu culture the majority of the NO_3^- is reduced to N_2 (Table 7-1).

Table 7-1 The rate for NO_3^- consumption and NO_2^-, N_2O and N_2 production in *A.xylosoxidans* chemostat cultures grown in 20 mM NO_3^-, 5 mM succinate *Paracoccus* media with either 50 µM, 18 µM or <0.5 µM Cu. The productivity rates were calculated at steady state (102h).

	Growth Yield	qcNO₃⁻	qpNO₂⁻	qcNO₂⁻	qN₂O	qN₂
	mg.ml.h^{-1}	mmol.g drymass^{-1}h^{-1}				
18 µM Cu	0.025 ±0.002	5.378 ±0.151	0.467 ±0.124	0.978	1.1x10^{-4} ±1.5x10^{-6}	0.504 ±0.209
<0.5 µM Cu	0.037 ±0.003	4.744 ±1.01	0.487 ±0.182	0.787	0.029 ±0.006	0.210 ±0.132
50 µM Cu	0.019	4.378 ±2.514	0.693 ±0.12	1.163	9.29x10^{-5} 5.3x10^{-8}	NA

Growth Yield -biomass production = Dx; where x = the biomass at steady-state.

NO_3^- consumption $(qcNO_3^-) = ([NO_3^-]R - [NO_3^-]r)\,D/x$; where $[NO_3^-]R$ is the NO_3^- concentration in the reservoir, $[NO_3^-]r$ is the residual NO_3^- concentration in the reactor vessel, D is the dilution rate and x is the protein concentration in the reactor vessel.

NO_2^- production $(qp\,NO_2^-) = [NO_2^-]\,r\,D/x$ where $[NO_2^-]r$ is the NO_2^- concetration in the reactor vessel.

NO_2^- consumption $(qc\,NO_2^-) = qcNO_3^- - qp\,NO_2^-$

N_2O production $(q\,N2O) = [N_2O]\,r\,D/x$; where $[N_2O]r$ is the N_2O concentration in the reactor vessel.

N_2 production $(q\,N_2) = [N_2]\,D/x$ where $[N_2]r$ is the N_2 concentration in the reactor vessel.

Negative (-) numbers indicate the consumption of the intermediate

NA – not applicable, 50 µM Cu chemostat cultures were not run with ^{15}N enriched NO_3^-

7.2.3 The effect on copper concentration on the transcription of *nosZ* in NO_3^- sufficient *A.xylosoxidans* chemostat cultures

Real Time PCR was used to report on the transcription of *nosZ* and *RpoB,* which is a house keeping gene and used to normalise the results. RT PCR is a quantitive form of PCR and can be used to identify increased expression of the target genes. Primers against *rpoB* and *nosZ* were used in the PCR reaction with a TAMERA reporter. RNA was isolated in the first 4 h of aerobic growth and then again at 102 h when the culture reached steady state. The RT PCR shows that *nosZ* is transcribed aerobically in 18 µM and <0.5 µM Cu (Figure 7.2-8). The expression of *nosZ* is not significantly different from the RNA isolated aerobically and anaerobically (Figure 7.2-8). This therefore shows that *nosZ* expression in *A.xylosoxidans* is not repressed by low Cu concentration; therefore N_2O can be reduced to N_2 in low Cu environments.

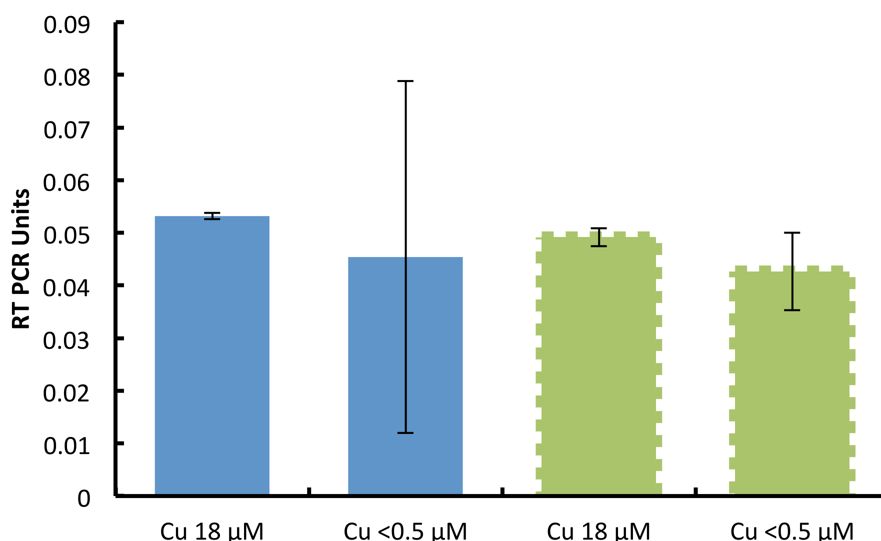


Figure 7.2-8 Real Time PCR showing the gene expression of *nosZ A.xylosoxidans* NO_3^- -sufficient succinate-limited chemostat cultures either with 18 µM or <0.5 µM Cu. The samples were taken aerobically (blue) at 4 h and at anaerobic steady state (green, dotted outline), at 102 h. The data was normalised with *RpoB* as the housekeeping gene. Each RNA sample isolated was analysed twice from each chemostat culture, the error bars represent the standard error.

7.2.4 The effect of Cu concentration on chemostat cultures of *A.xylosoxidans* grown in NO_3^- -limited succinate-sufficient media

7.2.4.1 The production of drymass in chemostat cultures of *A.xylosoxidans*

The media used in the previous section was NO_3^- -sufficient succinate-limited. To make the media NO_3^- limited the NO_3^- concentration was reduced to 5 mM, the succinate concentration was increased to 20 mM and the Cu concentration was either 18 µM or <0.5 µM. The first 24 h of growth in the chemostat cultures is an aerobic batch culture and as seen in the previous *A.xylosoxidans* culture. After 24 h of aerobic growth the 18 µM Cu culture shows 35 % less growth density than the <0.5 µM Cu culture (Figure 7.2-9). The drymass of the <0.5 µM Cu culture at 24 h was 0.58 ± 0.03 $mg.ml^{-1}$, which is nearly double the mass observed in at 24 h in the NO_3^- -sufficient succinate-limited <0.5 µM Cu culture. This is due to an increased yield from aerobic respiration because of the high succinate and oxygen concentration. At 24 h the air was removed and the feed was added, after 30 min the vessel had become anaerobic. In both cultures after 3 h of anaerobic growth the drymass increased slightly. The drymass begins to drop off and after 80 h both the 18 µM and the <0.5 µM Cu culture enter steady state. At 102 h the 18 µM Cu cultures drymass is approximately 20 % below the <0.5 µM Cu culture (Figure 7.2-9). This was also observed in the NO_3^- sufficient cultures confirming that high Cu concentrations may be toxic to *A.xylosoxidans.*

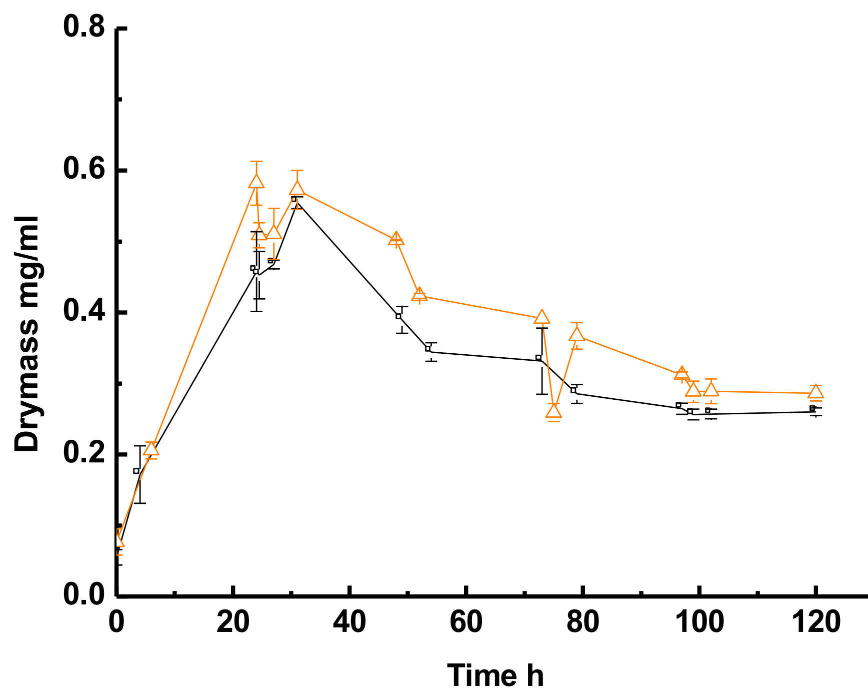


Figure 7.2-9 The drymass of *A.xylosoxidans* chemostat cultures grown in NO_3^- -limited succinate-sufficient *Paracoccus* minimal media with either 18 µM (□) or <0.5 µM Cu (△). At 24 h the air supply was removed and the feed was added (D=0.06 h^{-1}). The chemostat cultures were repeated three times for each, the error bars represent the standard error.

7.2.4.2 The consumption of nitrate in chemostat cultures of A.xylosoxidans

There was no NO_3^- reduction observed during the aerobic phase of the chemostat cultures. Once the air supply was removed at 24 h NO_3^- reduction began immediately in both cultures. The cultures became completely anaerobic after 30 minutes, and by then approximately 1.5 mM of NO_3^- is consumed in both the 18 µM and <0.5 µM Cu cultures. Therefore Cu concentration shows no significant affect on the reduction of NO_3^-, when NO_3^- is limited. After 31 h (7 h of anaerobic growth) 95 % of the NO_3^- was being consumed, and after 48 h 99 % of all the NO_3^- was being consumed (Figure 7.2-10). Thereafter the NO_3^- was unable to be detected and was presumed to completely consumed throughout the rest of the chemostat cultures (i.e. the rate of input is equal to the rate of consumption).

The specific rate of NO_3^- consumption ($qcNO_3^-$) between the 18 µM and <0.5 µM Cu chemostat cultures show no significant difference, due to both cultures consuming the same amount of NO_3^- (Table 7-2). Due to the NO_3^- being limited the $qcNO_3^-$ observed in the NO_3^- sufficient cultures is significantly higher (Table 7-1).

The specific activity of the NO_3^- reductase enzyme at steady state also showed no significant difference between the 18 µM and <0.5 µM Cu chemostat cultures (Figure 7.2-11). When the Cu was reduced in the NO_3^- limited culture there was little affect on Nar activity which was 32 ± 6 $nmol^{-1}min^{-1}mg\ drymass^{-1}$. This is 10 times higher than the rate observed in the NO_3^- sufficient culture (Figure 7.2-4 and Figure 7.2-11). Therefore when NO_3^- is limited the concentration of Cu has no significant affect on the specific rate of NO_3^- reduction, contrary to that observed in the NO_3^- sufficient chemostat cultures. This is potentially due to the culture being e^- acceptor limited and therefore the maximum amount of NO_3^- is utilised in both the 18 µM and <0.5 µM Cu cultures.

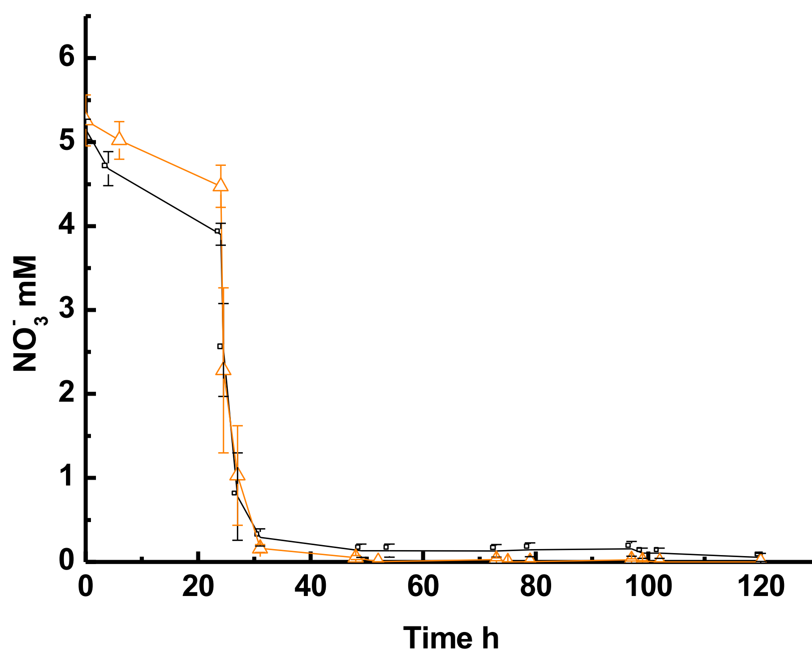


Figure 7.2-10 The consumption of NO_3^- in *A.xylosoxidans* chemostat cultures grown in NO_3^- -limited succinate-sufficient *Paracoccus* minimal media with 18 µM (□) or <0.5 µM Cu (△). At 24 h the air supply was removed and the feed added (D= 0.06 h^{-1}). Each sample from the chemostat cultures was analysed twice, the error bars represent the standard error.

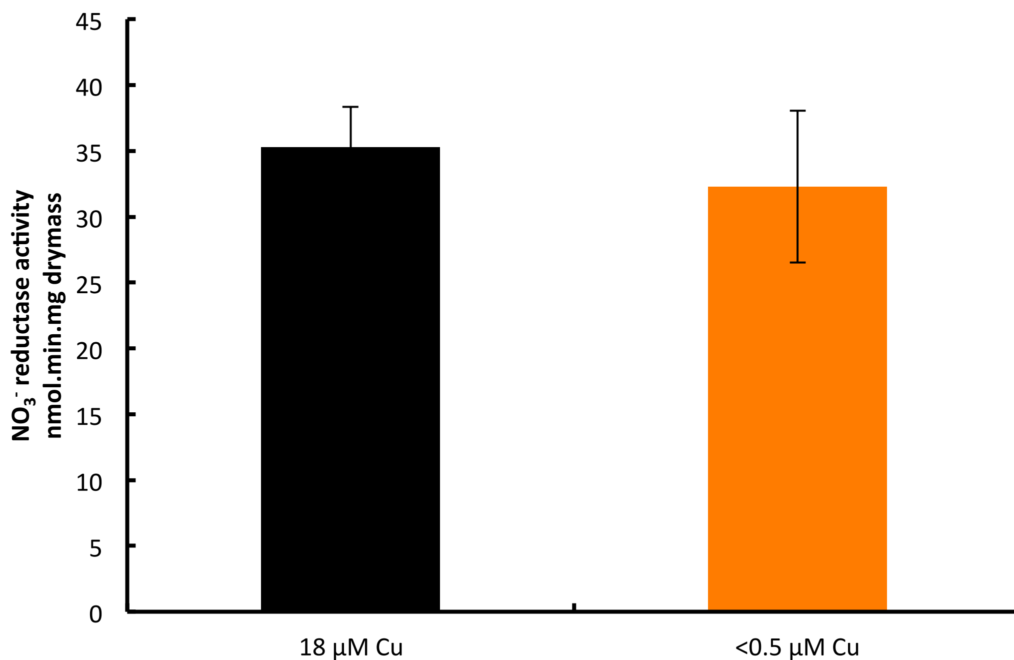


Figure 7.2-11 The activity of NO_3^-reductase at a 102 h in chemostat cultures of *A.xylosoxidans* grown in NO_3^- -limited succinate-sufficient *Paracoccus* media with

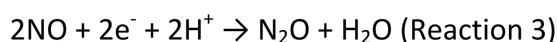
either 18 µM (black) or <0.5 µM Cu (orange). Each sample isolated from the chemostats were analysed twice, the error bars represent standard error

7.2.4.3 Nitrite production kinetics in chemostat cultures of A.xylosoxidans

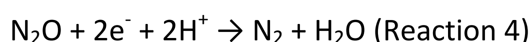
A.xylosoxidans uses a Cu dependent enzyme, NirK, to reduce NO_2^- to NO. This research has shown that NirK is active in the <0.5 µM Cu NO_3^- sufficient chemostat culture, but at a lower rate than 50 µM and 18 µM Cu cultures (section 7.2.2.3). In the first 24 h of growth there was a small amount of NO_2^- present which is from contamination of the media, or a small amount of carry over from the inoculum. Little to no NO_2^- was consumed or produced during the aerobic phase. After the culture became anaerobic at 24.5 h, NO_3 consumption began and NO_2^- then started to be produced, and therefore Reaction 1 is faster than Reaction 2:

$$2NO_3^- + 4e^- + 4H^+ \rightarrow 2NO_2^- + 2H_2O \quad \text{(Reaction 1)}$$

$$2NO_2^- + 2e^- + 4H^+ \rightarrow 2NO + 2H_2O \quad \text{(Reaction 2)}$$



$$2NO + 2e^- + 2H^+ \rightarrow N_2O + H_2O \quad \text{(Reaction 3)}$$



$$N_2O + 2e^- + 2H^+ \rightarrow N_2 + H_2O \quad \text{(Reaction 4)}$$

In the 18 µM Cu culture the NO_2^- is consumed by 54 h, and the NO_2^- then begins to accumulate at 96 h to 0.23 ± 0.1 mM at 102 h. This is significantly lower than the NO_2^- produced in the NO_3^- sufficient 18 µM Cu culture. The production of NO_2^- in the <0.5 µM Cu culture initially occurs at 24.5 h, once NO_3^- consumption begins, similar to the 18 µM Cu culture. The NO_2^- is then completely consumed by 51 h, the NO_2^- then begins to accumulate again at 74 h.

The specific production rate of NO_2^-, $qpNO_2^-$, in the 18 µM Cu chemostat culture is a negative value which represents NO_2^- consumption is higher than NO_2^- production (Table 7-2). Therefore by steady state the NO_2^- concentration is lower than the initial trace NO_2^- concentration in the medium feed, When the Cu is reduced to <0.5 µM the production of NO_2^- is increased compared to the 18 µM Cu culture,

however this is <1 % of the rate observed in the <0.5 µM Cu NO_3^- sufficient chemostats.

The specific activity of NirK was monitored at steady state in both the 18 µM and <0.5 µM Cu chemostat cultures. In the <0.5 µM Cu chemostat culture NirK activity was unable to be detected throughout the chemostat culture using MV+ aassays. NirK activity was detected in the 18 µM Cu culture, reaching 116 ± 29 nmol^{-1}min^{-1}g drymass at steady state, this is a similar rate to that observed in the NO_3^- sufficient 18 µM Cu culture (128 ± 22 nmol^{-1}min^{-1}mg drymass). NO_2^- reduction occurs in the <0.5 µM Cu culture as the NO_2^- is consumed between 52-78 h, but due to the low NO_2^- concentrations NirK could potentially be active but at lower rate, which is undetectable by MV + assays.

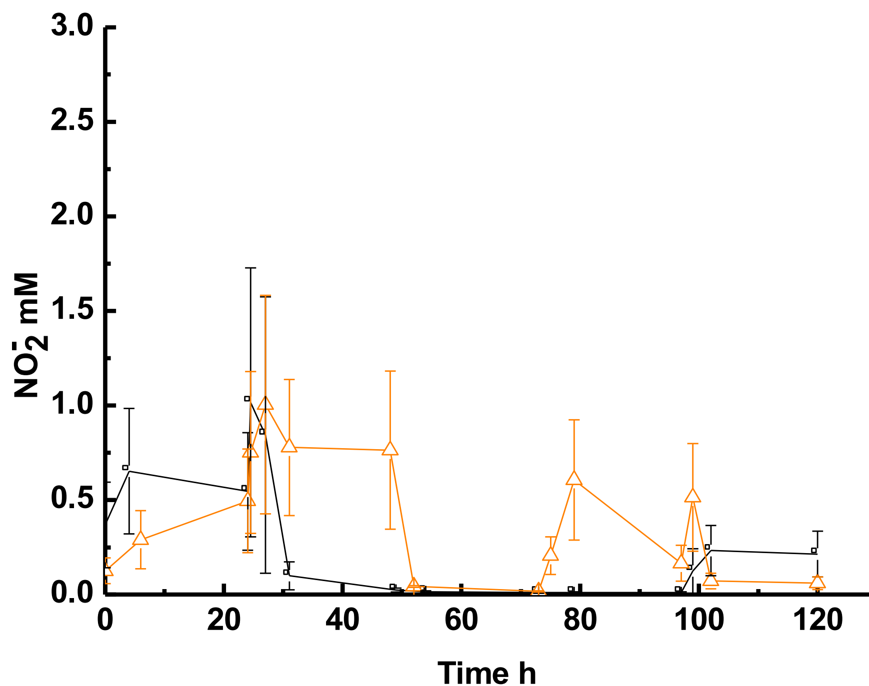


Figure 7.2-12 The production of NO_2^- in *A.xylosoxidans* chemostat cultures grown in NO_3^- -limited succinate-sufficient *Paracoccus* minimal media with 18 µM (☐) or <0.5 µM Cu (△). At 24 h the air supply was removed at 24 h and the feed was added (D= 0.06 h^{-1}). Each sample from the chemostat cultures was analysed twice, the error bars represent the standard error.

7.2.4.4 Nitrous oxide production kinetics in chemostat cultures of *A.xylosoxidans*

During the aerobic phase of the NO_3^- -limited succinate-sufficient chemostat culture no N_2O was produced in either the 18 µM or <0.5 µM Cu cultures. Once NO_3^- consumption began at 24.5 h the accumulation of N_2O also begins, with N_2O reaching 4.7 ± 2 µM in the 18 µM Cu culture and 64 ± 15.7 µM in the <0.5 µM Cu culture by 28 h (Figure 7.2-13). The production of N_2O in the 18 µM Cu chemostat culture at steady state is approximately 10 times lower than that observed in the <0.5 µM Cu chemostat culture (Figure 7.2-12 B). Therefore NirK must be active as NO_2^- is being reduced to NO and subsequently N_2O, the production of N_2O occurs as the reduction of N_2O to N_2 (reaction 4) is slower than the reduction of NO_3^- to N_2O (reactions 1, 2 and 3). Once N_2O stops accumulating, NosZ is reducing N_2O to N_2 in the <0.5 µM Cu cultures.

The specific rate of N_2O production, qpN_2O, in the NO_3^- limited chemostat cultures is significantly lower than the rates observed in the NO_3^- sufficient chemostat cultures. The qpN_2O in the 18 µM Cu culture is lower than the qcN_2O observed in the <0.5 µM Cu chemostat culture (Table 7-2). The rates observed in the NO_3^- limited cultures are also lower than the qcN_2O in the *P.denitrificans* NO_3^- limited chemostat cultures (Table 6.2-1). It appears that Cu concentration in *A.xylosoxidans* NO_3^- limited chemostat culture does not significantly affect the activity of NosZ. By completely reducing NO_3^- to N_2 the maximum concentration of electron acceptors available are consumed maximising energy conservation under these e^- acceptor limited culture conditions.

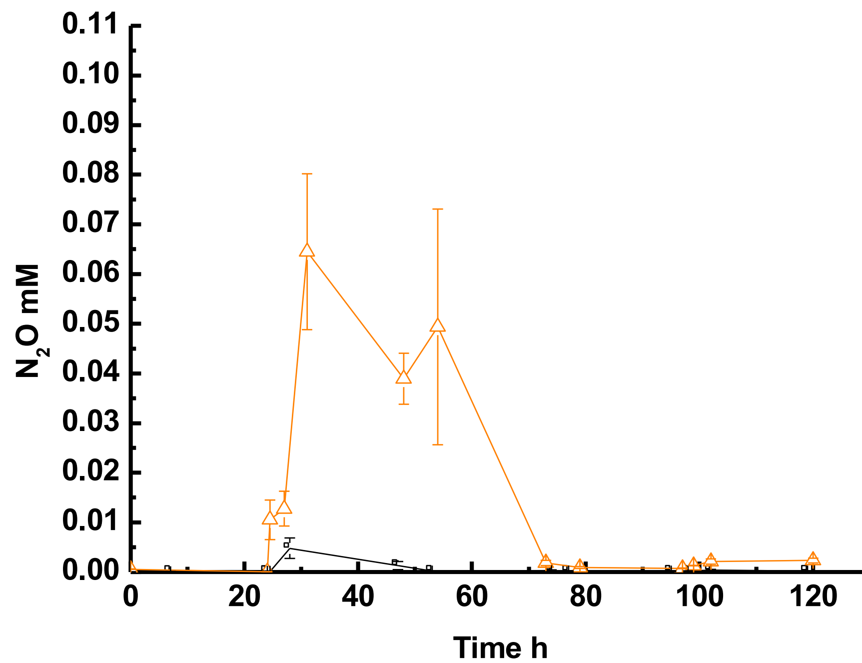


Figure 7.2-13 The production of N_2O in *A.xylosoxidans* chemostat cultures grown in NO_3^- -limited succinate-sufficient *Paracoccus* minimal media with 18 µM (☐) or <0.5 µM Cu (△). At 24 h the air supply was removed at 24 h and the feed was added (D= 0.06 h^{-1}). Each gas sample from the chemostat cultures was analysed twice, the error bars represent the standard error.

7.2.4.5 The production of dinitrogen in chemostat cultures of *A.xylosoxidans*

The production of N_2 is dependent on the activity of NosZ. In the 18 µM Cu chemostat culture a total of 586 ± 87 µM of N_2 was detected at steady state, which accounts for 23 % of the total NO_3^- consumed. When the Cu was reduced to <0.5 µM the N_2 produced marginally decreased to 443 ± 181 µM which accounted for 17.5 % of the NO_3^- consumed. Therefore NosZ is active despite the Cu concentration. The specific rate of N_2 production is 60 % higher in the 18 µM Cu culture than the <0.5 µM Cu culture (Table 7-2); this could potentially be due to a decrease in NosZ activity. Only 20 % of the total $^{15}N\text{-}NO_3^-$ was recovered as $^{15}N\text{-}N_2$. In theory 2.5 mM of N_2 should have been detected, however due to continuous cultures being an open system gases are able to diffuse and so the N_2 recovered may therefore be lower. NO_3^- is also a source of N which is used in amino acid synthesis and so a proportion of ^{15}N may have been used for NO_3^- assimilation.

7.2.4.6 Flux of NO_3^- to N_2 at steady state

The flux of N though the NO_3^- -limited succinate-sufficient chemostat cultures, $qcNO_3^-$, is lower than that observed in the NO_3^- sufficient chemostat cultures, therefore the N flux is sufficiently low to be accommodate the Nir system. This is also illustrated by the production rate of NO_2^- being significantly lower than in the NO_3^- sufficient chemostats. This indicates that there is a Cu hierarchy where by Cu is incorporated into the Nir system before the Nos system. This can be supported by the production of N_2O in the <0.5 µM Cu culture during the first 40 h of anaerobic growth, the NosZ then becomes active and N_2O is reduced.

Table 7-2 Productivity rates for NO_3^- consumptions and NO_2^-, N_2O and N^{15}-N_2 accumulation from the *A.xylosoxidans* chemostat cultures grown in 5 mM NO_3^-, 20 mM succinate *Paracoccus* media with either 18 µM or <0.5 µM Cu. The productivity rates were calculated at steady state (102h).

	Growth Yield	$qcNO_3^-$	$qpNO_2^-$	$qcNO_2^-$	qN_2O	qN_2
	$mg.ml.h^{-1}$	$mmol.g\ drymass.h^{-1}$				
18 µM Cu	0.01 ±0.0003	0.52 ±0.005	-0.016 ±0.005	0.504	$5x10^{-5}$ ±$4.4x10^{-5}$	0.061 ±0.001
<0.5 µM Cu	0.012 ±0.0008	0.46 ±0.008	$4.9x10^{-6}$ ±$4.3x10^{-6}$	0.46	$4.87x10^{-6}$ ±$4.3x10^{-6}$	0.038 ±0.0005

NO_3^- consumption $(qcNO_3^-) = ([NO_3^-]R - [NO_3^-]r)\ D / x$; where $[NO_3^-]R$ is the NO_3^- concentration in the reservoir, $[NO_3^-]r$ is the residual NO_3^- concentration in the reactor vessel, D is the dilution rate and x is the protein concentration in the reactor vessel.

NO_2^- production $(qp\ NO_2^-) = [NO_2^-]\ r\ D / x$ where $[NO_2^-]r$ is the NO_2^- concetration in the reactor vessel.

NO_2^- consumption $(qc\ NO_2^-) = qcNO_3^- - qp\ NO_2^-$

N_2O production $(q\ N2O) = [N2O]\ r\ D / x$; where $[N_2O]_r$ is the N_2O concentration in the reactor vessel.

N_2 production $(q\ N_2) = [N_2]\ r\ D / x$ where $[N_2]_r$ is the N_2 concentration in the reactor vessel.

Negative (-) numbers indicate the consumption of the intermediate

7.2.5 Real time PCR from *A.xylosoxidans* chemostat cultures grown in NO_3^- -limited succinate-sufficient media

The RTPCR was carried out on RNA samples isolated from NO_3^- -limited succinate-sufficient 18 µM Cu and <0.5 µM Cu cultures after 4 h (aerobic) and 102 h (anaerobic). The expression of *nosZ* is normalised by using a house keeping gene, *RpoB*. Overall there is a tenfold increase in the transcription of *nosZ* in the NO_3^--limited chemostats compared to the NO_3^- sufficient chemostats (Figure 7.2-8 and Figure 7.2-14). The transcription of *nosZ* occurs during the aerobic growth, the transcription of *nosZ* in the <0.5µM Cu culture is nearly 5 times higher than in the 18 µM Cu culture (Figure 7.2-14). The transcription of *nosZ* during the anaerobic phase is also increased in the <0.5 µM Cu chemostat culture compared to the 18 µM Cu chemostat, there is approximately a 2.5fold increase. In both cultures the expression of *nosZ* is lower during the anaerobic phase than the aerobic phase, which suggests that *nosZ* is not solely regulated by FNR in *A.xylosoxidans*. *nosZ* is upregulated in the NO_3^--limited succinate-sufficient <0.5 µM Cu cultures which suggests that Cu may play a role in the transcription of *nosZ*, however under NO_3^- -sufficient succinate-limited cultures *nosZ* transcription is not upregulated (Figure 7.2-8). The increase in *nosZ* expression under aerobic conditions was not expected and so potentially the RNA obtained from the chemostat cultures was not of high enough quality or the RTPCR was not carried out correctly. RNA isolation from chemostat cultures is more difficult than from batch cultures and so although the RNA was not degraded it may have been of a lower quality.

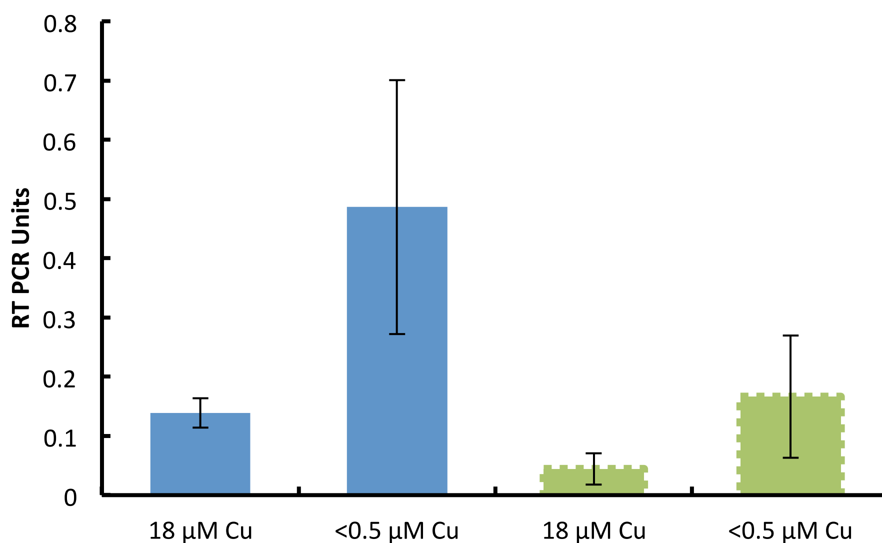


Figure 7.2-14 Real Time PCR showing the gene expression of *nosZ* in NO_3^- -limited succinate-sufficient *A.xylosoxidans* chemostat cultures either with 18 µM or <0.5 µM Cu. The samples were taken aerobically (blue) at 4 h and at anaerobic steady state (green, dotted outline), at 102 h. The data was normalised with *Rpo* as the housekeeping gene. Each RNA sample isolated was analysed twice from each chemostat culture, the error bars represent the standard error.

7.3 Discussion

The production of N_2O in chemostat cultures of *A.xylosoxidans* is significantly increased in NO_3^- sufficient and Cu limited conditions. The Cu concentration also showed a significant effect on the activity of NirK despite the NO_3^- concentration. The production of NO_2^- is constant throughout the anaerobic phase in the NO_3^- sufficient chemostat cultures, and in the NO_3^- -limited succinate-sufficient cultures NO_2^- production was approximately 10 fold lower, therefore Reaction 2 is consistently slower than Reaction 1 in *A.xylosoxidans* chemostat cultures. Previous work by Tocheva *et al.* (2008) suggests that NO_3^-, NO and N_2O can bind to the Cu ions in the NirK protein. When NO_3^- was bound to the Cu ions the catalytic activity of NirK was inhibited, therefore in higher NO_3^- environments the rate of NO_2^- could potentially be lower. It was thought that N_2O does not inhibit the activity as it binds similarly to the Cu centres as NO.

Due to the accumulation of NO_2^- the available e^- acceptors, NO and N_2O, will therefore be lower in concentration, ie. the flux of N ($qcNO_2^-$ Table 7-1) is reduced in the in the NO_3^- sufficient *A.xylosoxidans* chemostat culture is lower than that observed in the *P.denitrificans* NO_3^- sufficient cultures (Table 3.2-3). Consequently there was significantly less N_2O released from the *A.xylosoxidans* cultures. Therefore the release of N_2O in low Cu conditions fluctuates between bacterial species. Despite denitrification in *A.xylosoxidans* being heavily dependent on Cu, as it is involved in e^- transfer, azurins, NirK and NosZ as Cu concentration increased there was a negative effect on drymass. This implies that Cu has more of a toxic affect on *A.xylosoxidans* than *P.denitrificans.* Previous research suggests that Cu concentration in soils has no significant affect the microbial biomass until Cu concentration reaches > 1mM (Wang, Shi et al. 2007; Hamzah, Arifin et al. 2009). However the microbial diversity may change, as *P.denitrificans* is more tolerable to Cu than *A.xylosoxidans.*

NO_3^- limitation has the most significant affect on N_2O mitigation in the *P.denitrificans* and *A.xylosoxidans* chemostat cultures. Due to limited electron acceptors being available, the complete catabolism of 20 mM succinate can release a

maximum of 320 mM electrons. Therefore in the 5 mM NO_3^- media the reservoir-feed provides a potential sink for the consumption of 25 mM electrons. Therefore the cultures are very electron acceptor limited, therefore most of the intermediates/electron acceptors are used as to maximise energy conservation. Agricultural soils are high in NO_3^- and therefore electron acceptor sufficient, if Cu concentration is limited then the release of N_2O from the soils could increase significantly. Another significant factor when investigating the rate of denitrification and the accumulation of intermediates is pH, further work will be carried out to investigate the effects of pH on N_2O production. Preliminary work has been carried out and is discussed in the following Chapter (Chapter 8). The production of NO_2^- in the NO_3^- -sufficient succinate-limited chemostat cultures is significantly higher, than the NO_2^- concentrations observed in the *P.denitrificans* cultures (Chapter 3). In soils the release of NO_2^- as an intermediate can act as a substrate for nitrification, where NO_2^- is oxidised to NO_3^-. Therefore if NO_2^- accumulation is high and Cu is limited potentially *nitrobacter* spp. can oxidise NO_2^- to NO_3^- at the soil anoxic interface. Denitrifying bacteria can then reduce the NO_3^- to NO_2^-, therefore short cutting the denitrification process.

Chapter 8: Discussion and Future Prospects

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8.1 Overview of the key results of this thesis

This research has shown that NO_3^- and Cu concentration have a significant effect on the synthesis of proteins involved in denitrification in the model denitrifying species *P.denitrificans* and *A.xylosoxidans.*

The net consumption of NO_3^- in both the *P.denitrificans* and *A.xylosoxidans* NO_3^- sufficient cultures was increased as the Cu concentration became limited. Under NO_3^- -limited succinate-sufficient conditions, after 24 h of anaerobic growth NO_3^- was unable to be detected and therefore presumed to be completely consumed. The growth yield of *A.xylosoxidans* was on average 20 % lower in the NO_3^- -sufficient succinate-limited chemostat compared to the *P.denitrificans* NO_3^- sufficient cultures. Under NO_3^- limitation succinate sufficient conditions there was no significant difference in the growth yields. The $qcNO_3^-$ observed in all the chemostat cultures was higher in the NO_3^- sufficient *A.xylosoxidans* cultures (Figure 8.1-1).

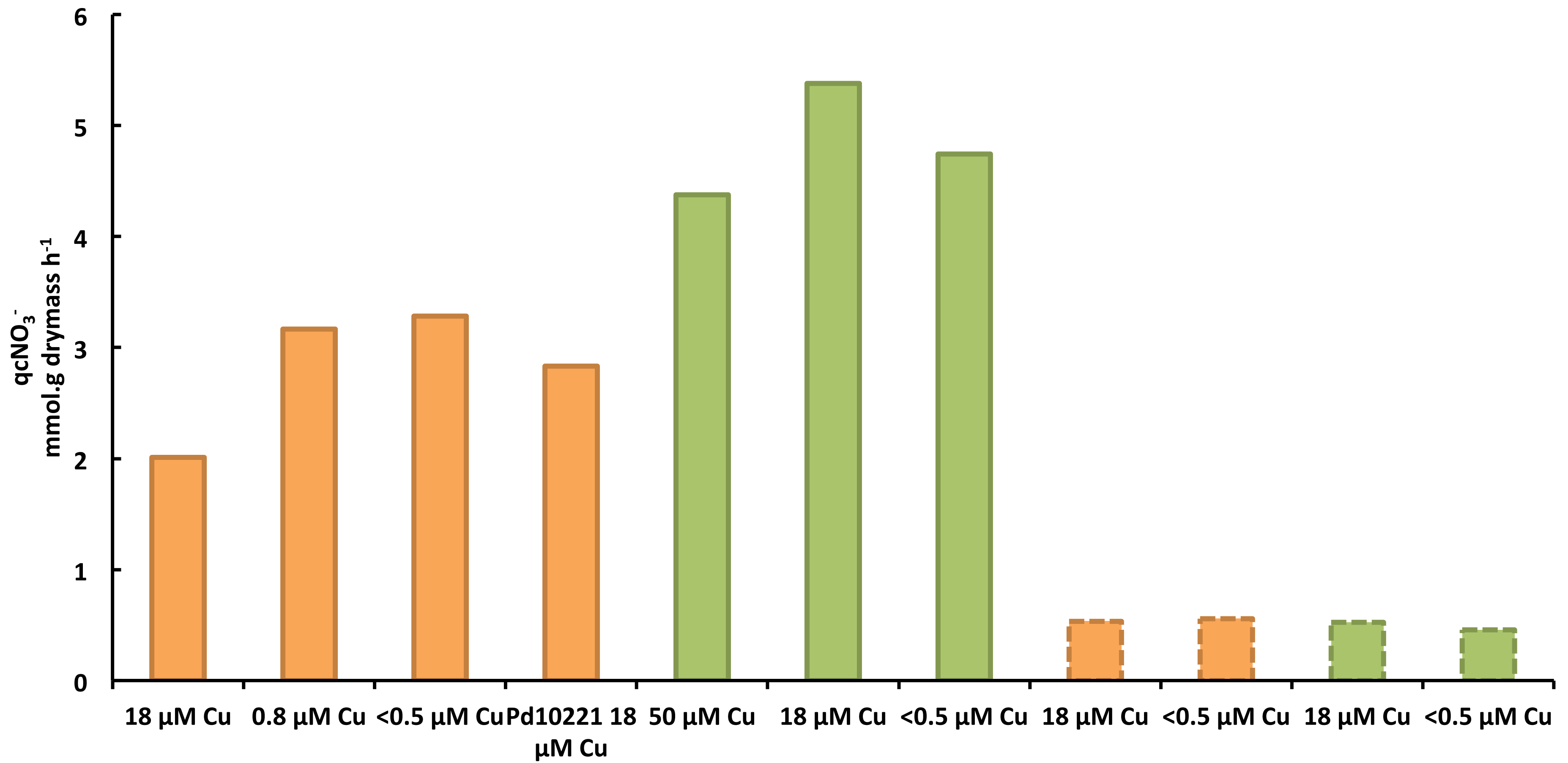


Figure 8.1-1 The rate of NO_3^- consumption ($qcNO_3^-$) in chemostat cultures of *P.denitrificans* (orange) and *A.xylosoxidans* (green) grown in NO_3^- sufficient (—) and NO_3^- limited (----) media with various Cu concentrations.

The production of N_2O was significantly increased in Cu limited cultures in both the *P.denitrificans* and *A.xylosoxidans* chemostat cultures, however the greatest production was observed in the *P.denitrificans* NO_3^- -sufficient succinate-limited <0.5 µM Cu culture (Figure 8.1-2). When Cu becomes limited the production of N_2O dramatically increases, which is due to the Cu centres in NosZ not being inserted correctly. Due to the NosZ polypeptide being less active in the <0.5 µM Cu cultures, the production of N_2 (qpN_2) is lower than in the 18 µM Cu cultures, this is true for both the NO_3^- sufficient and NO_3^- limited cultures. The magnitude of net N_2O and N_2 production is less in the NO_3^- limited cultures, due to the cultures being e^- acceptor limited therefore all nitrogenous intermediates are reduced through to N_2 to maximise the energy consumption. Under e^- acceptor sufficient conditions Cu limitation causes and increase in qpN_2O (Figure 8.1-2).

The research carried out in this thesis has shown that as Cu concentration has a significant effect on the rate and synthesis of proteins involved in denitrification in both *P.denitrificans* and *A.xylosoxidans*. The presence of the Cu containing Paz was absent in the Pd1222 <0.5 µM Cu cultures, and NosZ was also found to be degraded, however N_2O was still able to be reduced to N_2. Therefore it is likely that the NosZ polypeptide in the *A.xylosoxidans* periplasmic fractions is also likely to be degraded in Cu limited cultures. *A.xylosoxidans* encodes two Cu containing Azurins in its genome, therefore the demand for Cu was hypothesised to be higher in the *A.xylosoxidans* cultures. However as Cu increased, to 18 µM Cu, the drymass decreased suggesting that Cu has a toxic affect on the culture, this was confirmed by the 50 µM Cu culture having the lowest final biomass (Chapter 7).

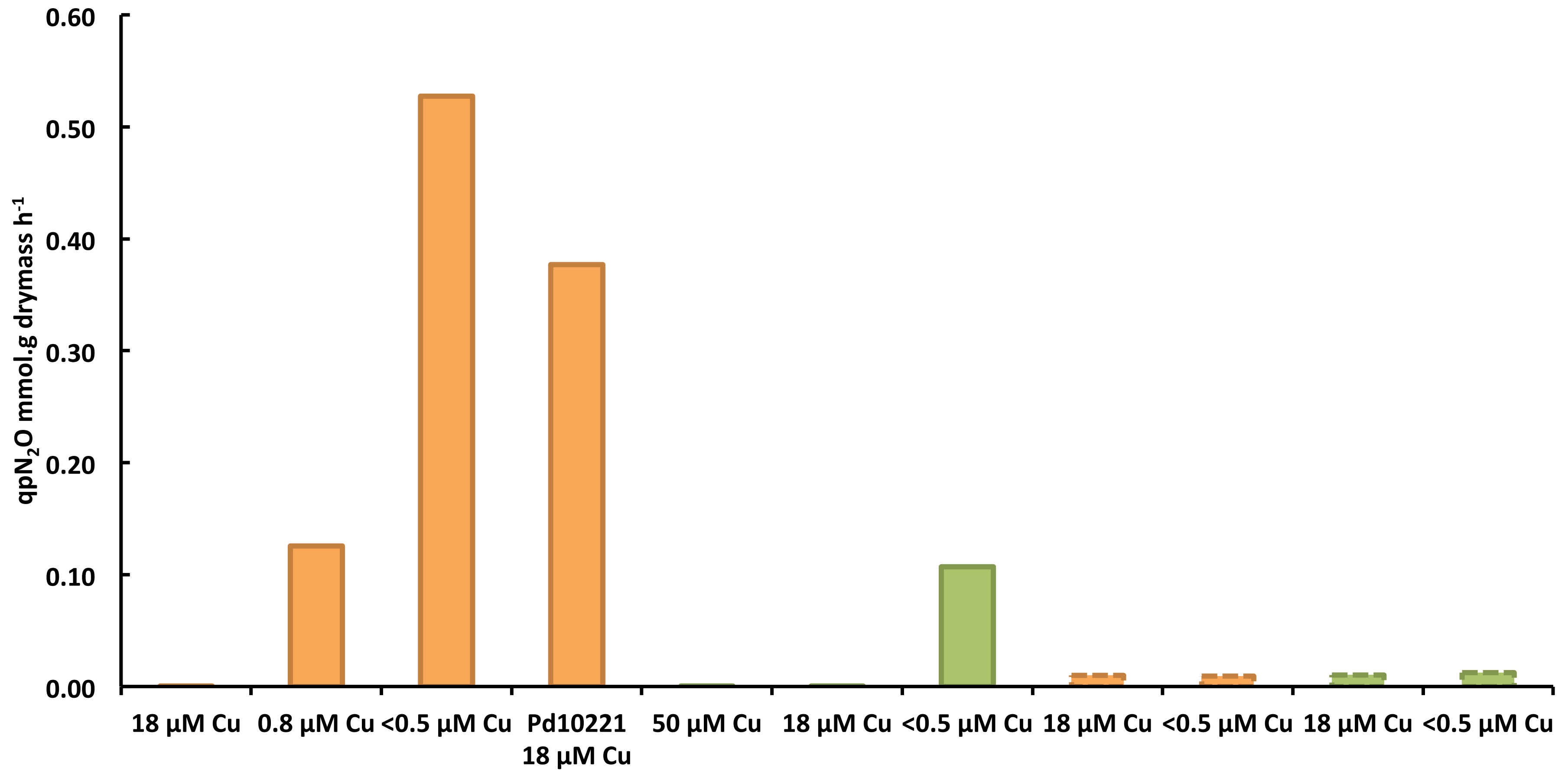


Figure 8.1-2 The rate of N_2O release (qpN_2O) by chemostat cultures of *P.denitrificans* (orange) and *A.xylosoxidans* (green) grown in NO_3^- sufficient (—) and NO_3^- limited (----) media with various Cu concentrations

The presence of the complete NosZ was identified as a 66 kDa band, however in the NO_3^- -sufficient succinate-limited chemostat cultures a 50 kDa band was observed. On further investigation in the limited Cu *P.denitrificans* cultures, the NosZ polypeptide was found to be expressed as a truncated form. As yet it is unknown if this 50 kDa NosZ polypeptide observed is capable of reducing N_2O and further work purifying the polypeptide and characterising the activity and catalytic centre is required. This truncated NosZ polypeptide has not been previously identified, however in work by Wunsch and Zumft (2005) into the synthesis of NosZ in *P.stutzeri* NosR mutants does not discuss a band of a similar molecular weight. Western blotted SDS PAGE gels of *P.st*utzeri cell extracts probed with Anit NosZ Abs showed that NosZ was expressed in various NosR mutants, and that a smaller NosZ band was also observed (Figure 8.1-3). In my view the band observed could potentially be a truncated form of NosZ, the NosR mutations resulted in an inactive form of Nos, and so degradation of the polypeptide may therefore occur, rendering a smaller band. The NosZ polypeptide observed in the WT (wild type) and strains expressing two complimentary NosR fusions expressed an active form of the 66 kDa NosZ, and were therefore capable of reducing N_2O.

In the Pd1222 NO_3^--sufficient succinate-limited cultures a truncated form of NosZ polypeptide was detected (50 kDa) rather than the full NosZ polypeptide (66kDa). Despite the absence of the full NosZ polypeptide being present N_2O was still being reduced to N_2, but at a reduced rate. Therefore NosZ must be active, but even more interestingly potentially the 50 kDa is active. In *Wolinella succinogenes* the NosZ polypeptide is extended, and encodes a mono haem *c* type cytochrome (Simon, Einsle et al. 2004). The function of this is yet unknown, however it potentially could shuttle electrons in the the Cu centres. Could c_{550} therefore shuttle electrons in the Cu_Z centre in the truncated NosZ? If the Cu_Z centre is formed, then the enzyme could potentially be active, the binuclear Cu_A site shuttles electrons to Cu_Z. Preliminary work suggests that periplasm isolated from the Pd1222 <0.5 μM Cu chemostat cultures are slightly more active at reducing N_2O when c_{550} is present, however it is not known whether or not the c_{550} directly interacts with the truncated NosZ. Further work will have to be carried out to restore this issue.

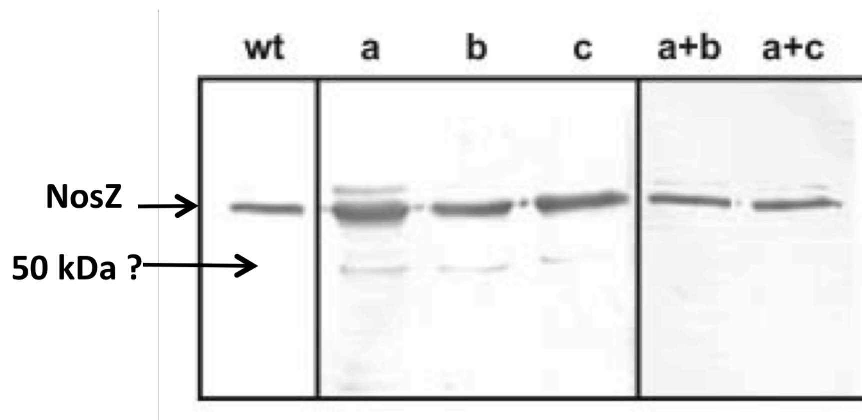


Figure 8.1-3 Figure 3 A from Wunsch and Zumft (2005) showing a Western blotted SDS PAGE gel probed with anti NosZ Abs against periplasmic fractions isolated from *Pseudomonas stutzeri* wild type (WT) and NosR mutants (a) NosR lacks the periplasmic domain and flavin-binding motif, (b) NosR has a deletion of the transmembrane helices TM3 to TM6, (c) NosR has a deletion of transmembrane helices TM5 and TM6. Mutants a+b and a+c are coexpressions of the mutations in a and b, and a and c.

8.1.1 Future Work –The Effect of pH on N_2O release

So far my research has been driven by the effects that Cu concentration has on the accumulation of N_2O in various denitrifying gram negative bacteria. The periplasm is exposed to numerous changes in the environment, one being pH. The effect of soil pH has been investigated numerous times and the main conclusions are that N_2O accumulation is elevated in more acidic soils (Stevens, Laughlin et al. 1998; Šimek and Cooper 2002) and the rate of denitrification is also lower in more acidic soils (Čuhel, Šimek et al. 2010). The activity of NosZ is at maximum at pH 8, where the bioavailability of Cu is decreased (Haltia, Brown et al. 2003). There is therefore a contradiction in turn, with the Cu bioavailability decreasing as the activity of NosZ and the denitrification pathway increases in high pH soils.

Soil pH is not only vital in the accumulation of greenhouse gases, but it is also essential in agriculture. Soil pH is vital in getting high yields of crops and agricultural activities such as fertilising and liming fields all affect the final pH of soils. Therefore

future work should involve work into investigating how pH effects the accumulation of denitrification intermediates in denitrifying bacteria. In preparation for this some preliminary work has been carried growing *P.*denitrificans in chemostat cultures and varying the pH, the findings are described in the following section.

A series of chemostat cultures were set up in NO_3^- sufficient (20 mM) succinate limited (5 mM) *Paracoccus* minimal media (18 µM Cu). Once the chemostat cultures had entered steady state the pH was changed from 7.5 to either pH 9 or pH 5 using 1 M NaOH or 0.1 M H_2SO_4 respectively. The pH change was maintained for 4 h and then was returned to pH 7.5, the consumption of NO_3^- and the production of NO_2^-, NO and N_2O were all monitored as to observe how a change in pH affects the denitrification pathway. The first 24 h of culture is an aerobic batch culture and so in the first 24 h of the chemostat culture the drymass increases. At 24 h the air supply is removed and the feed is added (D = 0.6 h^{-1}), the vessel becomes anaerobic by 24.5 h. From 24 h the drymass decreases until it reaches a steady state at approximately 70 h (Figure 8.1-4). The pH 5 burst chemostat culture initially shows, approximately, a 10 % decrease in biomass compared to the pH 9 burst culture, however this is within the error seen in the previous chemostat cultures of *P.denitrificans* (See Chapter 3). At 100 h the pH was changed from 7.5 to either pH 5 or pH 9, this sudden change in pH resulted in a drop in drymass in both cultures. This decrease in biomass was due to cell death which was identified by an increase in DO, ≥ 10 %. The decrease in biomass could potentially be due to stress as *P.denitrificans* has not synthesised enzymes to adapt to the change in pH. The decrease in drymass (an increase in cell death) was seen in both chemostats, however it was more evident when the pH was decreased to 5 as the drymass decreased by 20 %,compared to a 15 % decrease in drymass in the pH 9 burst (Figure 8.1-4).

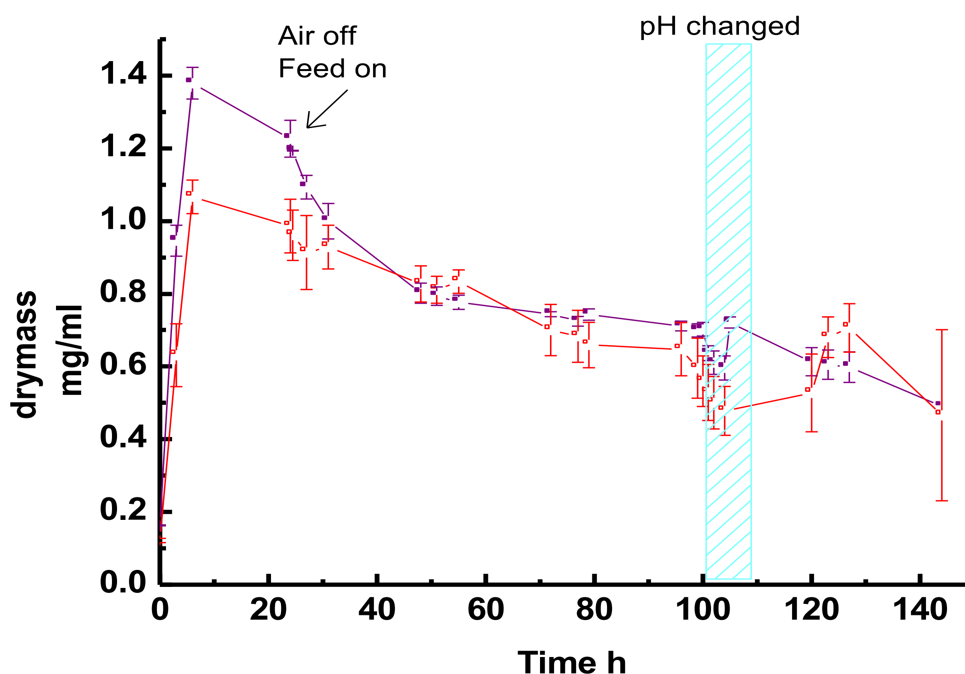


Figure 8.1-4 The drymass of *P.denitrificans* chemostat cultures grown in 20 mM NO_3^-, 5 mM succinate and 18 µM Cu *Paracoccus* minimal media. The pH was maintained at 7.5 until 100 h where it was changed to either pH 9 (■) or pH 5 (□) for 4 h (///).

The first 24 h of growth was aerobic and no consumption of NO_3^- was detected, the consumption of NO_3^- began when the cultures became anaerobic at 24.5 h. The pH5 burst and pH 9 burst chemostat cultures show a marginal difference in the amount of NO_3^- consumed, however due to the difference in the drymass between the two cultures the specific rate of NO_3^- consumption (qNO_3^-) shows no significant difference (Figure 8.1-5). The $qcNO_3^-$ in the pH 5 culture at 96 h was 0.63 ± 0.083 nmol.mg drymass^{-1}h^{-1} compared 0.67 ± 0.079 mmol.mg drymass$^{`1}$h^{-1} in the pH 9 culture. These rates are similar to that observed in the NO_3^- -sufficient succinate-limited 18 µM Cu chemostat culture pH 7.5 (Chapter 3).The change in pH affected the rate of NO_3^- consumption, when the pH was increase to 9 (at 100 h) the concentration of NO_3^- began to increased, implying that the rate of NO_3^- reduction had decreased. The concentration of NO_3^- began to decrease again once the pH was returned to 7.5. The rate of NO_3^- consumption was also affected by the pH being decreased to 5 as

when the pH was decreased to 5 the rate of NO_3^- consumption increased immediately (Figure 8.1-5).

The NO_3^- reductase activity was monitored during the pH change, when the pH was decreased to 5 at 100h the NO_3^- reductase activity showed a five fold increase from 8.3 ± 1.3 nmol.min.mg drymass to 25.9 ± 12.5 nmol.min.mg dry mass after an hour of the culture being at pH 5, the NO_3^- reductase activity remained elevated throughout the pH 5 burst. When the pH was increased to pH 9, initially the NO_3^- reductase activity did not change in the first hour, 2.27 ± 0.35 nmol.min^{-1}mg drymass^{-1}. The activity then increased three fold after the culture had been at pH 9 for 2 h, 7.17 ± 1.4 nmol.min^{-1}mg drymass^{-1}. The activity remained elevated for the reminder of the chemostat culture, even after the pH was decreased to 7.5.

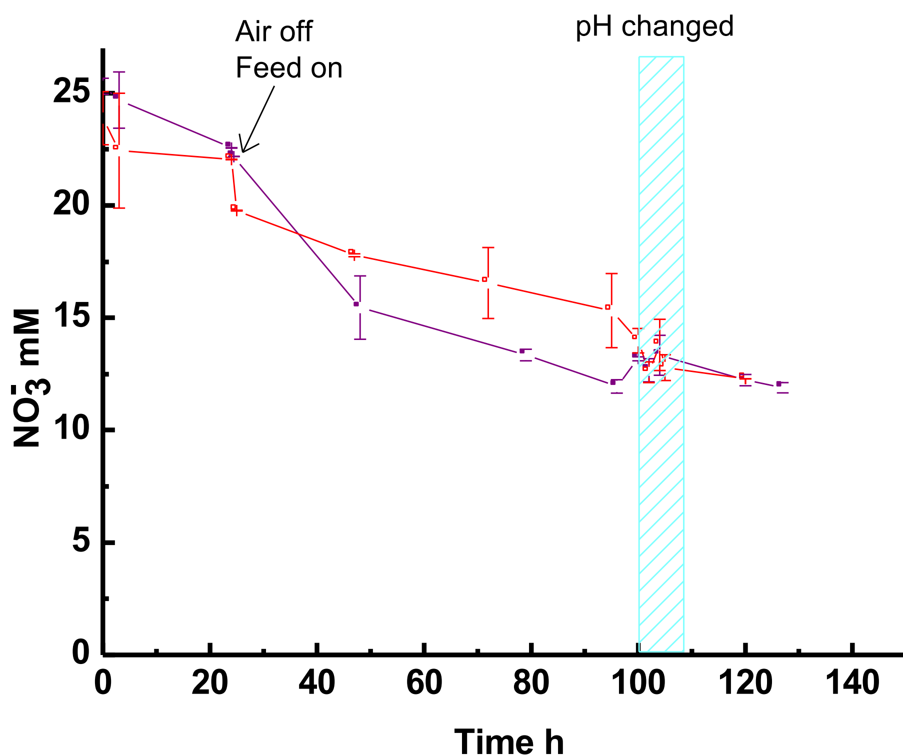


Figure 8.1-5 The consumption of NO_3^- by *P.denitrificans* chemostat cultures grown in NO_3^- -sufficient succinate-limited and 18 µM Cu *Paracoccus* minimal media. The pH was maintained at 7.5 until 100 h where it was changed to either pH 9 (■) or pH 5 (□) for 4 h (///).

Previous chemostat cultures of *P.denitrificans* (wild type) have shown that NO_2^- does not accumulate to more than 80 µM. The first 24 h of growth show a small amount of NO_2^- present due to carry over from the inoculum (Figure 8.1-6). In both the pH 5 burst and pH 9 burst chemostat cultures at steady state NO_2^- was detected at 37 ± 3 µM and 74 ± 1.6 µM respectively. When the pH was increased to 9 at 100 h no NO_2^- was detected extracellularly contrary to that observed when the pH was dropped to 5, where the NO_2^- concentration initially increased to 77 ± 4 µM (Figure 8.1-6). After 2 h of the culture being at pH 5 the NO_2^- concentration then decreased to 12 ± 7 µM.

The accumulation of NO_2^- when the pH is decreased to 5 could potentially be due to a decrease in the NO_2^- reductase activity. The NO_2^- reductase activity immediately decreased 200 %, to 35.2 ± 19 $nmol.min^{-1}mg\ drymass^{-1}$, when the pH was decreased. The activity then continually increased from 101 h up to 164 ± 36.9 $nmol.min^{-1}mg\ drymass^{-1}$ by 120 h. The NO_2^- reductase activity did not significantly change between the pH changing from 7.5 to 9. The NO_2^- reductase activity averaged 141.3 ± 11.89 $nmol.min^{-1}mg\ drymass^{-1}$ throughout steady state and through the pH 9 burst.

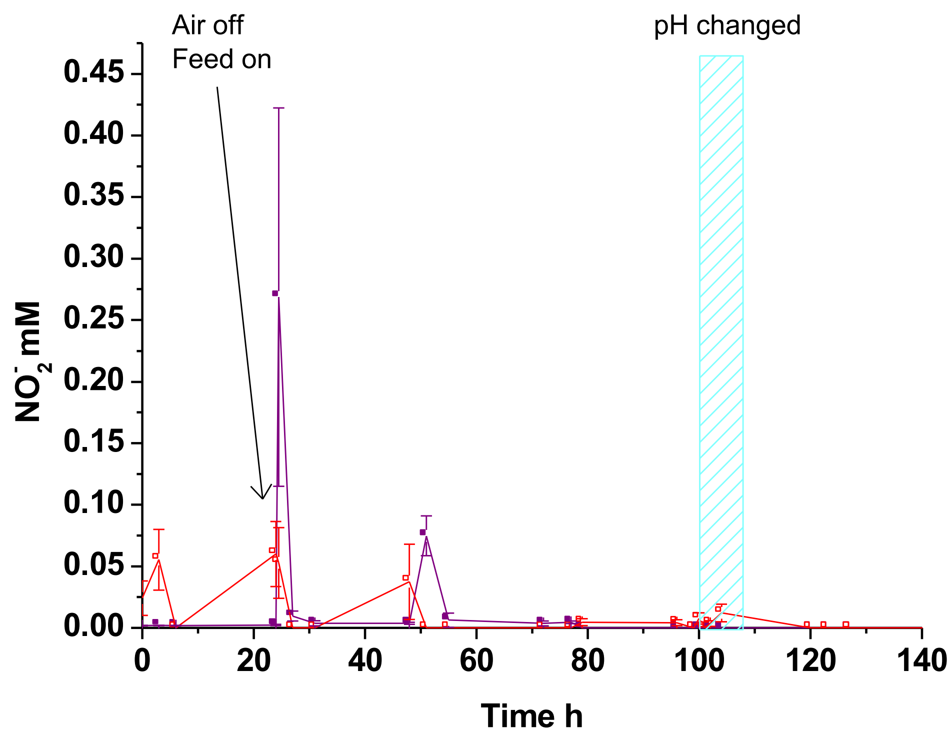


Figure 8.1-6 The accumulation of NO_2^- of *P.denitrificans* chemostat cultures grown in NO_3^- -sufficient succinate-limited and 18 µM Cu *Paracoccus* minimal media. The pH was maintained at 7.5 until 100 h where it was changed to either pH 9 (■) or pH 5 (□) for 4 h (///).

The release of N_2O in previous NO_3^- sufficient 18 µM Cu chemostat cultures was not found to exceed 0.6 µM, the same was also observed in the chemostat cultures prior to a change to either pH 5 or pH 9 (Figure 8.1-7). Once the cultures entered steady state and the pH was decreased to 5 at 100 h after 30 minutes there is an increase in N_2O released and after 3 h of the culture being at pH 5 the N_2O accumulated to 2.64 ± 0.53 µM, which is 10 times as much released when the culture was at pH 7.5 (Figure 8.1-7). The N_2O released remains elevated even when the culture is returned to pH 7.5. When the culture pH was increased from 7.5 to 9, the N_2O released decreased by a 50 %, after 2 h the N_2O detected was 0.04 ± 0.19 µM which is lower than the atmospheric N_2O concentration. When the pH was returned to 7.5 the rate of N_2O released from the culture increased to 0.1 ± 0.004 mM, which is a similar

concentration observed at steady state prior to the change in pH. The decrease in the rate of N_2O released during the pH 9 burst could potentially be due to an increase in the activity of NosZ.

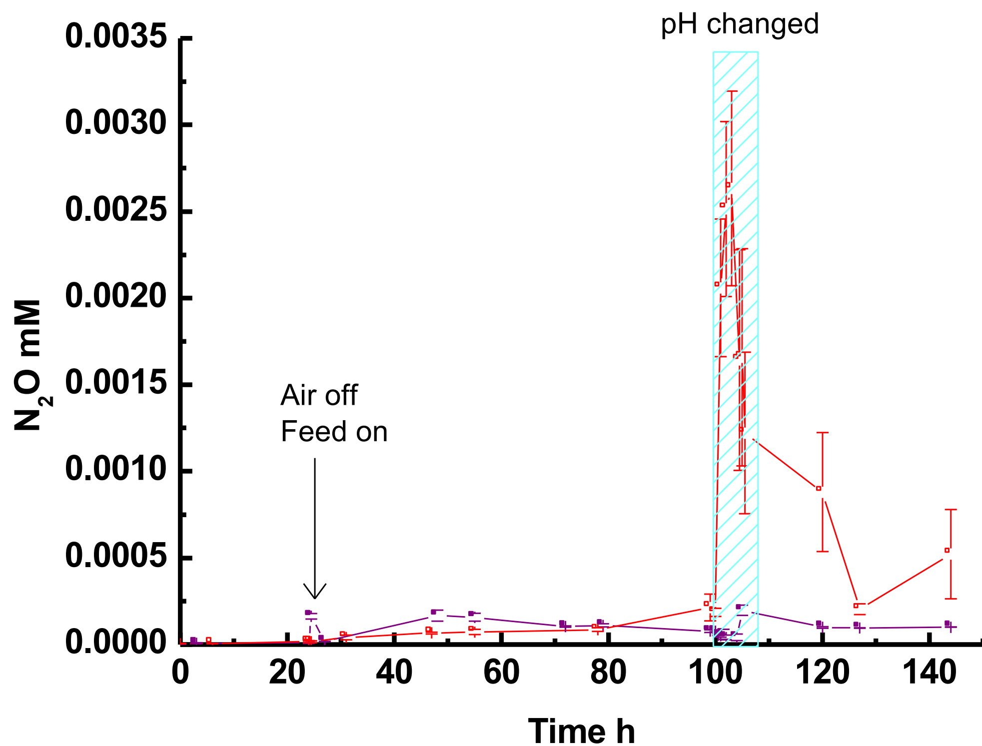


Figure 8.1-7 The accumulation of N_2O of *P.denitrificans* chemostat cultures grown in NO_3^- -sufficient succinate-limited and 18 µM Cu *Paracoccus* minimal media. The pH was maintained at 7.5 until 100 h where it was changed to either pH 9 (■) or pH 5 (□) for 4 h (///).

8.2 How can this research help in reducing global N_2O emissions?

In both *P.denitrificans* and *A.xylosoxidans* NO_3^- sufficient cultures, as the Cu becomes limiting the consumption of NO_3^- increases the release of N_2O is significantly greater that the levels detected under NO_3^- limitation and therefore when relating this to the environment and agricultural soils, when there is excess of NO_3^- due to too higher fertiliser application, this can potentially drive denitrification and consequently increase N_2O release from soils. When relating the research presented in this thesis, to the environment there are many factors which could be monitored so as to reduce the release of N_2O from soils. Cu concentration is vital for crop yields and health, and is also vital for microbial respiration, aerobic and anaerobic. Soils which tend to be low in Cu are clay soils as they absorb/chelate Cu, therefore only a fraction of the total Cu content is biologically available for plant and microbial uptake (Alloway 2008). In England the main areas with Cu deficient soils are the South-West, South-East and East Anglia, due to the high chalk and clay content of these areas (Alloway 2008). Therefore managed agricultural soils could potentially cause high N_2O emissions as the final step of denitrification can be missed out, therefore nitrates and nitrites will be reduced to N_2O, rather than the inert N_2. The addition of Cu to agricultural soils is currently only used when crops are showing characteristics of Cu deficiency, however the bioavailability to microorganisms maybe low and therefore the microbial community are Cu deficient prior to crops.

The research presented in this thesis signifies that in e^- acceptor limited environments result in lower N_2O emissions in Cu limited and sufficient conditions. Therefore in agricultural soils prone to Cu limitation, a way of reducing N_2O emissions would be applying less fertiliser to fields. However, to make this an effective method, knowledge of N requirements for crops will have to be identified. Reducing fertiliser applications would not only potentially reduce N_2O emissions form agricultural soils, but also reduce expenses. Therefore further work on assessing the impact of Cu and NO_3^- concentrations in soil on N_2O emissions will be carried out by collaborators from the University of Aberdeen. By observing N_2O emissions from various soils, which

contain various microbial communities, the patterns observed can then be applied to agricultural soils.

The observations made in the pH 5 and pH 9 burst chemostat cultures imply that the pH affects the denitrification pathway, especially in the reduction of N_2O. Literature has previously stated that the activity of NosZ is increased in high pH cultures, which corresponds to the decrease in the N_2O released when the pH is reduced to 9. Therefore the release of N_2O from soils could also be affected by changes in pH.

pH is vital in many aspects of life, it is vital in agriculture as the pH of soils greatly affects crop yield and quality. N_2O emissions have been found to be elevated in low pH soils, about pH 6.5, and emissions are reduced in higher pH soils, about pH 8-9 (Thomsen, Geest et al. 1994; Stevens, Laughlin et al. 1998). The desired pH of agricultural soils is between pH 6-7, which is achieved by liming the fields. Between pH 6-7 nutrients essential for crops are chemically available to crops and the toxic aluminium becomes biologically less available. High pH soils cause essential nutrients such as Cu, manganese and iron to precipitate and therefore become biologically unavailable to crops, which may affect the quality or quantity of the yield (Dawson 2006).

The type of crop can have an indirect effect on N_2O emissions from agricultural soils as they can influence the pH. The pH of agricultural soils has to be controlled as to gain optimum conditions for growing crops. Many crops are grown in lower pH soils, see Table 8-1, and therefore this could potentially lead to an increase N_2O released from the soils. The chemostats cultures show that even after a short and sudden decrease in pH, the N_2O released increased by nearly 4 -fold. When the pH is increased to pH 9 the N_2O released is greatly reduced to below the atmospheric concentration.

Table 8-1 Optimum soil pH for various crops (Crozier and Hardy 2003)

Crop type	Optimal pH
Asparagus, tomato.	6.5
Beans, potato, spinach, sweet potato, blackberry, grape, strawberry, corn, small grains, soybeans.	6.0
Pine trees, fir/northern spruce, Christmas trees.	5.5

The activity of NosZ is impaired under acidic conditions, hence an increase in N_2O released from acidic soils, low pH is favoured by autotrophic and heterotrophic nitrifiers which can form intermediates capable of being reduced by the denitrification pathway (Martikainen and de Boer 1993; Thomsen, Geest et al. 1994). NosZ is located in the periplasm in many denitrifying bacteria and is therefore exposed to changes in the immediate environment; cytoplasmic enzymes are less exposed to changes in pH due to regulation in the cytoplasmic membrane. Therefore as the pH decreases in soils the activity of NosZ decreases, potentially causing N_2O to accumulate. Sudden changes of pH are not common in agricultural soils, so burst of N_2O are rarely seen. On agricultural soils the maximum N_2O emissions are found to occur after 2-3 weeks after fertilisation. This is due to an increase in NO_3^- and NH_4 concentration promoting denitrification and nitrification (Skiba, McTaggart et al.). N_2O reductase has on optimum pH of pH 9 (with a pKa of pH 9.2), at lower pH the enzyme become less efficient at reducing N_2O to N_2 (Thomsen, Geest et al. 1994; Ghosh, Gorelsky et al. 2007). NosZ has been identified in many agricultural soils from numerous denitrifying bacteria, such as *Achromobacter spp., Pseudomonas spp., Bosea spp.* and *Sinorhizobium spp.*. However only 5 % of the total bacteria isolated in soils have been shown to contain NosZ (Henry, Bru et al. 2006), therefore the effects observed on pH

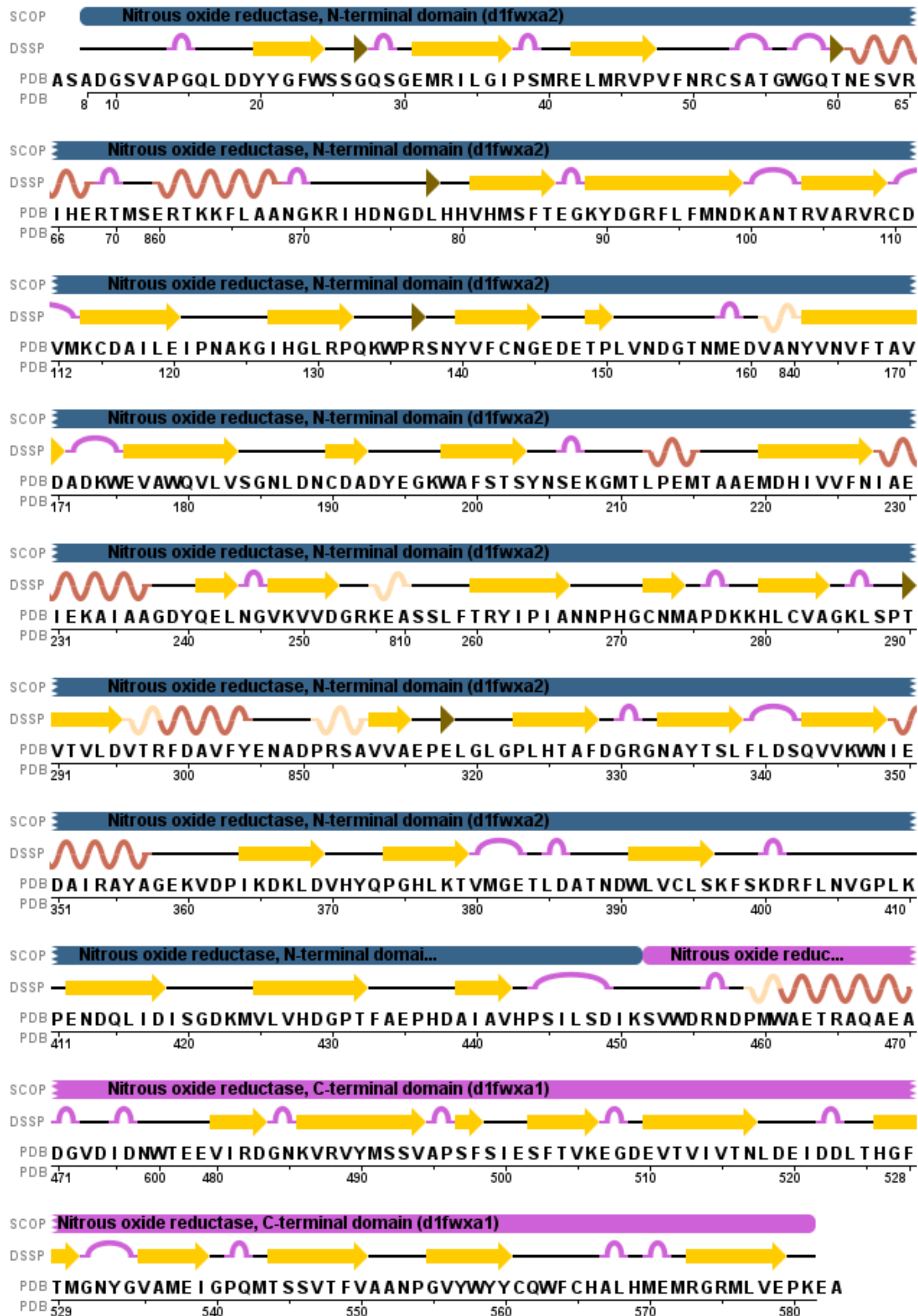
change and the release of N_2O from *P.denitrificans* may be the same affect observed in other denitrifiers.

Another factor pH has on soils is the availability of metals/micronutrients. In low pH soils Cu availability is significantly reduced, therefore the bioavailability of Cu can be reduced after liming fields and is high salt containing soils. pH also affects the bioavailability of many other metals such as Boron (B), Iron (Fe), Manganese (Mn), Molybdenum (Mo) and Zinc (Zn). Deficiencies in any of these micronutrients (Alloway 2008). Methods to improve micronutrient availability would involve the use of high micronutrient fertilisers and better management of agricultural soils, which would increase crop yield as well as potentially decreasing N_2O emissions from high NO_3^- containing soils.

With an increasing demand for food crop production, climate change and pressure on water resources, crop production and yields will be greatly affected in many countries. Heat, drought and high light intensities cause stress on the crops, especially with regards to the uptake of micronutrients. Therefore further research into soil composition, metal availability as well as investigation the effect of Cu and NO_3^- limitation on other denitrifying bacteria will have to be undertaken as to understand the complex biochemistry of denitrification in soil microorganisms and the interactions they have with agricultural soils and crops.

9 Appendix

Figure 8.2-1 Obttained from Brown, Djinovic-Carugo et al. 2000 (Brown, Djinovic-Carugo et al. 2000)



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