The Development of a Method Using Denitrifying Bacteria *Ochrobactrum anthropi* for δ^{15}N analysis of Nitrate

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“ When you get into a tight place and everything goes against you, till it seems you could not hold on a minute longer, never give up then, for that’s just the time and place that the tide’ll turn.”

Harriett Beecher Stowe.

Abstract

Nitrogen (N) is a major element in protein synthesis and is often the limiting nutrient in aquatic environments. As N availability is tightly coupled with carbon (C) uptake, it also has the potential to impact on species diversity, ecosystems and climate should levels be altered. Fuel combustion and synthetic fertilizers are now increasing N levels and perturbing N cycles, highlighting the need to manage anthropogenic N in order to reduce any negative effects.

Tracing N levels back to source point can be achieved by the study of stable N isotopes that pool in unique $^{15}N/^{14}N$ ratios during biogeochemical processes. Isotope ratio analysis is considered a powerful tool but can involve complicated chemical procedures resulting in fractionation and erroneous results when sample nitrate (NO_3^-) and nitrite (NO_2^-) is extracted for $\delta^{15}N$ measurements. Such problems may now be avoided by recently developed bacterial methods that manipulate bacteria into denitrifying NO_3^- and NO_2^- to nitrous oxide (N_2O) which can be analysed directly on a mass spectrometer.

The current project trialled two bacterial methods for denitrification of three NO_3^- standards with known $^{15}N/^{14}N$ ratios using the bacterium *Corynebacterium nephridii* which produced unsatisfactory results. This was attributed to culture conditions affecting denitrification rates, as the methods had been developed using two denitrifying *Pseudomonas* species, *P.aureofaciens and P.chlororaphis.*

A further complication arose when the candidate bacterium *C.nephridii* was found to be a totally different species of denitrifier, *Ochrobactrum nephridii,* but work continued using this species as the bacterium could potentially denitrify N-oxides from polluted and saline samples.

This resulted in the development of a new method that produced $^{15}N/^{14}N$ values as well as $^{18}O/^{16}O$ values which were in good agreement with known values of standards, demonstrating that the method is suitable for isotope analysis of $\delta^{15}N$ as well as $\delta^{18}O$ in freshwater samples.

Preface

Chapter 1 consists of two separate sections, A & B. Part A provides an insight into the complexity of natural biogeochemical cycling of nitrogen (N), local and global implications when these cycles are perturbed and the methods used for $\delta^{15}N/^{14}N$ isotope analysis.

Part B focuses on microbiology and includes the manipulation of microbial cultures as well the biochemical processes which occur during denitrification. Characteristics of the candidate bacteria are also investigated..

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Abbreviations

A	adenosine
ADP	adenosine diphosphate
ATP	adenosine triphosphate
C	cytosine
CABRI	Common Access to Biological Resources Information
CuNir	copper containing nitrite reductase
DNA	deoxyribonucleic acid
DSMZ	(Deutsche Samlung von Mikroorganismen und Zellkulturen
G	guanine
Nar	nitrate reductase
NF	nitrogen fixation
NH_2	amino group
Nir	nitrite reductase
Nor	Nitric oxide reductase
Nos	nitrous oxide reductase
RNA	ribonucleic acid
rpos	rna polymerase subunit
T	thymine
VBNC	viable but non culturable cells
ZPE	zero point energy

Chapter 1

Introduction

Part A

Nitrogen (N) is fundamental to all life forms on earth as it is a major element in amino acids and proteins, with differing levels of N affecting species composition, diversity and ecosystem function (Campbell, 1999; Gruber & Galloway, 2008; McLelland et. al., 1997; Spanning et. al., 2005; Vitousek et al., 1997).

Biologically available N is normally the limiting nutrient in ecosystem functions and is tightly coupled with carbon (C) cycles, but anthropogenic fertilisers have now increased this form of N, perturbing C:N ratios with unknown impacts (Gruber, 2005; Gruber & Galloway, 2008; Hyvönen et al., 2008; Wedin & Tilman, 1996).

Longterm use of fertilisers can, for example increase increase leaf litter, affecting biogeochemical processes involving N_2O efflux and methane consumption as observed in North European forests (Hyvönen et al., 2008).

Increased biogenic emissions of nitric (NO) and nitrous oxides (N_2O) cause chemical transformations and cascade effects within the transport pathway resulting in ozone loss and photochemical smog in the atmosphere before N reenters the biosphere as nitric acid in precipitation (Anderson & Levine, 1986; Gruber & Galloway, 2008).

As atmospheric depositions of N increase, non agricultural systems experience altered C levels as observed in Minnesota grasslands, where native plant species are decreasing in favour of non native grasses because of increased N mineralization, high N losses, decreased biomass C:N ratios and corresponding changes to foodweb structures (Wedin & Tilman, 1996). N driven eutrophication is also evident in terrestial and aquatic systems (Gruber & Galloway, 2008; Wedin & Tilman, 1996).

Nitrogen levels in N- deficient surface waters of oceanic subtropical gyres are also being altered by Diazotroph (phytoplankton) activity in response to climatic temperature

increases. Providing iron is available for the nitrogen fixing NF enzyme structure and function, (Gruber, 2005; Howard & Rees, 1996; Lenes et. al., 2001), new N is fixed from atmospheric reservoirs, generating a major source of biological N compared to vertical mixing of deep waters which has historically been considered the chief source of N for these areas (Gruber, 2005).

This response contradicts predictions that stratification of warmed surface water decreases vertical mixing, N availability and primary productivity (Doney, 2006). It also opposes the hypothesis that NO_3^-, remineralised from organic N in the dark ocean in a tight bidirectional process of denitrification/fixation, is still the dominant form of fixed N (Gruber, 2005).

As fertilizer use and climate responses have altered tight biological processes controlling N availability that cause unknown consequences to ecological and biogeochemical functions (Gruber & Galloway, 2008; Jenkins & Doney, 2003), it is important to track N transformations so that these changes can be understood.

This is now possible by using stable isotope ratio analysis, a powerful tool for establishing the abundance of stable isotopes in ecological materials of environmental systems (West, 2006). The power of the isotope ratio method is in its ability to distinguish chemically-identical forms of nitrogen from different sources but a difficulty lies in the need to convert sample nitrogen to a gas prior to analysis.

The current project aimed to apply a method developed by Casciotti et al. (2002) using the bacterium *Coryneform nephridii* to denitrify NO_3^- to gaseous N_2O for $^{15}N/^{14}N$ ratio analysis of environmental N (reported as $\delta^{15}N$,defined in section A.2.1 (ii.)

However DNA analysis revealed that *C. nephridii* was a different species of bacterium, *Ochrobactrum anthropi* which could denitrify in saline conditions, tolerate heavy metals, xenobiotic compounds and chromium (Kesseru et al., 2002; Laura et. al., 1996; Li et. al., 2008; Ozdemir et. al., 2003), indicating that this bacterium could be used for $\delta^{15}N$ ratio analysis of environmental nitrates from fresh, preserved, polluted, and saline samples.

Work therefore continued with this microbe, but as culture conditions of existing methods caused adverse responses and affected denitrification rates, adjustments were made which resulted in the development of a new method.

A.1. Nitrogen and the biogeochemical cycle

Inorganic N exists primarily as an inert, stable, atmospheric gas dinitrogen (N_2), with minor quantities of nitrous oxide (N_2O), nitric oxide (NO) and nitrogen dioxide (NO_2) occurring from extreme heat events like lightning. Small amounts of N are also present in the crust of the earth as minerals such as sodium nitrate ($NaNO_3$) (Campbell, 1999; Kendall et. al., 2007; Maier et. al., 2000).

In natural systems, some volatised N generated by microbial activity may enter an ecosystem as ammonia (NH_3) in precipitation, but most inorganic N enters the biosphere/food chain after microbial fixation has reduced inorganic N to reactive N which is the only form of N that can be accessed up by other living things (Lajtha & Michener, 1994).

This process is balanced by microbial remineralisation, when reactive N is decomposed and oxidized to inorganic N before returning to the physical environment (Brock et. al., 1994; Kendall et. al., 1998) as shown in Fig. 1.1 below.

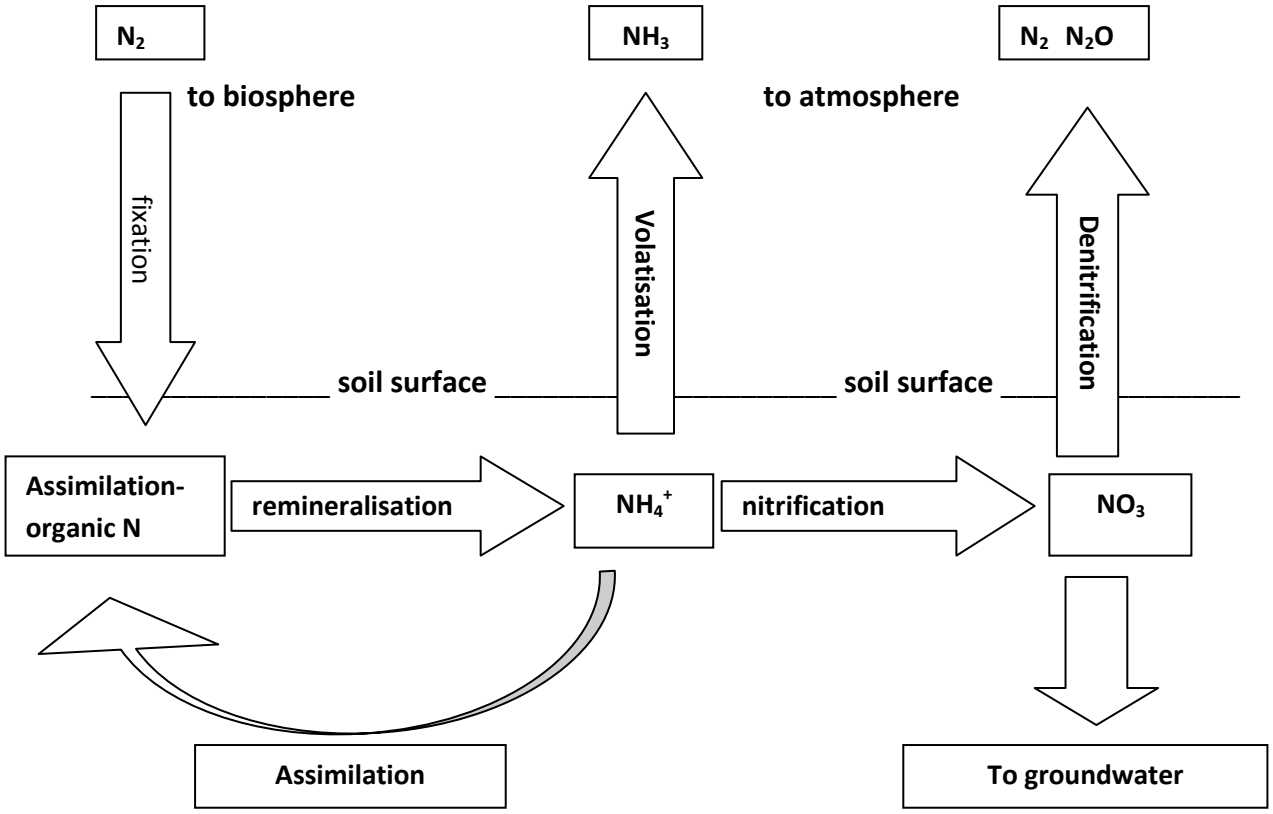


Figure 1.1 Scheme of the natural terrestial N cycle

Adapted from a diagram of nitrogen cycle (Lajtha & Michener, 1994)

Biogeochemical cycling of N thus involves the transfer of inorganic and organic N between the physical environment and living systems by chemical reactions and microbial microbial processes during which the natural variations of δ^{15}N ratios are formed.

A.1.1. Bacterial cycling of N

Bacterial cycling of N involves six microbial processes: fixation, assimilation, remineralisation, nitrification, denitrification and annamox which are discussed below.

(i) Fixation

N fixation is a major process in global N cycling where inert atmospheric N_2 is converted to reactive NH_3 for protein synthesis and growth by living organisms at the base of the food chain. N fixation occurs within a diverse group of prokaryotes and cyanobacteria which can be free living, loosely associated or directly in symbiosis with plants (Brock et. al., 1994; Gruber & Galloway, 2008; Howard & Rees, 1996; Kendall et. al., 1998; Lajtha & Michener, 1994).

The process reduces a stable N-N triple bond to NH_3 as described by the following formula:

$N_2 + 8H^+ + 8e^- + 16$ Mg ATP => $2\ NH_3 + H_2 = 16$ Mg ADP + $16P_1$ (Glass & Silverstein, 1998).

Thus microbial fixation of N, (coupled with denitrification), is one of the most important natural processes that could alter the earth system, as changes to the reactive N budget could alter the global carbon cycle and impact on climate (Gruber & Galloway, 2008).

(ii) Assimilation

Assimilation of N occurs at the base of the food chain when reactive N (NH_3) is taken up by surrounding plants and microorganisms for protein synthesis and growth (Brock et. al., 1994). NH_3 is converted to amino groups (NH_2) which are attached to synthesised carbon skeletons, forming amino acids that then assemble into peptides, the monomers of bacterial and plant proteins.

Proteins are then consumed, cycling N through the food chain as organic N while microbial remineralisation converts organic waste back into NH_3, providing a continuous source of

reactive N for reassimilation (Brock et al., 1994; Kendall et al., 1998; Lajtha & Michener, 1994).

(iii) Remineralisation

Aerobic bacterial decomposers remineralise organic N of excreted matter, urea and dead organisms generated at every level of the food chain (Brock, 1994). Fungi catabolise large protein molecules into peptones and peptides, enabling further degradation by extracellular bacterial proteases and peptidases which break the carbon skeleton/amino link by hydrolysis. Amino groups are then deaminated internally by bacterial cellular hydrolysis, oxidation or reduction and synthesised into bacterial amino groups, releasing excess N into the environment as NH_3 for ongoing uptake (Swatek, 1967).

At neutral pH, ammonia converts to ammonium (NH_4^+) and is rapidly assimilated by plants (Brock et al., 1994; Griffiths, 1998). In anaerobic conditions such as fine, organically enriched sediments, ammonium is usually stable, existing in this state as the predominant form of N although recent studies have revealed NH_4^+ may be oxidised to N_2 (Dalsgaard et. al., 2003). In oxic conditions such as well oxygenated soils, ammonia is also stable but can be oxidised to nitrogen oxides by bacterial nitrification (Brock et. al., 1994).

(iv) Nitrification

Nitrification is the oxidation of reactive N to NO_3^- by the combined activity of ammonia oxidising bacteria and nitrifying bacteria, with some intermediate species of nitrogen oxides, nitrate radicals and acids also occurring (Brock et. al., 1994; Kendall et. al., 1998; Lajtha & Michener, 1994).

Oxidation of NH_4^+ to nitrite (NO_2^-) is a two step reaction by ammonia oxidising chemolithotrophs, eg. *Nitrosomonas.* (Brock et. al., 1994; Kendall et. al., 1998).

The first step requires no energy as an electron donor enzyme, monooxygenase and NADH oxidise NH_4^+ to hydroxylamine (NH_2OH), (Brock et. al. 1994).

$$NH_4^+ + H_2O \longrightarrow NH_2OH + H_2 + H^+ \text{ (Kendall et. al., 1998)}$$

The second step uses energy generated by a cytochrome electron transport system, phosphorylating ATP which then oxidises NH_2OH to NO_2^- (Brock et. al. 1994).

$$NH_2OH + O_2 \longrightarrow NO_2 + H_2 + H^+ \text{ (Kendall et. al., 1998)}$$

Nitrification is completed by reduction of NO_2^- to NO_3^- by nitrite oxidising bacteria eg. *Nitrobacter* in a single step reaction involving cytochrome transport systems and ATP phosphorylation of the nitrite oxidase system.

$$NO_2^- + H_2O \longrightarrow NO_3^- + H_2 \text{ (Kendall et. al., 1998)}$$

Although nitrifying bacteria are ubiquitious in NH_3 rich soils and waters, nitrification is most extensive in neutral or alkaline aerobic conditions that counteract acid buildup compared to anaerobic conditions, where low O_2 levels inhibit further oxidation, NH_3 levels are increased and toxic, mutagenic nitrous acid forms (Brock et.al., 1994).

Nitrification may also occur during autotrophic fixation of carbon dioxide (CO_2), when NH_3 acts as an electron donor that provides energy in the absence of light or chlorophyll. Most NO_3^- in forest soils is produced in this way (Brock et. al., 1994).

(v) Denitrification

Denitrification, (coupled with nitrification), is the other major process of N cycling where fixed nitrogen is lost to the atmosphere in a one way process. Denitrification (and nitrification) is kept in balance by carbon coupling and climate as rapid variations of atmospheric N_2O measurements over the last 60,000 years show. (Gruber & Galloway, 2008; Granger et. al., 2008).

Denitrification can be assimilatory, when NO_3^- is reduced to NH_4^+ for uptake, or dissimilatory, during which energy is conserved as substrate nitrogen oxides NO_3^- and NO_2^- are transformed to gaseous N_2 by microbial anaerobic respiration. Nitrate is an ideal compound for alternative respiration during anaerobic conditions as it readily accumulates in waterlogged soils.

Assimilatory denitrifiers include species of *Enteriobacteraceae, Bacilli* and *Clostridia* (Anderson et. al. 1986; Brocket.al., 1994; Griffiths, 1998; Zumft, 1997) and is not considered denitrification in the true sense as little gaseous nitrogen is produced (Knowles, 1982).

Dissimilatory denitrifiers use nitrogen oxides as terminal electron acceptors during anaerobic respiration (Knowles, 1982) and is considered the true form of denitrification (Zumft, 1997) where NO_3^- is reduced to dinitrogen (N_2) (Sigman et. al., 2001; Zumft, 1997).

A typical bacterial reduction pathway of NO_3 is therefore:

$$NO_3^- \longrightarrow NO_2^- \longrightarrow NO \longrightarrow N_2O \longrightarrow N_2$$

It is the process required for the current project providing the bacterium cannot reduce N_2O to N_2.

Dissimilatory denitrifiers are heterotrophs, although species within other trophic groups also occur. They are usually predominant in the alpha and beta classes of proteobacteria but no recognizable distribution pattern is evident (Zumft, 1997). Species include *Pseudomonas*, *Acaligenes, Achromobacter* , *Agrobacterium*, *Bacillus* and *Corynebacteria* (Knowles, 1982).

(vi) Annamox

The anammox reaction is a recently discovered process thought to return 33% - 50% of global oceanic N to the atmosphere. Anammox reactions are driven by the activity of microbes in the *Planctomycetes* family which have been found in diverse anaerobic sediments ranging from sewage sludge to aquatic sediments (Dalsgaard et. al., 2005).

The anammox reaction occurs in anaerobic conditions where microbes use NO_2^- to oxidise NH_4^+ to yield N_2 in a 1:1 ratio (Dalsgaard et al., 2003) as shown in the following equation:

$$3NO_3^- + 5NH_4^+ \longrightarrow 4N_2 + 9H_2O + 2H^+$$

(Dalsgaard et.al., 2005)

Fixed nitrogen in upwelling oceanic waters also decreases when NO_3^- rich waters are exposed to anoxic sediments where sulphur oxidising bacteria reduce NO_3^- to NH_4^+ which then converts to N_2 by anammox processes. (Dalsgaard et. al., 2003).

A.1.2. N as a contaminant

Natural N inputs originating from biological nitrogen fixation have now been exceeded by synthetic fertilizers, causing chemical transformations and cascade effects within the transport pathway.

(i) Effects of excess N

Anthropogenic N has doubled turnover rates, causing reactive N to exceed biologically fixed N and alter autotrophic productivity, ecosystem function and the global N cycle as input of reactive N exceeds natural N cycling by microbial denitrification.

Excess N from terrestrial systems is transported laterally to freshwater systems where nearly half of the global terrestrial denitrification occurs. Normally little reactive N remains for transport to the ocean, but critical changes occur as N levels increase. Chemical transformations of N-oxides along the N transport pathway may now cause cascade effects where one molecule of NO in the atmosphere produces photochemical smog before converting to HNO_3 and returning to the biosphere to cause acidification and eutrophication in ecosystems (Gruber & Galloway, 2008).

Excess N also contributes to global warming effects (Anderson & Levine, 1986) when trophospheric N_2O diffuses up to the stratosphere, destroying ozone (O_3) and absorbing infra red radiation emitted from earth.

(ii) Sources of excess N

Reactive N is increased by the use of anthropogenic fertilizers urea, potassium nitrate (KNO_3) and ammonium nitrate (NH_4NO_3) which are fixed from inert atmospheric N_2 by the Haber process (Gruber & Galloway, 2008; Kesseru et. al., 2002). This raises inorganic N to unnatural levels that load the environment and skew natural N cycles (Gruber & Galloway, 2008; Kendall et. al., 1998; Lajtha & Michener, 1994).

N pollution is also emitted to the atmosphere by non point atmospheric deposition during fossil fuel combustion (Carpenter et. al., 1998).

(iii) Excess NH_4-N

Agricultural lands with concentrations of manure or high soil pH or produce NH_4^+ which volatises to the atmosphere as NH_3, only to re-enter the biosphere as dissolved NH_4^+ in precipitation for biological uptake (Brock et. al., 1994; Campbell et.al., 1999). Nitrogen has now increased in natural systems by precipitation from 0.5 to current values of 2.5 $N.m^{-2}.year^{-1}$ in North America and 0.5-6 $gN.m^{-2}.year^{-1}$ in North Europe over the last 40 years (Wedin & Tilman, 1996).

Ammonium in precipitation is a unidirectional inorganic reaction, causing fractionation that enables N isotope variation in the biological environment to be studied (Heaton, 1986) when nitrifiers and ammonia oxidisers convert excessive NH_4^+ to highly soluble NO_3^- that leaches out of the immediate ecosystem (Brock et. al., 1994; Lajtha & Michener, 1994).

(iv) Excess NO_3-N

Fertilisers and biological waste generate serious pollutants from nitrified NH_3 as freshwaters become acidic by the formation of nitrous acid (HNO_3) while surrounding estuarine waters are eutrified, causing hypoxic conditions and fish kills (Camargo & Alonso, 2006; Gruber & Galloway, 2008; Kendall et. al., 2007; McClelland et. al., 1997). Locally, New Zealand coasts are under pressure from agricultural practices that have increased N levels in many river systems, permitting excess N to enter coastal ecosystems (Ford & Taylor, 2006).

Highly soluble NO_3^- migrates across ecosystems through soils, humus, deeper soil profiles and groundwaters, where it continues to be utilised by denitrifying bacteria, forming NO_3^- pools progressively enriched in ^{15}N (Fry, 2006; Lajtha & Michener, 1994)

Because microbial denitrification is rate limited, up to one quarter of applied fertiliser can leach into groundwater as NO_3^- instead of returning to the atmosphere as N_2.

Nitrate concentrations are now rapidly rising in groundwaters of developing nations (Heaton, 1986), presenting potential health risks if ground water is used for drinking water as it can cause infant methemoglobinemia or carcinogenic nitrosamines in human digestive systems (Böhlke et. al., 2007; Camargo & Alonso, 2006; Ford & Taylor, 2006; Glass & Silverstein, 1998; Kendall et. al., 2007; Kesseru et. al., 2002; Lajtha & Michener, 1994).

A.2.1. Stable isotopes, a powerful tool for isotope fingerprinting

Based on the processes described above, biogeochemical cycling of N is a highly complex series of events where unique natural $^{15}N/^{14}N$ isotope ratios form during unidirectional driven kinesis of ^{15}N in an open system.

These ratios are a challenge to study but provide an important means for identifying relationships and pathways amongst various N substrates and pools in ecosystems, including sources of N pollution or disturbances in clearing of land (Chang et. al., 2002; Fry, 2006, Griffiths, 1998; Lajtha & Michener, 1994).

Studies using stable isotope signatures have also validated the occurrence of N fixation in oceanic surface waters, where half of the organic N is sourced from atmospheric N_2 with fixation rates based on N and P ratios (Gruber, 2005)

(i) Isotopes

Isotopes originate in planetary systems of nuclear stellar processes, changing in terrestrial environments by radioactive decay and spontaneous disintegration to form other isotopes (Kendall & McConnell, 1998).

They are variants of the same element which have different masses due to a different number of neutrons. Stable isotopes consist of the light elements H, C, N, O and S where the number of neutrons (*N*) and protons (*Z*) is similar and no further disintegration occurs (Fry, 2006; Sulzman, 2007). Although stable isotopes have a proton number ≤ 20, the relative mass difference is large enough to produce measurable fractionation during physical or chemical reactions (Kendall et.al., 1998; North, 2006).

(ii) Notation

Isotopes of the same element are known as nuclides and are notated as the elemental symbol plus the atomic weight. Nitrogen nuclides are therefore notated as ^{15}N and ^{14}N where *Z* is 7 and *N* is 8 or 7 (Clark, 1997; Kendall et. al., 1998; North, 2006).

A delta (δ) symbol represents the ratio of the two most abundant nuclides. For nitrogen the δ^{15}N is defined as;

$$\delta^{15}\mathrm{N} = \left(\frac{\left({}^{15}\mathrm{N}/{}^{14}\mathrm{N}\right)_{\mathrm{SAM}}}{\left({}^{15}\mathrm{N}/{}^{14}\mathrm{N}\right)_{\mathrm{AIR}}} - 1 \right)$$

where the subscripts SAM and AIR refer to the sample and the international reference (air) respectively. Atmospheric air is considered homogenous in its N isotope composition with a ^{15}N abundance ratio of $3.677*10^{-3}$ (Kendall et al., 1998).

δ-values are normally multiplied by 1000 and expressed in units of permille (‰). Positive δ values show a sample ratio is higher than the standard while negative values show a sample ratio is lower than the standard (Kendall et. al., 1998).

Should one material be enriched or depleted with a nuclide, it is important to state if the enrichment/depletion is in ^{15}N or ^{14}N (Kendall et. al., 1998).

(iii) Kinetic isotope effects

Kinetic isotope effects occur when heavy and light nuclides of an element undergo the same chemical reaction at a different rate because of energy characteristics and ratios of heavy or light nuclides in a molecule (Kendall et. al., 1998; Mariotti et.al., 1981).

Light nuclides have a dissociation energy with a lower zero point energy (ZPE) compared to heavy nuclides. Lighter ^{14}N nuclides in a compound consequently have a faster reaction rate compared to heavier ^{15}N nuclides of the same compound. As less energy is required for a bond to be broken (Kendall et al., 1998), lighter isotopes will readily fractionate into products while heavy isotopes become enriched in the residue during incomplete reactions (Criss, 1999).

Irreversible kinetic effects are driven by low temperatures where light nuclides accumulate in one compound while heavy nuclides accumulate in another (Kendall et al., 1998).

Alternatively, unidirectional fractionations will disappear in a closed system if all substrate has been converted into a product and the reaction has reached completion.

(iv) Isotope effects and equilibrium reactions

Under certain conditions reversible processes reach a state of equilibrium where the rate of a proceeding chemical reaction is equal to the rate of the reverse reaction, with no further tendency for either reaction to move forward or backward (Goates, 1981; Mortimer 1983).

a) Chemical equilibrium reactions cannot occur in general conditions as energy differences that decrease ZPE differences are approximately 1000 times greater than chemical reactions that drive isotope kinetic effects and fractionation (Kendall et.al., 1998).

b) In a closed, well mixed system, nuclides of an element are redistributed in a constant ratio among various species, compounds or phases as forward and back reaction rates of any nuclide are identical during chemical equilibrium reactions at a given temperature. The heavier nuclide generally accumulates in a liquid phase or compound with a higher oxidation state while the lighter nuclide resides in a gas phase (Kendall et al., 1998).

c) In an open biosphere system, nuclides of an element are distributed in varying ratios during chemically and biologically driven forward and back reactions, but kinetic isotope reactions can be unidirectional if products are physically isolated from residues (Kendall et. al., 1998).

d) Although NO_3^- is the end product, biological nitrification in an open environment is a multistep oxidation process causing N nuclides to fractionate as different reactions of biological N produce various N oxides at differing rates (Kendall et al., 1998).

These aspects need to be considered when analyzing the $\delta^{15}N$ of environmental samples.

(v) Isotopes and biological systems

Gradual fractionation of N isotopes takes place during deamination and mineralization of organic N, as microbes incorporate peptides enriched in ^{15}N and release excess NH_3 depleted in ^{15}N into the surrounding soil for plant uptake. (Lajtha & Michener, 1994).

Pools of reactive ^{15}N may alter during bacterial turnover when organic N enriched in ^{15}N is released back into soil matter (Lajtha & Michener, 1994), providing a means to trace N through the environment by $\delta^{15}N$ analysis.

A model of Isotopic fractionation in the soil of a forest system is shown in Fig. 1.2 below.

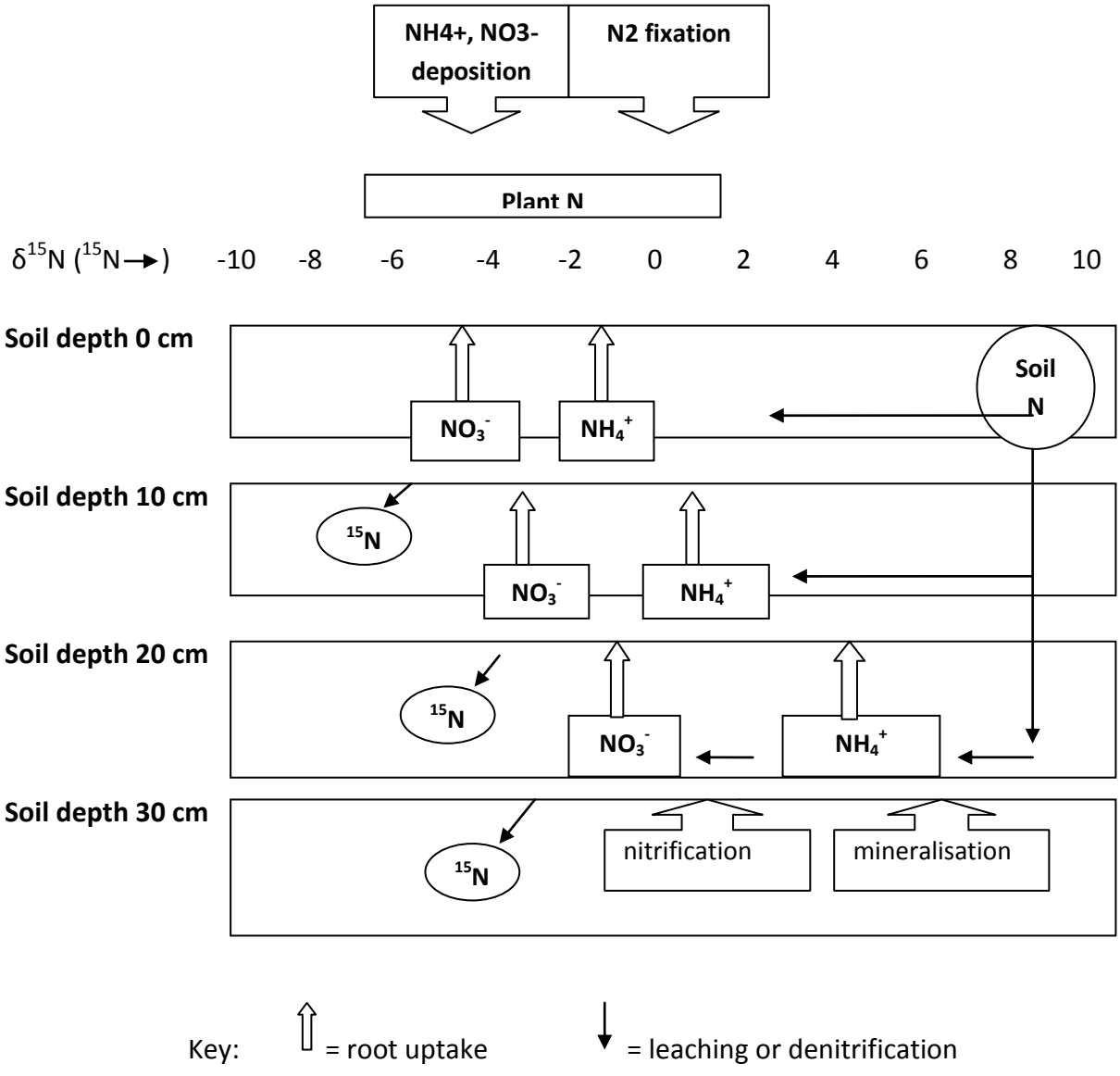


Figure 1.2. **A hypothetical model for isotope fractionations in a forest system based on a model of Lajtha & Michener (1994)**

A.2.2. Fingerprinting, chemical methods

Determination of $^{15}N/^{14}N$ ratios requires quantitive conversion of substrate N into N_2O or N_2 gas for analysis and is achieved by chemical methods involving conversion of inorganic N to N gas which is purified, ionised and analysed for $^{15}N/^{14}N$ ratios on a mass spectrometer (Kendall et. al., 1998).

Samples collected for $\delta^{15}N$ determination are normally filtered, preserved with sulphuric acid, mercuric chloride or chloroform and kept in cold storage until analysis. Alternatively, samples can be concentrated on anion or cation exchange resin columns for NO_3 and NH_3 respectively (Kendall et. al., 1998).

(i) Steam distillation

Steam distillation is suitable for extracting low levels of NO_3^- from large quantities of water, including seawater, by making the pH of sample water basic and trapping NH_3 in an acid trap. Samples are then extracted by distillation for analysis. (Lajtha & Michener, 1994). The method can be used to analyse NO_3^- after reduction to NH_3 with Devarda's Alloy (Lajtha & Michener, 1994; North, 2006).

(ii) Passive distillation

This method involves making the pH of sample water strongly basic and distilling NH_3 into an acid trap before extracting with zeolite. NH_3-N is then combusted in a sealed Dumas tube, producing N gases for analysis (Lajtha & Michener, 1994).

(iii) Passive diffusion

Other methods such as passive diffusion techniques function in a similar manner. Sample water pH is made basic, yielding NO_3^- which is then trapped onto an acidified filter fibre wrapped in a Teflon envelope which is either floated in, or suspended above the solution in a closed container. The filters are dried and combusted to convert NH_3-N to N gases for analysis on an IRMS spectrometer (North, 2006, Sigman et. al., 1997).

(iv) Ion exchange columns

Ion exchange columns enable the concentration of NO_3^- from large volumes of sample in the field. NO_3-N is trapped on anion exchange columns before transporting to the laboratory where NO_3^- is eluted with hydrochloric acid (HCl), neutralised with silver oxide (Ag_2O) and filtered to remove the silver chloride precipitate. Solid silver nitrate ($AgNO_3$) is obtained by freeze drying and combusted, producing N_2 for $\delta^{15}N$ analysis. The method avoids transporting large volumes of sample and the use of hazardous preservatives (Silva et al., 2000).

(iv) NO_3-N extraction

Devarda's Alloy is first used to reduce NO_3^- to NH_3. The solution pH is made basic and diffusion takes place over 3-5 days. Following diffusion, filter papers are dried and combusted using an automated CN-Mass spectrometer (Lajtha & Michener, 1994). Samples containing low levels of N need to be concentrated down to 100 mL by boiling. This can cause volatisation if samples are not acidified with H_2SO_4 first. Alternatively, samples can be concentrated on ion exchange resin columns.

A.2.3. Problems with the chemical methods

Chemical methods have provided a successful means for isotope fingerprinting but have associated problems which limit application or affect accuracy.

(i) Volume of sample

Because distillation and diffusion processes have limited efficiency, sample (N) needs to be at 2-3 µM, a problem if only millilitres of sample is available and the methods are very labour and time intensive (Sigman, et. al., 2001).

Logistic problems also prevail if large sample volumes need to be transported to the laboratory (Lajtha & Michener, 1994; North, 2006; Vitousek et. al., 1997).

(ii) Preservation

Samples often need to be acidified or preserved with toxic chemicals such as mercuric chloride ($HgCl_2$) to inhibit microbial action on organic and inorganic nitrogen which could

cause fractionation prior to analysis of the compound (Corriveau et. al., 2008; Kattner, 1999; Kotlash & Chessman, 1998; MacDonald & McLaughlin, 1982; Silva et. al., 2000). Using toxic chemicals always has associated hazards and is banned in some countries.

(iii) Steam distillation

Problems with steam distillation arise when samples contain organic N (NH_2) which will also reduce during conversion of NO_3^- to NH_3 and cause inaccurate results (Casciotti et al., 2002; Kendall et al., 1998; Lajtha & Michener, 1994; Sigman et al., 2001).

If analyte splashes into condensate head during the volatile reactions of steam distillation, residues can remain to contaminate the next sample. The method is therefore limited by sample volume of 150 ml to make it a time consuming process, as only one sample can be processed at a time, requiring constant attendance (North, 2006).

(iv) Passive diffusion

Fractionation will occur with passive diffusion methods if reactions are not complete, as heavy isotopes can pool in liquid or gas phase following Rayleigh's equation, to produce an enrichment of ^{14}N in ammonium salts trapped on the acidified disc (North, 2006)**.**

(v) Ion exchange columns

The use of ion exchange columns is effective for freshwater samples but excludes seawater samples or other samples with high specific conductivity as ions compete for sites on the exchange column (Casciotti et. al., 2002; Silva et. al., 2000).

(vi) Use of reagents

Inefficient extraction by chemical methods and the presence of a significant reagent blank from Devarda's reducing agents can cause inaccurate $\delta^{15}N$ measurements (North, 2006). The methods are also labour intensive and time consuming (Sigman et. al., 1999; Sigman et. al., 2001).

A.2.4. Analysis of δ^{15}N, bacterial method (batch, closed system)

Bacterial denitrification is now being used to quantitively reduce sample NO_3^- and NO_2^- to nitrous oxide (N_2O) for direct isotopic analysis on a mass spectrometer.

Denitrification occurs naturally under anaerobic conditions when bacterial respiration mechanisms utilise NO_3^- as an alternative electron acceptor in the absence of O_2, (Brock, et.al., 1994; Knowles, 1982; Zumft, 1997). Denitrifying bacteria which lack the enzyme N_2O reductase are ideal organisms for δ^{15}N analysis as they cannot reduce N_2O to N_2. This enables reduction of NO_3^- and NO_2^- to product N_2O for direct analysis (Aldsworth et. al., 1999; Schimel et. al., 2007; Sigman et. al., 2001).

(i) Advantages

The method is preferable to chemical processes as only N-oxides are reduced and contamination from organic N species in the sample does not occur (Sigman et al., 2001). Providing all substrate is respired, potential fractionation is avoided as all light and heavy N isotopes will reside in the product to yield an accurate fingerprint. Samples of NO_3-N at nM levels can be analysed without the need for prior concentration, thus avoiding logistic problems involved with transferring large samples to the laboratory (Sigman et. al., 2001).

(ii) Disadvantages

Potential problems of the bacterial method involve establishing predictable growth for optimal denitrification and total conversion of NO_3^- and NO_2^- to N_2O. This requires knowledge of the candidate organism, as laboratory conditions may elicit unforeseen metabolic processes which can impact negatively on denitrification. Some of these responses are outlined in part B.

Part B

B.1. General aspects of bacterial cultures

Application of the bacterial method for δ^{15}N analysis may be problematic, as microbes have evolved different strategies to survive changing environments which can drive bacterial responses in a direction other than denitrification (Arber, 2000; Booth, 2002; Fux et. al., 2005; Kolter et. al., 1993).

Bacterial growth and survival is not driven by tightly controlled mechanisms of supply and demand, but rather by adaptability to stressors, governed by synthesis of eg. ribosomal proteins in excess to demand and new protein synthesis in response to change (Booth, 2002).

Because organic compounds of high molecular weight do not cross the semipermeable bacterial membrane, exoenzymes have evolved to catabolise large molecules into accessible compounds, resulting in the evolution of microbes that occupy specific ecological niches. Microbial activity is therefore limited by the availability of metabolic products. (Willett, 1976).

Ecological niches may not necessarily occur in the unnatural conditions of a laboratory as temperature, pH and nutrients are manipulated to establish parameters for a desired outcome (Brock et.al., 1994; Jefferson, 2004; Kolter et. al., 1993; Roszak & Colwell, 1987).

Cultures are constantly mixed to ensure cells are homogenized and in equilibrium with the gas phase (Monod, 1949), as well as preventing cells from settling and forming biofilms which could impact on denitrification rates (Deziel et. al., 2001).

Provision of optimal growth conditions in the laboratory usually creates an environment favouring a long period of exponential growth. This is counter to bacteria in natural environments that exist in a starvation state, producing short bursts of exponential growth when nutrients become available (Kolter et. al., 1993).

Conditions for this project will differ from conditions favouring optimal exponential growth, as N_2O production occurs under oxygen starvation, but as controlled conditions are not

natural conditions, unexpected bacterial strategies may occur. Some of these events are discussed below.

(i) Age of culture

Different metabolic events will occur if bacteria are grown in a batch culture over an extended period of time, as bacteria have naturally evolved responses to depletion of nutrients, accumulation of metabolic toxins and changes in ion equilibria, usually pH (Monod, 1949).

Long incubation times may stress cultures and risk altering bacterial metabolism towards strategies other than anaerobic denitrification such as shutting down into a state of dormancy until conditions improve (Schimel et.al., 2007).

Cultures grown in a rich medium over 2-5 days incubation experience cell counts which decrease by one or two orders of magnitude (Kolter et al., 1993) and can also promote mutation events. These aspects need to be considered for the current project as bacterial methods for δ^{15}N analysis require up to 10 days incubation (Casciotti et.al., 2002; Sigman et. al., 2001).

(ii) Temperature

Maximum, minimum and optimal temperatures for microbial growth and division were recognised by early microbiologists. Organisms fell into three groups: psychrophils, mesophils and thermophils, having cardinal temperatures of 0-18^{o}C, 10-45^{o}C and 40-90^{o}C respectively with optimum growth temperatures found towards the warmer maximum (Brock et.al., 1994; Swatek, 1967).

Temperatures for optimum denitrification may differ from temperatures favouring growth, as the culture adapts to the stresses of a changing environment caused by decreased oxygen availability and an altered growth medium (Booth, 2002; Monod, 1949). In these conditions, temperatures favouring optimal growth could drive metabolism in a different direction.

(iii) Nutrients

All life, including bacteria, exist on seven key elements: oxygen (excluding obligate anaerobes), carbon, hydrogen, nitrogen, phosphorus, potassium, and sulphur. Other nutrients include magnesium, copper, iron, sodium and chlorine and biological compounds such as vitamins (Brock et. al., 1994; Gottschalk, 1986; Swatek, 1967). Micronutrients cobalt, zinc, molybdenum, copper, manganese nickel, tungsten and selenium are also critical for bacterial growth (Brock et. al., 1994).

Nutritional needs vary amongst species as microbes have adapted to utilise metabolic products of other microorganisms. Consequent studies found non synthetic media to be a good general medium (Brock et.al., 1994; Swatek, 1967) as it provided amino acids, organic carbon and growth factors which can be accessed by most heterotrophic organisms.

The media used in this study was Bacto ^{TM}Tryptic Soy Broth (TSB), a general, non-synthetic media containing pancreatic digest of casein, papaic digest of soybean, dextrose, sodium chloride and dipotassium phosphate which meet the needs of most heterotrophic bacteria.

B.2. The growth curve of bacteria

To understand the growth cycle of an organism in the laboratory, a growth curve established under optimum growth conditions will show the lag, log and stationary phase of a culture over time, providing a quick easy estimate of cell mass by measuring turbidity. Absorbance is proportional to cell number and mass which can be predicted at a given temperature and time. A drop in turbidity signals a reduced cellular count and cell lysis (Brock et. al., 1994; Reed et. a.l, 1988).

(i) Lag phase

Lag phase represents the period when cells adjust to a new environment and the cytoplasm enlarges in preparation for, but beyond the requirements of cell division. Cell division is enhanced by a build up of carbon dioxide and temperature which directs chemical activity of bacterial enzymes (Swatek, 1967).

Depending on the age of the inoculum, various cells will die, repair themselves or divide. Division may be delayed in fresh media as it lacks signal metabolites (Aertsen & Michiels,

2004; Brock et.al., 1994; Swatek, 1967). Bacteria at this stage are also sensitive to changes in temperature when a drop in optimum temperature will disrupt synchronised metabolic rhythms established during incubation at a constant temperature (Swatek, 1967).

ii) Log phase

Log (exponential) phase evolves from lag phase and represents the shortest generation time when cells have readjusted to minimal cytoplasmic synthesis for maximum cell division. Cell size is small and contains no vacuoles, (Swatek, 1967).

Exponential growth is influenced by genetic characteristics of the organism as well as temperature and medium and all biochemical constituents are synthesised at the same time (Brock et. al., 1994).

If culture is inoculated (stepped up) into a richer medium, RNA synthesis, particularly ribosomal, is increased. DNA and protein synthesis increases later (Brock et. al., 1994).

Should log phase cultures be inoculated into a poorer medium, (stepped down) RNA synthesis decreases and DNA and protein synthesis continues at a faster rate, although this is dependent on the amount of ribosomes present in the cell, as ribosome efficiency is constant at different growth rates (Brock et. al., 1994).

(iii) Stationary/decline phase

Stationary phase represents a slowing of growth due to depletion of nutrients. Under laboratory conditions remaining energy is directed towards completing cell division and cells become normal or abnormally large from inclusions or vacuoles which form and coalesce in the cytoplasm. Crowding, decreased nutrients and accumulation of metabolic by products begins to affect cells (Swatek, 1967).

Cell functions may continue, secondary metabolites and antibiotics specific to late log phase are synthesised and survival genes are upregulated, producing proteins which protect the starving cell from oxidative damage (Brock et. al., 1994).

Continued stationary phase heralds an increased death rate until death rate and multiplication reach equilibrium. At maximum stationary phase, cells are subjected to

growth factors and antagonists of dead and dying cells. Maximum stationary phase represents the point when the largest numberof living cells exist, competing for limiting nutrients such as carbon or nitrogen while changes in pH occur to inactivate extracellular enzymes. Chelating agents may remove ions of trace elements including Fe^{3+}, Mg^{2+} and Cu^{2+}, altering monovalent and divalent cation concentrations which affect the permeability of the plasma membrane (Swatek, 1967).

Escherischia coli cells undergoing slow starvation have exhibited slowed metabolic activity, size reduction and production of cell walls that favour adhesion and cause aggregation or clumping (Kolter et. al., 1993).

(iv) Death phase

Continued incubation of batch cultures produce stresses such as crowding, lack of nutrients (particularly carbon and nitrogen), pH changes, precipitation of ions and accumulation of wastes that cause cell death (Aertsen & Michiels, 2004; Swatek, 1967).

Viable cell counts drop, cells lyse and turbidity decreases as the population dies, although individual cells may survive these conditions (Brock et.al., 1994). Moribund cells are in a state of oxidative injury as free radicals destroy periplasmic proteins (Aertsen & Michiels, 2004). Dormancy and death occur when stresses are too extreme for cells to reallocate resources from growth to survival (Schimel et. al., 2007).

In minimal media, lowered availability of C and salts**,** particularly PO_4, always result in faster kinetics of death compared to cultures grown in nutrient rich media such as Luria broth, when viable cell counts can drop to a low level but remain constant over weeks, suggesting mutations have taken place (Kolter et. al., 1993).

(v) Some manipulations during different phases

A new inoculation into depleted media produces less cell growth, suggesting nutrient levels are too low for metabolism of cell mass. Conversely, addition of exponential phase cells into fresh media will produce a burst of growth (Brock et. al., 1994; Swatek, 1967).

When bacteria at different stages of the growth cycle are inoculated into fresh media, they continue in that phase for a while before readjusting (Swatek, 1967). Lag phase may be

prolonged if cells are inoculated into poor medium as cellular constituents and co enzymes first need to be synthesised for essential metabolites (Brock et. al., 1994). Addition of supernatant from a log phase culture will shorten the lag phase (Aertsen & Michiels, 2004). This suggests the presence of signalling factors such as AHLS (*N*-acylhomoserine lactones), (Dunne Jr, 2002).

Temperature changes have the greatest impact on cell division during lag and log phase as certain enzymes are slowed by temperature fluctuations that are too high or low for optimum metabolism (Swatek, 1967).

Based on the information above, a growth curve was established to correlate temperature and time for log, lag and stationary phases of the candidate bacterium. During experiments, final cell density was recorded and correlated with N_2O yields to identify which growth phase produced optimal denitrification and exclude stress responses which are discussed below.

B.3. Stress responses

Stress is defined as physiological challenges that threaten microbial function and survival Schimel et. al. (2007).

In natural conditions, each organism has evolved strategies to survive in an environmental niche that is governed by stresses such as nutrient availability, temperature, salinity, solar radiation and oxygen saturation (Aertsen & Michiels, 2004; Aldsworth et. al., 1999; McDougald et. al., 1998; Schimel et. al., 2007).

Responses to environmental stress include cross feeding, mutation, polysaccharide production/biofilm formation, sessile cells, production of viable but non culturable cells (VBNC) , dormancy and death (Doebeli, 2002; Dunne Jr., 2002; Fux et. al., 2005; Pfeiffer & Bonhoeffer, 2004; Schimel et. al., 2007) and are discussed below.

(i) Biofilms

Biofilm slime is not slime of lysed cells which consists of exopolysaccharide mixed with cytoplasmic polysaccharide and nucleic acids (Costerton et. al., 1981; 1994; 1995; Hall-Stoodley et. al., 2004).

Bacteria have specialised mechanisms for biofilm formation, suggesting strong survival advantages as biofilms remove microbes from a competitive environment and act as protectants against environmental stresses (Dunne Jr., 2002). Biofilms occur during nutrient favourable conditions and may be regulated by population densities generating CO_2 or cell to cell signalling molecules such as acylated homoserine lactones (AHLS) (Dunne Jr., 2002).

Cells immersed in biofilms settle into a vegetative state, existing in a microniche that has a basic form of homeostasis, metabolic cooperation and circulation (Costerton et. al., 1994; 1995) which generates different patterns of bacterial growth including resistance to antibiotics and near dormancy (Dunne Jr., 2002).

Once the biofilm has reached a critical mass, vegetative cells shed planktonic cells for dissemination into and colonisation of new areas (Costerton et. al., 1981; 1995; Dunne Jr., 2002). Cells below surface become quiescent or die from nutrient depletion, decreased pH, O_2 or toxic metabolic byproducts (Dunne Jr., 2002).

Denitrification occurs within anaerobic conditions in biofilms where cells are reliant on diffusion of nutrients including electron donors and receivers (Jefferson, 2004; Nielsen et. al., 1990). Metabolism within biofilms cause environmental factors including altered pH, pCO_2, divalent cation concentration, and pyrimidine concentration which produce negative effects of acidic, anaerobic conditions in the deepest biofilm layers (Dunne Jr, 2002; Jefferson, 2004).

For the purpose of the current project, biofilm formation should probably be avoided as:

1) Bacteria immersed within a biofilm access sample nitrate diffused at an unknown rate that could impact on denitrification rates. As interiors of biofilms are also acidic, diffused nitrates could be reduced to other N species.

2) Laboratory cultures may upregulate polysaccharide production and divert energy from denitrification if excessive metabolic byproducts alter the growth medium. Assessment of culture age and cell density is therefore required, as a certain cell density may generate signals for biofilm production. Time, temperature, nutrients and cell density therefore need to be established for minimised biofilm production and maximised denitrification.

(ii) Cross feeding

Cross feeding involves the production of an intermediate energy source by one strain of bacteria which is utilised by another strain of the same bacteria when nutrients are limited (Pfeiffer & Bonnhoeffer, 2004).

Cross feeding was evident in chemostat cultures subjected to a limited primary nutrient, glucose. Doebeli (2002) suggested the secondary metabolite produced, acetate, allowed conditions for a specialist consumer (phenotypic variant) to proliferate, generating a tradeoff between uptake efficiency and physiological constraints on catabolic pathways.

Another study proposed cross feeding events were maximised by ATP production with minimised concentrations of pathway enzymes and intermediates (Pfeiffer & Bonhoeffer, 2004).

Although cross feeding seems to be restricted to chemostat cultures, prolonged growth of a batch culture may produce similar metabolic events as cells cope with limited nutrients and altered pH. Trade-off between uptake efficiency and constraints on catabolic pathways may easily occur due to a depletion of energy during log phase growth of the culture.

Cross feeding might affect this study if energy sources are depleted during an extended incubation time, as maximised ATP production could drive minimised pathway enzymes for specialised consumption of nutrients and decrease denitrification rates.

(iii) Mutation

Mutation is driven by environmental effects such as osmotic stress, radium emanation or solar radiation in bacteria as well as higher organisms (Braun, 1947; Csonka, 1989).

These specific agents can be eliminated in laboratory conditions, but adaptive or spontaneous mutations may still occur within a bacterial population. Environmental changes such as altered pH, decreased nutrient and lowered redox potential can produce unstable mutants which lessen when the environment reverts to the original state (Braun, 1947). Undirected spontaneous genetic variation has been attributed to small local changes in genetic sequences, intragenomic reshuffling, insertion of extraneous DNA and horizontal gene shuffling (Arber, 2000; Lawrence & Hendrickson, 2003).

This contrasts with mutants that appear in cultures which have been grown over long periods of time in chemostats and during serial transfer techniques (Kolter et al., 1993). Such mutations may be regarded as the result of adaptive dynamics towards a gradual evolutionary change in asexual populations (Doebeli, 2002) and could be inadvertently selected for during culture procedures (Kolter et. al., 1993).

For example, aged cultures were found to have acquired advantages in stationary phase conditions which enabled them to outgrow a parent population (Kolter et al., 1993). When 24 h and 10 d stationary phase cells were mixed in a rich spent medium, the 10 d culture outgrew the 24h culture, indicating phenotype changes from mutations which differ from a temporary physiological adaptation in direct response to starvation (Kolter et. al., 1993).

As methods for the current study use 6 – 10 d cultures, mutation affecting denitrification rates is possible.

(iv) Phase variation

In contrast to mutation, phase variation is dependent on physiological acclimation mechanisms during chronic or sudden changes in the environment (Schimel et. al., 2007) which may occur during sudden freezing, when cultures are stored for future use. Fitness of frozen sub cultures therefore needs to be evaluated for the current project and may depend on cell density of the stored culture.

(v) Viable but non-culturable cells (VBNC)

The existence of VBNC cells remains unclear (Kell et al., 1998). Orthodox microbiology considers inability of colony growth on agar to represent death (Panoff et.al.,1998) but may represent a state of injury leading to self repair or death (Aertsen & Michiels (2004).

Starvation produces VBNC cells which maintain some metabolic activity but cannot resume growth on nutrient agar plates and therefore differ from starved cells. (Aertsen & Michiels , 2004; Fux et. al., 2005; Oliver, 2005; Roszak & Colwell, 1987) and (Kolter et.al., 1993).

VBNC cells can occur in stationary phase cultures where oxidative stresses and metabolic toxins of a may act as signalling factors for younger cells to enter a VBNC state, (Aertsen & Michiels, 2004; Nilsson et. al., 1991; Oliver, 2005).

Light, low temperatures and quorum regulated biofilms can also induce VBNC cells (Faruque et. al., 2004; Oliver, 2005) which revive when conditions become favourable (Whitesides & Oliver, 1997).

As lengthy incubations may produce biofilms and VBNC (Costerton et.al., 1995; Dunne Jr., 2002; Trevors, 2011), current bacterial methods using 6 – 10 d cultures for δ^{15}N analysis may need to be examined should denitrification be unsatisfactory.

B.4. Physiology of bacterial denitrification

Denitrifiers can be (i) assimilatory or (ii) dissimilatory but not both as two different sets of genes are involved which generally occur in different classes of bacteria. (Knowles, 1982; Zumft, 1997).

(i) Assimilatory denitrification

Assimilatory denitrifiers often include species of *Enterobacteriaceae, Bacilli* and *Clostridia* (Knowles, 1982) that reduce NO_3^- to NO_2^- which is detoxified by acting as an electron sink to produce NH_4^+ (Knowles, 1982; Zumft, 1997).

Assimilatory denitrification is repressed in the presence of NH_3 but not by O_2 (Zumft, 1997) and differs from dissimilatory denitrification which proceeds with or without NH_3 (Knowles, 1982; Zumft, 1997). It is not the metabolic pathway of interest for the current project as no gaseous nitric or nitrous oxides are formed.

(ii) Dissimilatory denitrification

Predominant dissimilatory denitrifiers are often alpha and beta classes of proteobacteria with no recognizable distribution pattern evident (Zumft, 1997) although dissimilatory denitrification is often associated with *Pseudomonads* and *Alcaligenes* species (Knowles 1982).

Unlike assimilatory denitrification, dissimilatory denitrification is repressed in the presence of O_2, as dissimilatory NO_3^- reduction is catalysed by anaerobic conditions (Knowles, 1982; Zumft, 1997).

Dissimilatory denitrification occurs under low oxygen tensions when bacteria utilize nitrogen oxides as alternative terminal electron acceptors during respiration. This generates an electrochemical gradient across the cytoplasmic membrane as electrons transfer across several terminal oxidoreductases and allows conservation of energy during reduction of nitrogen oxides to gaseous nitric and nitrous oxides (Zumft, 1997). Total dissimilatory denitrification is a complex system involving four enzymes, NaR, NiR, NOR and NOS, being nitrate, nitrite, nitric and nitrous oxide reductases respectively, of which more than one type may occur. Each enzyme represents a specific respiratory process where the denitrifying cell transfers electrons over oxidoreductases that use different N oxides to sequentially transform N oxides from NO_3 to N_2 (Zumft, 1997).

B.5. Dissimilatory denitrifying enzymes

Nitrate (Nar) and nitrous oxide (Nos) reductases have the largest degree of independence and function autonomously while periplasmic Nitrite (Nir) and Nitric oxide (Nor) reductases function interdependently, both controlled at transcriptional and enzyme levels in a negative feedback system. The Nar enzyme is oriented to the cytoplasmic side while the Nir enzyme associated with NO_2 reduction is periplasmic (Zumft, 1997). Reduction of two oxyanions NO_3 and NO_2 occur at opposite faces of the membrane with NO_2 generated in the cytoplasm by the membrane bound Nar enzymes and NO in the periplasm (Zumft, 1997). External NO_3 is accessed and reduced to NO_2 (by Nar), NO_2 is then reduced inside the cell to NO (by Nor) and directed out of the cytoplasm and cell (Zumft, 1997). This would explain the siting of enzymes Nar and Nir as NO is toxic and needs to be moved away from the cytoplasm.

(i) Respiration of nitrate

Three types of nitrate reductases (Nar) have been recognised in nitrate respiration, one a soluble assimilatory type periplasmic reductase and two membrane-bound respiratory type

reductases. Many bacteria are found to have a combination of these enzymes which are all under the control of NO_3 (Zumft, 1997).

The periplasmic reductase is synthesised and activated in the presence of oxygen to possibly play a role in the transition to anaerobosis while membrane bound respiratory reductases are only expressed under anaerobic growth. A general conclusion is that the periplasmic reductase is confined to assimilatory denitrifiers (Zumft, 1997) and will not be considered further here.

The two membrane-bound respiratory Nar enzymes constitute a complex of γ, α and β subunits where the γ subunit is anchored to the cytoplasmic membrane, interacting with a larger second α subunit containing an active molybdenum site. A third small β subunit which has iron-sulphur (Fe-S) clusters with redox potentials of +80 and +60 mV to -200 to -400 mV then combines with the γ and α complex. Subsequent intra-molecular electron transfers are thought to involve γ and β subunit in reduction of NO_3 to NO_2 (Zumft, 1997).

(ii) Respiration of nitrite

Two different NO_2 reductases exist, but never together in the same cell:

a) Cytochrome *cd* $_1$ with an Fe center and

b) CuNir with a copper centre.

Both types of enzyme have different structures and prosthetic metals and are found in α, β and γ subclasses of proteobacteria (Zumft, 1997).

a) Cytochrome cd1

Synthesis of periplasmic cytochrome cd1 depends on the presence of NO_3^- and has a physiological function as an NO_2^- reductase by protonating NO_2^-, removing H_2O and producing NO.

$$NO_2^- + 2H+ + e- \longrightarrow NO + H_2O$$

It is a homodimer consisting of Fe prosthetic groups heme C and hemeD which form a tetra hemeprotein. It has electron acceptor specificity to C551 and pseudoazurin via hydrophobic patches which are complementary to a hydrophobic patch on the domain of cd_1, bringing

the metal centers within closest proximity for electron transfer. Nitrite and resulting product NO then bind via N atoms to heme D1, the catalytic site of cytochrome cd_1. Nitric oxide may then be discharged from the enzyme by a flexible conformation during redox cycling (Zumft, 1997).

Cytochrome cd_1 also catalyses ^{18}O exchange between NO_2 and H_2O, to alter the oxygen isotopic composition of NO_3^- (Casciotti et. al, 2002; Zumft, 1997) which needs to be considered if $\delta^{18}O$ isotopes of nitrogen oxides are to be analysed, as bacteria possessing the cd1 enzyme cannot be used.

b) CuNir

All periplasmic CuNir species are trimeric, having a primary structure of a single type subunit with hydrodynamic properties of rapid dissociation-reassociation equilibrium. The copper centre of Type 1 CuNir is tightly bound, forming a flattened hedron from adjacent residues at resting stage. Type 2 CuNir forms a pseudotetrahedral shape with H_2O where NO_2 displaces water and binds to Cu via an O_2 atom (Zumft, 1997).

During N_2O reduction, azurins and pseudoazurins donate electrons to type 1 CuNir which transfer electrons to Type 2 CuNir (Zumft, 1997).

Formation of a key intermediate Cu^+-NO^+ nitrosyl complex is suggested during the reaction of Cu and NO_2^-.

$2H^+$, $-H_2O$.

$$E\text{-}Cu^+ + NO_2^- \longrightarrow E\text{-}Cu^+ - NO_2^- \rightleftharpoons E\text{-}Cu^+ - NO_2^- \rightleftharpoons E\text{-}Cu^{2+} + NO$$

The amount of NO formed by bacterial denitrification depends on the organism and culture conditions such as low pH. Physiology and regulation is tightly coupled with NO_2 respiration in a –ve feedback system to control excess intermediate NO which is toxic to the cell (Zumft, 1997).

NO can act as a reductant where the presence of an unpaired electron allows membrane/intermembrane diffusion to cause NO reactions with O_2.

$$NO + O_2 \longrightarrow \text{superoxide radical } O_2^{*-}$$

Toxicity manifests by O_2^{*-} reacting with bacterial amines, thiols and metalloproteins heme Fe, non heme Fe and Cu containing enzymes. Altered enzymes then interfere with cellular processes.

NO is also mutagenic to bacterial DNA because of a nitrosating and deaminating reactivity where:

$$C \longrightarrow T, AT \longrightarrow GC \text{ and } GC \longrightarrow AT$$

The action of NO on DNA is complex, damaging cross links and forming strand breaks which no DNA repair system can cope with (Zumft, 1997).

During steady state denitrification, extracellular NO is kept at low nM concentrations. Lack of cell division attributed to NO toxicity is expected at or above 1 mM, while cellular protection systems cope at nM and µM levels. Nitrite can accumulate with a steady state NO concentration of 1 nM although this can increase to 0.5-31 nM within 40 min of reaction time depending on cell density (Zumft, 1997).

For the purposes of this project, cell density as well as NO_3^- concentration need to be assessed to avoid problems of mutation and incomplete denitrification by cells subjected to high levels of NO_2/NO during lengthy incubation times in NO_3^- amended media.

(iii) Respiration of nitric oxide

Nitric oxide was thought to be an intermediate molecule in the production of N_2O, as the isolation of a pure enzyme was difficult to achieve. This has now been resolved with the following model proposed.

NO reductase (Nor) catalyses NO – N_2O:

$$2NO + 2H^+ + 2e^- \longrightarrow N_2O + H_2O$$

This reaction requires 2 electrons to dimerise mononitrogen species to an N-N bond.

Nitric oxide reductase (Nor) is thought to be a complex of two or more subunits consisting of a cytochrome C and a large Nor B complex. The Nor B complex is possibly comprised of two or more subunits where:

a) The membrane-bound subunit cytochrome C of Nor C is bitopic with an N terminal directed towards the cytoplasm and

b) A large heme C binding domain is sited in the periplasm (Zumft, 1997).

Periplasmic azurin or cytochrome C are the proposed electron donors that interact with the heme C binding domain, enabling electron flow through Nor C across mid point potential hemes c + b to a large Nor B (Zumft, 1997).

Enzymatic studies have revealed Nor C having a low spin heme C and Nor B having a low spin heme B and a high spin heme B/non heme Fe binuclear site. The low spin heme B and high spin hemeB/non heme Fe binuclear site of Nor B is proposed to be the catalytic site where the binuclear heme/non heme Fe site is the proposed redox active site for the N-N bond of N_2O.

If electrons flow over Nor B from a low spin heme B to a high spin heme B, a reduced heme B is generated to form redox active catalytic sites that reduce NO to N_2O (Zumft, 1997).

The reduced heme B is generated with heme ferrous complexes that have a high affinity for NO with a redox potential of a Heme FE21-NO-/FE21-NO_2 couple which is too –ve to be accessed by a physiological reductant.

A proposed reaction involving the oxidation of heme FE21-NO is the oxidation to heme Fe2NO1-NO1 or the formation of a nonheme FE dinitrosyl complex at the active site for N-O-N bond formation.

(iv) Respiration of nitrous oxide

Nitrous oxide reductase is thought to be two identical subunits, each subunit containing an average of 4 Cu atoms. The electron entry site does not appear to be recognition specific, indicating receptivity to several electron carriers which are probably periplasmic.

The Cu sites are binuclear in each of the two subunits and labelled CuA and CuZ respectively.

Cu A is the site for electron entry where the transport competent form of the Nos reductase structure is stabilised by Cu A domain while Cu Z, the catalytic site, acts as the substrate bonding centre.

N_2O is reduced by two electrons to yield N_2 and water.

$N_2O + 2H^+ + 2e^- \longrightarrow N_2 + H_2O$

N_2O is usually inert but becomes an oxygen transfer reagent in the presence of a transition metal. It usually reacts by the extrusion of N_2 and formation of OXO complexes. In N_2O reductase, N_2O could bind end on to a Cu atom with unidentate binding to Cu occuring via nitrogen or oxygen. It could also bind as a bidentate ligand of a binuclear site, both ways are possible (Zumft, 1997).

B.6. Characteristics of the candidate bacteria

A denitrifying bacterium used by Casciotti et al. (2002) *Corynebacterium nephridii* ATCC 11425, was selected for δ^{15}N analysis of this study as it is a bacteria which will only reduce NO_3^- to N_2O (Knowles, 1982).

C. nephridii ATCC 11425, biosafety level 1, was isolated from the urine of a medicinal leech (Hart, Larson, & McCleskey, 1965) with an ATCC recommended culture medium of rabbit blood agar and a temperature of 37^{o}C, deemed (by the writer) unusual for a biosafety level 1 soil organism which should require a general purpose medium. It was also thought to be of uncertain taxonomic status by Knowles (1982).

Basic microbiological investigation and subsequent DNA analysis revealed the candidate bacteria to be a totally different bacterium, *Ochrobactrum anthropi* which concurred with findings by DSMZ (Deutsche Samlung von Mikroorganismen und Zellkulturen, a German resource centre for biological material) and CABRI (Common Access to Biological Resources Information) who stated *Corynebacterium nephridii* to be an invalid name (ref. appendix V).

DSMZ *O. anthropi,* 20150 (alternative identification number *C. nephridii,* number ATCC 11425) is grown on DSM media 53, at 30^{o}C (ref. Appendix IV) and is registered as a category risk group 2, capable of infecting immunocompromised humans (Alnor et. al., 1994; Deliere et. al., 2000; Kettaneh et. al., 2003; Leal-Klevezas et. al., 2005). This concurs with isolation of the species from a medicinal leech (Hart, et. al., 1965).

(i) Phenotype characteristics

O. anthropi, is a rod shaped, gram –ve, aerobic, non pigmented, motile organism which reduces NO_3 and NO_2 (Bathe et. al., 2006; Holmes, 1988).

It is classified as an $\alpha-2$ proteobacteria, a genetically diverse group, closely related to *Brucella* species (Graham et al., 2006; Jumas-Bilak et al., 2005; Leal-Klevezas et al., 2005; Lebuhn et al., 2000) that and only shares 163 of 284 common phenotype characteristics, (Leal-Klevezas et. al., 2005).

The bacteria is a free living species, ubiquitious in soil and water but capable of nosocomal infections, possibly by forming initial biofilms on fomites such as catheters (Bathe et. al., 2006; Graham et. al., 2006; Jumas–Bilak et. al., 2005; Laura et. al., 1996; Leal-Klevezas et. al., 2005; Lebuhn et. al., 2000). Strains have been isolated from various environments including hospitals, water, concrete, soils, termites, sewage sludges and oil spills (Bathe et. al., 2006; Kesseru et. al., 2002; Leal-Klevezas et. al., 2005; Lebuhn et. al., 2000) and is a mesophilic organism that can grow in temperatures between 4-40^{o}C in pH between 3-9, with optimum growth in a pH of 6-7 at 30^{o}C (Lebuhn et. al., 2000).

O. anthropi has been found to detoxify xenobiotic substances such as halobenzoates, chlorophenols, urea-formaldehyde, glyphosate and complex organic substances including crude oil and atrazine under denitrifying conditions (Graham et. al., 2006; Jumas-Bilak et. al., 2005; Kesseru et. al., 2002; Leal-Klevezas et. al., 2005; Shushkova et. al., 2009; Song & Ward, 2006).

It tolerates 0.21% acid extractable arsenic (As) (Chopra et. al., 2007) and high levels of reactive N oxides (Takaya & Takizawa, 2009). It can denitrify in the presence of 0.1 mM mercury (Hg,) 0.1 mM zinc (Zn,) 1.0 mM ead (Pb), 0.5 mM copper (Cu), 0.5 mM nickel (Ni) and 0.1 mM cadmium (Cd) (Kesseru et. al., 2002). It has also demonstrated heavy metal biosorption of chromium (Cr), cadmium (Cd) and sulphide oxidation (Li et al., 2008; Ozdemir et. al., 2003) and can grow in the presence of 40 g/L (40 psu) sodium chloride (Kesseru et. al., 2002).

(ii) Genotype characteristics

The tolerance of *O. anthropi* to a wide variety of environments can be explained by an adaptable genome consisting of two independent, complex, circular chromosomes with exceptionally high genetic diversity. This suggests potential adaptability to a variety of ecological niches (Grahame et al., 2006; Jumas-Bilak et. al., 2005; Leal-Klevezas et. al., 2005).

The megaplasmid content is highly variable amongst 9 strains of *O. anthropi* with a variation of 5060-8300 Mbp (Jumas-Bilak et al., 2005). Variation in genome size is due to acquisition of foreign DNA rather than chromosomal variation which explains the extensive variability of extrachromosomal elements which continue to be produced in cells (Jumas-Bilak et. al., 2005).

Analysis of a soluble subproteome reveals 249 proteins characterised as typical housekeeping genes as well as virulence factors relevant to human and plant disease. *O.anthropi* also has biotechnically useful enzymes, is resistant to all β lactams and possesses enzymes involved with cold shock proteins and extracellular polysaccharides (Grahame et. al., 2006; Jumas-Bilak et al., 2005).

One strain has a plasmid encoded for the degradation of parathion nitrophenol (Qiu et. al., 2006) and another has gene clusters for nitrate, nitrite, nitric and nitrous oxide reductases (Nar, Nir, Nor and Nos) respectively, exhibiting a capability to reduce N_2O to N_2 (Doi et. al., 2009).

Studies by Kesseru et. al. (2002) found *O.anthropi* completely reduced 12 mg NO_3 in 300 minutes, coinciding with a maximum concentration of NO_2. An increase in pH to 8.0 occurred after 80 minutes which required control. Nitrite accumulation indicated steady state NO concentration as regulation was tightly coupled with NO_2^- respiration to control excess NO (Zumft, 1997).

Other studies have shown *O. anthropi* to possess a copper containing dissimilatory Nir enzyme (Causey et. al., 2006; Doi et. al., 2009; Metz et. al., 2003; Witzel, 2000) while Kim et. al. (2006) reports the presence of a membrane bound nitrate reducing (Nar) enzyme, confirming the organism as a dissimilatory denitrifier. Halobenzoate degrading nirK genes are also associated with *O. anthropi* (Lee et. al., 2002).

B.6. Suitability of the candidate organism for the current project

Based on the studies above, *O. anthropi* is a promising candidate for the current project because it:

(a) Is a dissimilatory denitrifier

(b) Possesses copper containing Nir enzymes, therefore has potential to yield fingerprints for oxygen as well as nitrogen of NO_3^- and NO_2^-

(c) Is halotolerant and can therefore be used for fingerprinting saline sample

(d) Tolerates heavy metals and may therefore be suitable for δ^{15}N analysis of samples preserved with $HgCl_2$

(e) Tolerates anthropogenic toxins such as halobenzoates and chlorophenols, therefore may be suitable for δ^{15}N analysis of samples from polluted waterways

B.7. Aims

As the candidate bacterium *O.anthropi* appears to be a promising denitrifier for analysis of δ^{15}N in NO_3^- from fresh, saline and polluted water, culture protocols for optimal denitrification need to be established. These may differ from current protocols as *O.anthropi* has potential to adapt to a variety of ecological niches including the laboratory which may stimulate unexpected and undesirable responses. To achieve the aim of effective protocols for denitrification, the following objectives need to be met:

- To establish a growth curve of the candidate organism *O. anthropi*
- To establish that *O. anthropi*. can tolerate saline conditions
- To examine if current fingerprinting methods are suitable using *O. anthropi*
- To alter methods for accurate fingerprinting
- To simplify altered methods

Chapter 2

Materials and Methods

This project required work in several areas before achieving satisfactory N_2O yields and $\delta^{15}N$ of three standards.

Preparatory work included verification of the bacterial species by DNA analysis and confirmation that the candidate bacterium was halophilic. General microbiological procedures were also undertaken to characterise gross morphology, ensure culture purity and assess viability.

The production of a growth curve enabled cell titre to be correlated with temperature, incubation time and N_2O yields that revealed incomplete denitrification of KNO_3 amendments and $\delta^{15}N$ which were depleted or enriched in ^{15}N.

Careful examination of results led to method alterations which eliminated the need for KNO_3 amendments and long incubation times by producing anaerobic inocula in a candle jar, reducing cell titre and establishing a temperature for optimal denitrification.

All samples were submitted to the Stable Isotope Laboratory at Lincoln University for analysis on a PDZ Europa TGII/20-20 continuous flow mass spectrometer as the Stable Isotope Laboratory at University of Otago was not set up for detecting N_2O

2.0. Materials

A complete list of materials and equipment is presented in Appendix 1.

The candidate bacteria *Corynebacterium nephridii*, ATCC 11425 was purchased from ATCC: American Type Culture Collection (The Global Bioresource Center USA), and imported into New Zealand under compliance with ERMA (Environmental Management Risk Authority) requirements.

All microbiological work was undertaken using standard microbiological techniques as outlined in Reed et. al., (1998).

Media used for culturing the candidate bacteria was in accordance with denitrification and δ^{15}N analysis methods of Renner & Becker (1970), Hart & McClesky (1965), Casciotti et. al.,(2002), Christensen et. al., (1988), Sigman et. al.,(2001), Revesz & Coplen, (2007), Mørkved et. al., (2007).

(i) Rehydration and storage of subculture

A glass vial containing vacuum dried *C.nephridii* ATCC11425 was opened according to supplier's specifications and emptied into a glass universal containing 9 ml Difco ^{TM} TSB broth made up according to the manufacturer's specifications.

The universal was gently shaken to disperse the cells and 200 μl aliquots pipetted into universals containing 10 ml TSB which were then incubated in a shaker bath at 30 - 45 ^{o}C for 48 h.

Following incubation, each culture was transferred to a 20 ml centrifuge tube, spun for 5 mins on an MSE minor centrifuge at 3000 rpm and resuspended in 1 ml of supernatant. Cultures were then pipetted into to sterile 1 ml Eppendorf tubes and stored at at -80 ^{o}C.

Subcultures were prepared by rapidly thawing one vial of frozen culture with a flamed scalpel, inoculating 100 μl into each of several 12 ml polycarbonate tubes containing 8 ml TSB and incubating at 37^{o}C under varying incubation times to produce inocula of OD_{600} 0.300 – 0.600 (Pharmacia Biotech Novaspec II spectrometer). Subcultures were then stored for future use as described above.

(ii) Characterisation of *C. nephridii*, ATCC 11425:

a) Gross morphology : one loop of culture was streaked on TSA agar and incubated 24h at 36^{o}C. The resulting colonies were examined and their appearance noted.

b) Cell morphology: cells were Gram stained and examined under a microscope (oil immersion, magnification x100) for cell shape and colour

c) DNA analysis

Protocols for DNA extraction and 16S DNA analysis.

Unless otherwise stated, all centrifugation runs were performed in a SIGMA 1 – 15 centrifuge (Sigma Laboratory Centrifuge, Osterode amd Harx, Germany). Genomic DNA was extracted using a Qiagen DNeasy Tissue Kit, (Qiagen, Germany).

Culture for DNA extraction was prepared by thawing frozen stock with a cell absorbance of OD_{600} 0.300, using scalpel passed through a Bunsen flame (flamed) and pipetting 100 μl into 10 ml aliquots of Difco ™ TSB media dispensed into 12 ml glass exetainers with 2 ml headspace (HS). Cultures were incubated for 35 h at 36°C in a shaker bath until turbid (optical density not noted).

One ml of culture was pipetted into a 1.5 ml Eppendorf microcentrifuge tube and spun at 7,500 x g for 10 mins, (Beckman 212 M/E centrifuge, Beckman Coulter Inc. California, USA). The pellet was resuspended in 1 ml 10mM TE Buffer (10 mM Tris HCL, Bio Rad, 1 mM EDTA, (BOH Chemicals, Poole, England). The sample was spun for 2 mins at 12,000 rpm, the pellet resuspended in 180 μl Buffer ATI and 20 μl proteinase K, vortexed and incubated at 55°C for 3 mins.

Following incubation, 200 μl Buffer AL was added to the sample which was then vortexed and incubated at 70 °C for 10 mins. After incubation, a 200 μl volume of 100% ethanol was added, the sample vortexed and pipetted into a DNeasy minispin column. The column was spun for 10 mins at 12,000 rpm, the subsequent flow through discarded and 500 μl of AW1 added. The column was spun for 5 mins at 12,000 rpm, flow through discarded and 500 μl of AW2 added. Finally, the column was spun for 3 mins at 12,000 rpm, flow through discarded and the column respun for 3 mins to dry down membranes.

The spin column was transferred to a 1.5 ml Eppendorf microcentrifuge tube, 200 μl Buffer AE pipetted onto the membranes and incubated for 1 min at room temperature (RT). The column was spun for 1 min at 13,000 rpm and transferred to a new 1.5 ml Eppendorf tube, 200 μl Buffer AE pipetted onto membranes and spun for 1 min at 13000 rpm. These elutions, now containing sample DNA, were frozen at -20°C until required. The spin columns were discarded.

Genomic 16S rDNA of the candidate organism was amplified using the following protocol: 35.8 μl molecular grade water, 5 μl x10 Buffer, 5 μl dNTPs (2mM stock), 1.5 μl primer1:

forward 16S (10 μM stock), 1.5 μl primer2: reverse 16S (10 μM stock),1 μl DNA template and 0.2 μl Taq polymerase to a 0.2 ml microcentrifuge PCR tube (Eppendorf).

PCR amplification was undertaken using the following PCR programme on a Gene Amp PCR system 9600 (Perkin-Elmer corporation, Norwalk, USA) thermocycler:

Cycle 1: denaturation temperature 95 ^{o}C for 3 mins 10s, annealing temperature 33^{o}C for 2 min 30s and elongation temperature 65 ^{o}C for 3 mins; cycles 2-30: denaturation temperature 92^{o}C for 30s, annealing temperature 33^{o}C for 30 s and elongation temperature 65 ^{o}C for 1 min.

The PCR products were purified using the QIA PCR Purification Kit (Qiagen GmbH, Hilden, Germany) and the size of PCR products, around 800 kilo base pairs (Kb), confirmed by running 8 μl of purified product onto a 1% LE agarose gel and sequenced directly. DNA samples, mixed with agarose gel loading buffer, were separated on 0.8 % (w/v) agarose gels in TAE electrophoresis buffer containing 0.5 μg/ml ethidium bromide. Electrophoresis occurred at 70 V for 1 h or until the front dye had migrated down 2/3 of the gel. DNA was detected by UV transillumination and gel images obtained with a Gel Doc gel imager (Bio-Rad laboratories Inc., USA).

Sequencing reactions were carried out using a PRISM ready reaction DyeDeoxy terminator cycle sequencing kit (Applied Biosystems). Sequence analyses were undertaken using a Band- sequenamp (Massey University) and the program BLASTN (National Center for Biotechnology Information, Los Alamos, N.Mex.), available via the internet: (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&BLAST_PROGRAMS=megaBlast&PAGE_TYPE=BlastSearch&SHOW_DEFAULTS=on&LINK_LOC=blasthome).

(iii) Establishing a growth curve

Inocula for a growth curve was prepared by thawing a frozen subculture, OD_{600} 0.300 using a flamed scalpel. A 12 ml glass exetainer containing 10 ml TSB media was inoculated with 100 μl of thawed culture and incubated for 39.5 h at 36^{o}C in a shaker bath, reaching an OD_{600} of 0.612.

Exetainers containing 10 ml Difco ^{TM} TSB broth were then inoculated (in triplicate) with 100 μl of this culture over 2 h intervals and incubated at 36 ^{0}C in a shaker bath up to 33 h.

Optical densities of newly inoculated and incubating cultures were recorded at each inoculation with a final inoculation performed at 20.5 h. Recording culture density resumed after a 12 h interval and continued at 1h intervals over 11 h. All recorded measurements were then graphed.

(iv) Establishing salt tolerance of the candidate bacterium

The candidate bacterium was tested for tolerance to different salinities in order to establish if fingerprinting NO_3^- and/or NO_2^- in seawater at varying NaCl dilutions was possible.

Three NaCl concentrations were used:

a) S = 35: undiluted seawater (sw) filtered with a 0.45 μm nylon syringe filter (Membrane-Solutions).

b) S = 20.7 : Difco ^{TM} TSB broth amended with 1g/L KNO_3, 1g/L $(NH_4)_2SO_4$ and 4g/L K_2HPO_4 and 23 g /L NaCl.

c) S = 15.75: Difco ^{TM} TSB broth amended with 1g/L KNO_3, 1g/L $(NH_4)_2SO_4$ and 4g/L K_2HPO_4 and 17.5 g NaCl/L

An inoculum was prepared by thawing frozen stock, (OD unknown) using a flamed scalpel and pipetting 100 μl into a 12 ml exetainer containing 8 ml TSB media (4 ml HS) amended with 0.2 g KNO_3, 0.1g $(NH_4)_2SO_4$ and 1g K_2HPO_4. The culture was incubated 19 h at 34^o C in a shaker bath to reach an OD of 0.341.

Ten ml triplicates of each saline media were dispensed into 12 ml glass exetainers (2 ml HS) and inoculated with 100 μl culture. The exetainers were capped with VC 301 blue caps (butyl septa) and incubated on a shaker at 34 ^{o}C. The OD_{600} was recorded at varying intervals over 121 h and results graphed.

2.2. Overview of bacterial methods for denitrification

Because the candidate bacteria *C.nephridii*, [now recognised as *Ochrobactrum anthropi* (see 3.1)] has a highly adaptable genome, it is capable of adapting to changing conditions.

Culture methods were therefore reviewed to establish conditions for denitrification and subsequent δ^{15}N analysis.

(i) Denitrification methods using the candidate bacterium when known as *C.nephridii*

- Hart et. al, (1965) used *C.nephridii* for denitrification experiments under aerobic and anaerobic conditions. Cultures were maintained on semi solid agar and grown in TSB or peptone broth.

  Incubation with NO_3^- continued over several days at 28 ^oC on a shaker in 100 ml TSB media with 400 ml headspace (HS) which ensured aerobic conditions.

  Cultures were also incubated in capped tubes containing NO_3^- amended media with no headspace (for anaerobic conditions) over 24 h.

  Results showed *C.nephridii* to be a dissimilatory denitrifier as denitrification only occurred in anaerobic conditions (Hart et.al., 1965).

- Renner & Becker, (1970) trialled denitrification of growing and resting *C.nephridii* cultures in TSB media amended with 1% KNO_3 and 0.5% glucose.

  An inoculum of aerobic growing cells was incubated at 30 ^oC in anaerobic conditions with sample NO_3^- in a Warburg flask flushed with He. Growing cells utilised most NO_3^- after 36 h but accumulated NO_2^- after 72 hours.

  An inoculum of anaerobic cells was also grown in capped test tubes, amplified and incubated anaerobically over 38 h at RT. Cells were then enriched, resuspended in saline water and incubated in a Warburg flask containing 100 mM phosphate buffer, 30 mM sodium lactate and 13.5 mM sample NO_3^- or NO_2^-.

  This culture successfully denitrified all NO_3^- after 20 min and 13.5 mM NO_2^- after 30 minutes, confirming the bacterium did not reduce NO_3^- to N_2 as the end product of denitrification was N_2O.

(ii) Denitrification experiments using *O.anthropi*

Kesseru et. al., (2002) incubated *O.anthropi* cultures to midlog phase in DSM (Difco Sporulation Medium) at 30^oC on a shaker before enriching and incubating at 25 ^oC.

Total denitrification of 12 mg NO_3^- occurred after 300 mins with an NO_2^- maximum observed after 220 mins, showing NO_3^- reductase activity was twice as high compared to NO_2^- reductase activity. The pH was controlled after 80 mins as it increased to pH 8 after the addition of NO_3^-.

(iii) An overview of methods for δ^{15}N analysis, batch, closed system

Because *O.anthropi* is highly adaptable to changing conditions, (Grahame et. al., 2006; Jumas-Bilak et. al., 2005; Leal-Klevezas et. al., 2005) methods for δ^{15}N analysis were compared, as any variation could have a positive or negative effect on denitrification.

This was particularly relevant as several laboratories reported problems with existing methods (ref. appendix 3).

All methods of bacterial δ^{15}N analysis use the robust *Pseudomonas chlororaphis* (Sigman et. al., 2001) or *Pseudomonas aureofaciens,* (Casciotti et. al., 2002; Revesz & Coplen, 2007; Sigman et. al., 2001) with the exception of Casciotti et. al., (2002) who also trialled *P.aureofaciens* and *C. nephridii* for δ^{18}O analysis.

Variations of methods are summarised in table 2.1 below.

Table 2.1 Summary of bacterial methods for $^{15}N/^{14}N$ ratio analysis

Method	Christensen	Sigman	Casciotti	Reston	Mørkved
Culture	*P. chlororaphis*	*P.chlororaphis, P aureofaciens*	*P.chlororaphis, P.aureofaciens C.nephridii*	*P.aureofaciens*	*P.chlororaphis,*
1. Agar	TSA	TSA, 10mM KNO_3, 1mM $(NH_4)_2SO_4$	TSA 10mM KNO_3,	TSA 0.5g KNO_3, Incubate 2-3 days and subcultured	TSA
Incubation temperature	unspecified	RT	RT	unspecified	unspecified
2. inoculum	unspecified	5 ml TSB, 10mM KNO_3, 1mM $(NH_4)_2SO_4$	5 ml TSB, No amendments.	5 ml TSB (½ strength) 0.5g $(NH_4)_2SO_4$, 4.9 g K_2HPO_4,	One colony directly to working stock.
Incubation time	unspecified	24h, shaker	24h	24 h	NA
temperature	unspecified	RT	RT	unspecified	NA
3. Working stock	TSB with 0.1% KNO_3	TSB, 10mM KNO_3, 1mM $(NH_4)_2SO_4$	TSB, 10mMKNO_3, 7.5 mM NH_4Cl, 36mM K_2HPO_4,.	TSB, 1g KNO_3 0.5g $(NH_4)_2SO_4$ 4.9g K_2HPO_4,	TSB, 10mM KNO_3, 2mM NH_4 Cl,
Headspace	100ml:60 ml HS	400 ml:100 ml HS	130ml:30 HS	500 ml:80 ml HS	1L:1L HS
4. Incubation	5 days	6-10 days	6-10 days	1 week	6 days
Temperature	RT	RT	RT	unspecified	RT
NED test	NA	NA	NA	yes	He, 3h while stirred. Direct transfer to incubation bottles. He flush x5 while stirred. Sample 1 – 5 μg NO_3^-N. Incubation 12 – 14 h, room temp.
5. Centrifuging.	7400g, 10 min	7500g, 10 min	7500g, 10 min	75800 rpm, 15 min	
washing	no	no	no	no	
Resuspension	Supernatant	Supernatant	Supernatant	Inoculum medium	
6. Flushing	Ar, 10 min				
7. sparging	NA	N_2, 2^+ h	N_2, 3 h	He, 1h	
sample	5 – 50 nM NO_3,	10 – 20 nM NO_3,	10 – 20 nM NO_3,	10-40 nM NO_3.	
incubation	10-15 min	24 h	24 h	2 h	
Incubation temp	RT (usually 25 ^oC).	RT	RT	unspecified	
NaOH.	no	0.1 – 0.2 ml	0.1 ml 10N NaOH	0.1 ml 10N NaOH	no

Major steps of these methods are outlined in flowchart 2.2 below.

Table 2.2. Flowchart of all methods for bacterial denitrification

1. Revive bacteria	2. Produce inoculum	3. Produce culture	4. Extend incubation for anaerobic growth.	5. Enrich cells and resuspend in supernatant	6. Sparge out residual N_2O, add sample NO_3 -

Key :

1) Deep frozen bacteria are revived on TSA agar and incubated until colony formation.

2) Inocula are prepared by transferring one colony into amended TSB media and incubated over 24h.

3) Denitrifying culture is produced by pipetting an inoculum into amended TSB media and incubating to stationary phase.

4) Incubation time is extended to force anaerobic respiration of KNO_3 amendments.

5) Cultures are concentrated, resuspended in NO_3 - free media or spent supernatant.

6) Existing N_2O is sparged out before sample NO_3 is added

Of note was the omission of steps 2 and 5 by Mørkved et. al. (2007).

2.3. Denitrification experiments of the current project

(i) Sigman et. al., (2001) method

Frozen stock, OD_{600} 0.309 was thawed with a scalpel heated in a Bunsen flame, streaked onto TSA agar amended with 1g/L (10 mM) KNO_3 and incubated for 48 h at a RT of 22 ^oC which dropped to 12^oC overnight. As very little growth occurred [see Fig. 3.4. (i)], incubation of the culture continued for another 24 h in a water bath set at 22 ^oC, improving growth although colonies remained quite small.

An inoculum (in triplicate) was produced by transferring a colony into a 12 ml polycarbonate tube containing 5 ml TSB media amended with 1mM $(NH_4)_2SO_4$ and 10 mM KNO ,and incubating 24 h at 22 ^oC in a shaker bath. One inoculum (OD_{600} 0.662) was then selected for the denitrification experiment.

Four hundred ml TSB media (120 ml HS) amended with 1 mM $(NH_4)_2 SO_4$,10 mM KNO_3 and 1 ml antifoam was inoculated with two loops of culture and incubated at 22^{o}C on a shaker bath for denitrifying stock.

Incubation was halted after when the culture declined from an OD_{600} of 0.751 at 24 h to an OD_{600} of 0.518 at 96 h. One ml of culture was tested for NO_2^- by adding 40 µl 1 % sulphanilamide and 40 µl 1% NED. Results indicated the culture was NO_2^- – free as no pink colouration occurred.

Cells were enriched tenfold by decanting 10 ml aliquots culture into 12 ml polycarbonate tubes, spinning for 7 min at 3,000 rpm and discarding the supernatant. Each pellet (approximately 5 mm in diameter), was resuspended in 1 ml of retained supernatant, transferred to a 12 ml exetainer and capped with a VC 301 blue cap.

A 25G BD PrecisionGlide™ venting needle was then inserted into each septa before exetainers were placed on a manifold fitted with 26G BD PrecisionGlide™ sparging needles. The manifold, designed specifically for these experiments, was inverted and cultures sparged at 1.5 psi with nitrogen gas, std SM3 for 2 h.

Fig. 2.1. Cultures being sparged on an inverted manifold

Three sparged cultures were each injected with 1 ml of standard #1, using a 1 ml disposable syringe fitted with a 25G BD PrecisionGlide^{TM} needle and the procedure repeated with 1 ml distilled water (dw) for blanks. The standard had been diluted to 1 ppm NO_3-N for a yield of 100 ppm N_2O (ref appendix X).

All cultures were incubated 48 h at 22 ^{o}C before being destroyed by injecting with 1ml 1% Trigene, giving a total volume of 3 ml : 9 ml HS per exetainer. Product N_2O was analysed on a PDZ Europa TGII /20-20 mass spectrometer for N_2O concentration and δ^{15}N.

(ii) Sigman et al, (2001) method, heated gas sparge

Frozen stock,(OD_{600} 0.319) was thawed with heated scalpel, streaked onto TSA agar amended with 1g/L KNO_3 and incubated 24 h at 22 ^{o}C An inoculum was produced by transferring one colony into a 12 ml polycarbonate tube containing 5 ml TSB media amended with 1mM $(NH_4)_2SO_4$ and 10 mM KNO_3 which was incubated on a shaker at 22 ^{o}C for 24 h to reach an OD_{600} of 0.998.

Denitrifying stock was produced as in 2.3 (i) above, but incubation was halted at 48 h to avoid decline because the culture had reached stationary phase (OD_{600} 0.997). An NED test indicated the culture was NO_2^- - free and cells were prepared for sparging as in 2.3 (i) above.

Nitrogen gas was heated to 20 – 22 ^{o}C by flowing through 1.5 M of silicone tubing (internal diameter 7 mm) coiled in a pan of simmering water on a hotplate (temp 65 - 75 ^{o}C), before being connected to the sparging manifold. The manifold was inverted and enriched cultures were sparged with heated nitrogen gas 2 h at 1.5 psi.

Figure 2.2 Arrangement for heating gas. Nitrogen gas is ducted through red tubing to white silicone tubing immersed in a pan of simmering water. Warmed gas exits through red tubing (connection point not visible) to two inverted sparging manifolds, each holding six tubes.

When sparging was complete, blanks and standards (#1) were added and the samples incubated overnight at 22 ^{o}C. Following incubation 100 µl aliquots were withdrawn from three randomly selected samples using a 1 ml disposable syringe fitted with a 25G BD Precision Glide^{TM} needle, streaked onto Difco ^{TM} TSA agar amended with 1g/L K NO_3 and incubated overnight at 22 ^{o}C to assess viability and non contamination.

Remaining cultures were destroyed by injecting each sample with 1 ml 1% trigene. Product N_2O was then analysed on a PDZ Europa TGII /20-20 mass spectrometer for N_2O concentration and δ^{15}N.

(iii) Reston Method, (Revesz and Coplen, 2007), heated gas sparge

Frozen stock (OD_{600} 0.200), was hand held until thawed before streaking 100 µl onto TSA agars (in triplicate) amended with 1g/L KNO_3 and incubating at 30 ^{o}C for 48 h. Following incubation, triplicate subcultures were streaked onto TSA agar amended with 1g/L KNO_3 and incubated at 30 ^{o}C for 48 h.

Three polycarbonate tubes containing 5 ml NO_3^--free TSB medium, (15g/L Difco ^{TM} TSB broth amended with 0.25g/L $(NH_4)_2SO_4$ and 2.45 g/L K_2HPO_4) were each inoculated with one subcultured colony and incubated at 30 ^oC for 24 h.

Denitrifying stock was produced by decanting one 5 ml culture (OD_{600} 0.469) into a 500 ml Schott bottle containing 550 ml TSB media (85 ml HS) amended with 1g/L KNO_3, 0.5g/L $(NH_4)_2SO_4$ and 4.9 g/L K_2HPO_4).

The culture was incubated at 36 ^oC in a shaker bath, reaching an OD_{600} of 0.177 after 7 d and tested for NO_2^- as outlined in 2.3 (i). Results indicated the denitrifying culture was NO_2^- free and cells were prepared for sparging by adding 500 µl antifoam, centrifuging for 15 mins at 3,400 rpm and resuspending the pellet in 1 ml NO_3^- – free TSB media.

Resuspended cells were sparged with nitrogen gas heated to a temperature of 22 - 29 ^oC for 2 h as described in 2.3. (i) above before adding standards and blanks and incubating at 30 ^oC overnight.

Following incubation, cultures were destroyed as described in 2.3 (i) above and product N_2O analysed on a PDZ Europa TGII /20-20 mass spectrometer for N_2O yield and $\delta^{15}N$. Results were then analysed before any modifications to the method were made.

2.4. New method, heated gas sparge

Because the three denitrification experiments failed to produce good results, a new method was developed to achieve $\delta^{15}N$ values with greater precision. This involved producing an anaerobic inocula and incubating a denitrifying colony in non amended TSB media, thereby avoiding the risk of KNO_3 amendments being carried over and contaminating samples. A flow chart of the new method is shown in table 2.3 below.

Table 2.3. Flow chart of new method

1. Resuscitate bacteria In a candle jar	2. Incubate bacteria anaerobically without KNO_3	3. Sparge and add sample	4. Produce controls:	5. Analyse product N_2O

Key:

1) Cells are resuscitated in near anaerobic conditions of a candle jar to produce denitrifying bacteria.

2) Denitrifying stock is produced by transferring denitrifying cells into NO_3 – free media and incubating overnight with limited ambient oxygen.

3) Unenriched denitrifying stock is sparged with heated gas before adding three standards.

4) Unenriched denitrifying stock is sparged with heated gas before adding NO_2^- and tested with NED to confirm denitrification

5) product N_2O of standards is analysed

(i) New method using TSB media only, heated sparge

Frozen stock (OD_{600} 0.200) was hand thawed and 100 μl streaked onto TSA agar amended with 1g/L KNO_3 (in triplicate). Microaerobic conditions of a candle jar (Jensen et al., 1977; Martin et al., 1974), were created by placing agars in an airtight tin with a lit candle. The lid heated when the tin was sealed but cooled after the ambient O_2 had been combusted and extinguished the flame. Resuscitating stock was then incubated for 48 h at 30 ^oC to produce denitrifying colonies.

Denitrifying stock was produced by inoculating a denitrifying colony directly into each of two media:

a) non-amended Difco ^{TM} TSB broth

b) Difco ^{TM} TSB broth amended with 1.5 g/L K_2HPO_4

and incubated 24 h on a shaker at 30 ^oC to reach an OD_{600} of 0.208 in condition (a) and an OD_{600} of 0.124 in condition (b). Ambient atmospheric O_2 was restricted to 20 ml HS.

Following incubation, 100 µl of antifoam was added to each culture and 1 ml of unconcentrated culture (condition [a]) pipetted into 12 ml exetainers (x 12) and capped with VC 301 blue caps and butyl septa.

These steps were repeated for cultures in condition (b).

The aliquots of denitrifying stock were then sparged with gas heated to 29 – 33 ^{o}C for 30 mins before adding three standards (#1, #2, and #3) and blanks (in triplicate. Standards had been diluted to yield 100 ppm N_2O (ref. appendix 10).

Cultures were incubated for 24 h at 30 ^{o}C after which each was injected with 200 µl 2% trigene. Product N_2O was then was analysed on a PDZ Europa TGII /20-20 mass spectrometer for an N_2O yield and $\delta^{15}N$.

Controls were prepared (see 2.5 below) and the NED test applied to confirm denitrification by viable cells had occurred.

(ii) Repeat of New method, using TSB media amended with K_2HPO_4, heated sparge

Frozen stock (OD_{600} 0.200) was resuscitated in accordance with the new method and denitrifying stock produced in TSB media (20 ml HS) amended with 9 mM K_2HPO_4 to reach an OD_{600} of 0.177.

Following incubation denitrifying stock was prepared in accordance with the new method and cultures sparged with heated gas for 1 h at 29 – 33 ^{o}C before adding blanks and three standards (in triplicate)..

Cultures were then processed in accordance with the new method before analysis $^{15}N/^{14}N$ ratio.

Controls were prepared for NED tests as described in 2.5 below.

2.5. NED tests for the New method

A control standard was prepared (in triplicate) by combining 1 ml 0.014mM $NaNO_2$ and 1 ml of TSB before adding 40 µl 1 % sulphanilamide and 40 µl 1% NED. This produced a reference colour for unreduced 0.014mM $NaNO_2$.

One ml of unconcentrated denitrifying culture (OD_{600} 0.208) was sparged and incubated with 1 ml of 11.7 mM $NaNO_2$ for 24 h at 30 ^{o}C before adding 40 µl 1 % sulphanilamide and 40 µl 1% NED. Any colouration was then compared against the colouration control standards to assess if denitirification of NO_2 had occurred.

2.6. Final method

Results of the previous repeat (new method) led to a final method (see table 2.4) which eliminated the need to sparge cultures on a manifold.

Table 2.4. Flow chart of the final method

1. Resuscitate bacteria In a candle jar	2. Sparge denitrifying stock during incubation	3. Transfer exetainers containing sample	4. Analyse product N_2O

(i) Trial 1

Samples were prepared by pipetting triplicates of blanks (dw) and standards #1, #2 and #3 into exetainers which were capped with VC 301 blue caps and butyl septa. The tubes were then flushed with He gas and stored in a fridge until required. Controls containing 1g/L $NaNO_2$ were prepared in the same way.

Inocula was produced by hand-thawing frozen stock (OD_{600} 0.200) and streaking 100 µl thawed stock onto triplicate TSA agars amended with 1g/L KNO_3. Plates were then placed in a candle tin and incubated for 48 h at 30 ^{o}C, producing a large spreading, semi-translucent colony.

A large clump of colony was inoculated into 110 ml TSB media (20 ml HS) to which 100 μl of antifoam had been added and culture bottle capped with an aperture cap and silicone septa before inserting a 25G BD PrecisionGlide™ venting needle and 0.9 x 90 mm Terumo spinal needle which almost reached the base of the flask.

A gas ducting tube was connected to the Terumo needle and unheated gas sparged through the medium at the lowest achievable pressure of 1 psi overnight on a shaker at 30 ^{o}C, producing denitrifying stock with an OD_{600} of 0.419.

Fifteen ml of culture was rapidly drawn up into a 15 ml disposable syringe fitted with a 26G BD PrecisionGlide™ needle and 1 ml aliquots injected into the prepared samples and controls which had been prewarmed to 30 ^{o}C. The samples were then incubated overnight at 30^{o}C.

An NED test was performed on the controls to detect if denitrification of NO_2^- had occurred which confirmed a readtion had taken place. Following this confirmation, cultures containing standards were each injected with 200 μl of 2 % Trigene and product N2O analysed on a PDZ Europa TGII /20-20 mass spectrometer.

(ii) Trial 2

Samples and controls were prepared as described in 2.5 (i) above.

Frozen stock with an OD_{600} of 0.100 was resuscitated in a candle tin as described above and a large clump of culture inoculated into 110 ml TSB media (20 ml HS) with 100 μl of antifoam. Denitrifying stock was incubated at 30 ^{o}C as described in 2.6 (i) above, to reach an OD_{600} of 0.100.

Fifteen ml of culture was rapidly drawn up into a 15 ml disposable syringe fitted with a 26G BD PrecisionGlide™ needle and 1 ml aliquots injected into the prepared samples and controls which had been prewarmed to 30 ^{o}C. The samples were then incubated overnight at 30 ^{o}C.

An NED test was performed on the controls which confirmed viable denitrifying cells as a quantity of NO_2^- had been reduced. The remaining samples were then prepared for analysis as described in 2.6 (i) aboveand analysed.

The candidate bacterium was found to respond differently to culture protocols of bacterial methods using a *Pseudomonas* species. This justified a move away from original methods for δ^{15}N analysis and led to the development of a new method that was based on evaluation and interpretation of results which are shown in Chapter 3.

Chapter 3

Results

3.0. Summary of Results

Gross morphology of the candidate bacterium *Corynebacterium nephridii* showed culture purity and typical colony growth, but DNA analysis revealed the bacterium to be another species of denitrifier, *Ochrobactrum anthropi* that is halotolerant and can potentially denitrify N-oxides in seawater. Work therefore continued using this species where a growth curve was produced to show typical growth phases that were subsequently linked with temperature and denitrification rates.

An accidental drop in temperature during the 1st experiment attenuated growth of a resuscitating culture and led to a finding that the sparging gas temperature affected denitrification rates.

The 2nd experiment used a heated sparging gas which increased denitrification rates, but showed a considerable amount of N-oxides had been carried over to contaminate results.

During experiment three, cultures were incubated for an extended time and resuspended in NO_3-free media to eliminate contamination by N-oxides. This produced N_2O yields which were close to expected concentrations, but δ^{15} N results showed fractionation or contamination had occurred.

A new approach was therefore taken to produce a denitrifying inoculum in a candle jar which produced morphologically different colonies in microaerobic conditions.

An anaerobic colony, amplified in NO_3-free media was then incubated with NO_2^- and tested with NED which showed this unenriched culture could denitrify a quantity of NO_2^-. Three NO_3^- standards of known isotopic composition were consequently trialled in a fourth experiment using this method for producing a denitrifying culture. Results indicated problems of a technical nature as $\delta^{15}N$ values were enriched but increased in accordance with values of standards.

A repeat experiment revealed that replacement of a blocked needle during the sparge caused changes that affected denitrification rates. The method was therefore adjusted to eliminate such risks by sparging the entire culture instead of individual aliquots. Resulting cultures produced δ^{15}N ratios as well as δ^{18}O values that were in good agreement with the known values of standards. All results are reported in detail below.

3.1. Characterisation of the candidate bacteria

(i) Fig. 3.1 shows the normal morphology of a culture incubated at 36^{o}C on TSA agar. Colonies were small, creamy and mucoid, merging into a heavy growth in the 1st and 2nd streaks.

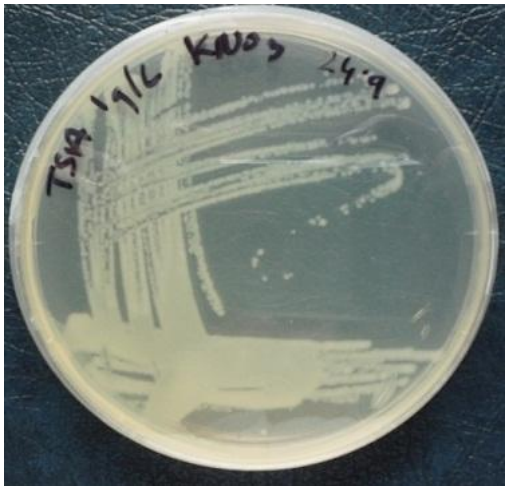


Figure 3.1. **Agar plate showing gross morphology of *O. anthropi* culture resuscitated at 36^{o}C for 48h.**

(ii) Microscopic examination under oil immersion revealed Gram negative fat rods in clumps, singles and pairs, showing the species was not a *Corynebacterium*.

(iii) Fig 3.2 showed Genomic DNA from the candidate bacteria lane (N), produced bands with a molecular size of approximately 800 kilo base pairs (Kb) when compared with the Kb Plus DNA Ladder on the left but a band in the negative control lane indicated a contamination in either reagents or the DNA sample.

Figure 3.2 Extracted DNA of *C. nephridii* against +ve and –ve controls.

Left hand (LH) box: DNA ladder and Kb sizes. Right hand (RH)box: 16S rRNA of *C. nephridii.*

RH box from left to right: Lanes 1 & 8 marker DNA, lane 2 & 3 +ve controls, lane 4 & 5 –ve controls, lane 6 blank, lane 7 PCR amplified 16S rRNA gene of *C. nephridii,*), showing the molecular weight of approximately 800 kilo based pairs (Kb).

(iv) Fig. 3.3 shows a rerun of samples using new reagents, confirming initial reagents were contaminated and that the genomic DNA for 16s rRNA has a molecular size of approximately 800 kilo base pairs.

Genomic DNA of *Pseudomonas chlororaphis* was included in lane 2 as an extra control.

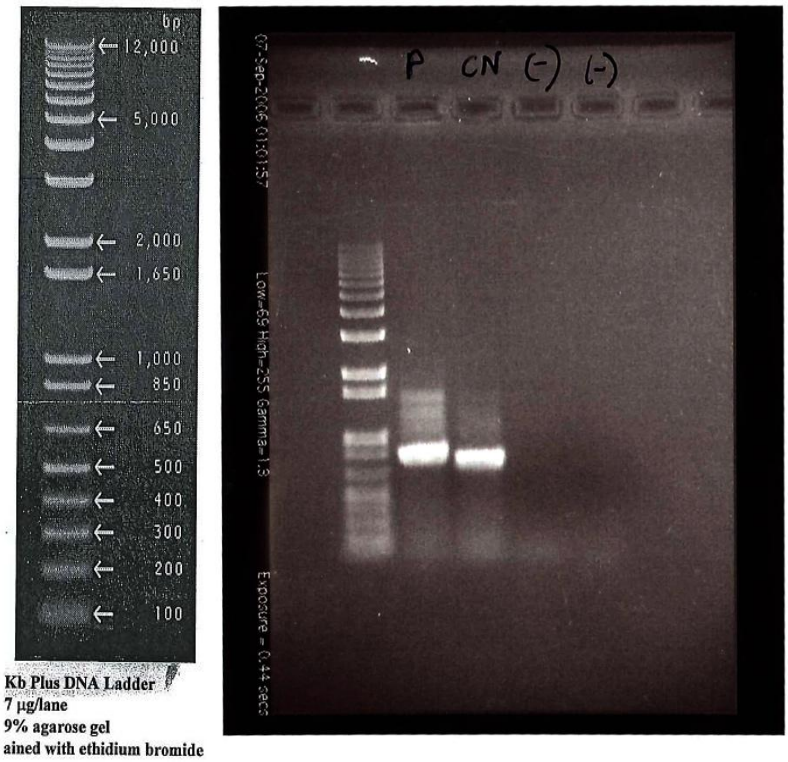


Figure 3.3. Rerun of extracted DNA of *C. nephridii* against –ve controls.

LH box: DNA ladder and Kb sizes, RH box: genomic 16S rRNA of *C. nephridii.* Lane 1 marker 16S rDNA, lane 2 genomic 16S rRNA of *P.chlororaphis*, lane 3 genomic rRNA of *C. nephridii*, lane 4 & 5 –ve controls.

(v) DNA analysis using BLASTN revealed a 99% homology with *Ochrobactrum anthropi* which is a different genus and species of bacterium from *Corynebacterium nephridii*. This concurs with findings by DSM, (Deutsche Sammlung von Mikroorganismen und Zellkulturen) the German resource centre for biological material (ref. Appendix 12).

3.2. The growth curve of *O.anthropi*

Mean OD_{600} values of triplicate inoculations were graphed, showing a typical growth curve with lag, log, stationary and decline phases (see Fig 3.4).

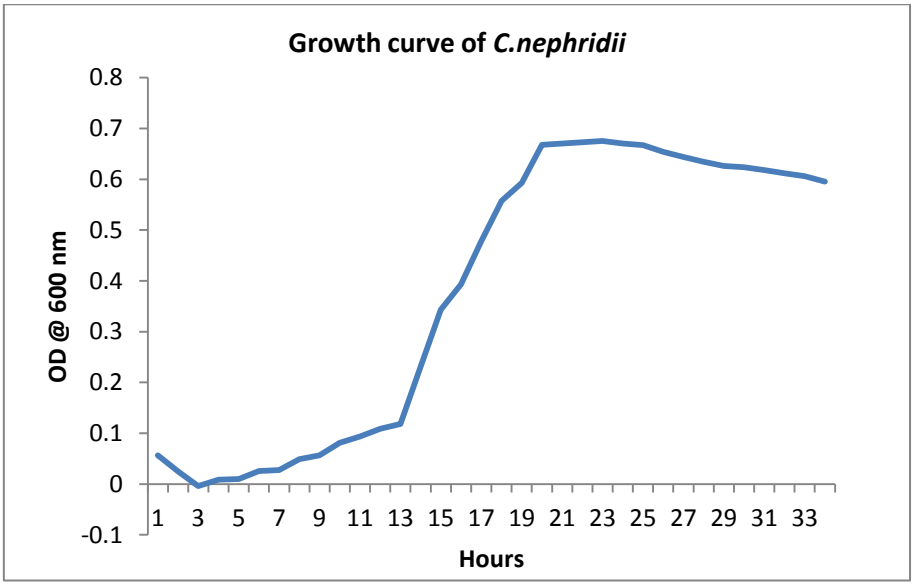


Figure 3.4. Growth curve of candidate bacteria *O.anthropi.*

a) Lag phase is marked by a decline of initial density (OD_{600} 0.06) during the first 3 h of incubation after which cell density increases slowly over 10 h to OD_{600} 0.12.

b) The exponential increase in optical density at 13 h shows log phase growth that continues over 7 h, peaking with an OD_{600} of 0.700 at 20 h.

c) A stationary phase appears between 20-24 h with an OD_{600} of 0.700 and continues over 4 h.

d) The decline/death phase begins at 24 h with a decrease in optical density to an OD_{600} of 0.600 at 33 h after which recording ceases.

3.3. *O.anthropi* tolerance to salinity

Fig. 3.5 shows mean OD_{600} values of triplicate inoculations in three saline media. All inoculations increased over time, indicating growth and a tolerance to salinity.

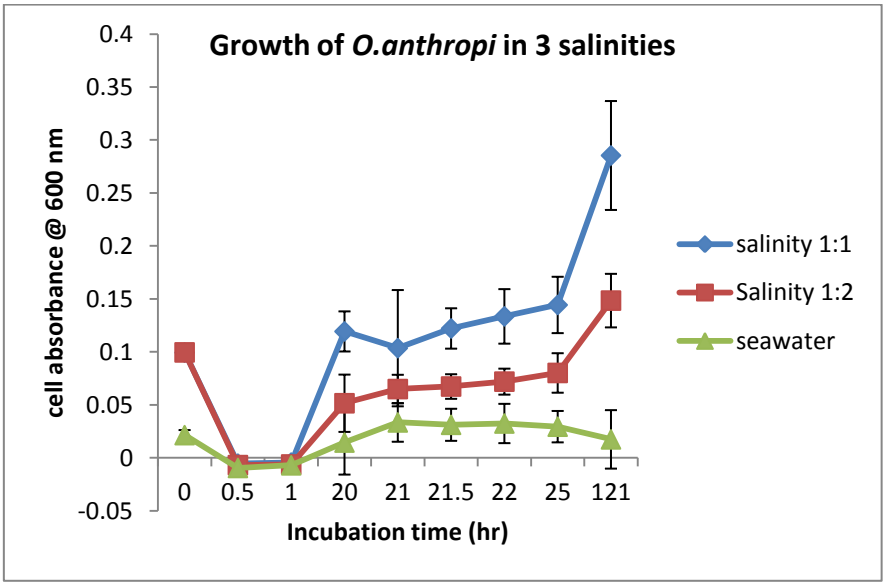


Figure 3.5. *O. anthropi* growth in 3 saline conditions.

Key

a) Seawater (S = 35.0) unamended filtered seawater

b) 1:2 salinity (S = 20.7) media emulating NaCl salinity in 2 ml seawater to 1 mL culture.

c) 1:1 salinity (S = 15.75) media emulating NaCl salinity in 1 ml seawater to 1 mL culture.

All media exhibited a drop in OD after 30 mins incubation, as cells rapidly adjusted to the new environment. Lag phase and exponential phase occurred during 1 – 20 h and a stationary phase was evident during 21-25 h after which growth in condition (a) ceased at an OD_{600} of 0.026 and the media cleared while conditions (b) and (c produced another growth event to reach an OD_{600} 0.29 and 0.102 respectively at 121 h.

Filamentous white biofilms were observed in the base of all the sample tubes at 121 h .

3.4. Denitrification experiments of the current project, observations and results

(i) Temperature effects on resuscitating bacteria

Compared to normal growth (see Fig. 3.1), resuscitating stock showed atypical, white colonies after the ambient laboratory RT dropped from 22 ^{o}C to 12 ^{o}C overnight (see Fig. 3.6). This shows low temperatures affected growth and could also impact on denitrification rates.

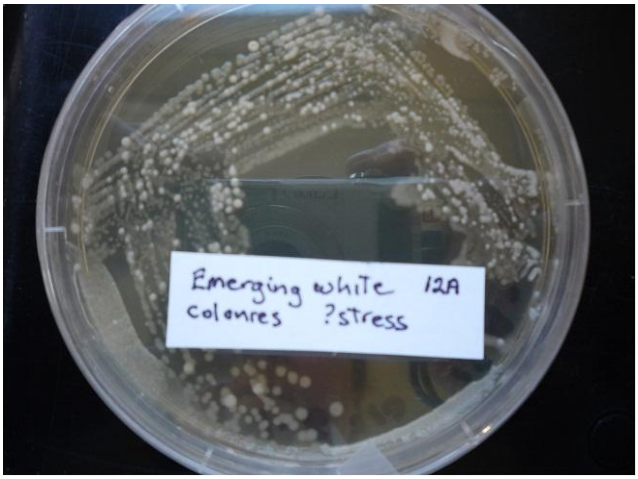


Figure 3.6. Gross morphology of *O.anthropi* after a drop in temperature to 12 ^{o}C

Growth of normal appearance resumed when the cultures were reincubated at 22 ^{o}C for an extra 24 h.

(ii) N_2O yield of the Sigman et. al. (2001) method, unheated sparge

Fig. 3.7 shows a culture that had been resuspended in supernatant and sparged with unheated N_2 gas (12 ^{o}C) produced mean N_2O concentrations of 425 ppm N_2O in blanks and 435 ppm N_2O in standard #1 (raw data, appendix 4). The standard had been diluted to 1 ppm NO_3 - N for a yield 100 ppm N_2O. Results are the mean values of triplicate samples.

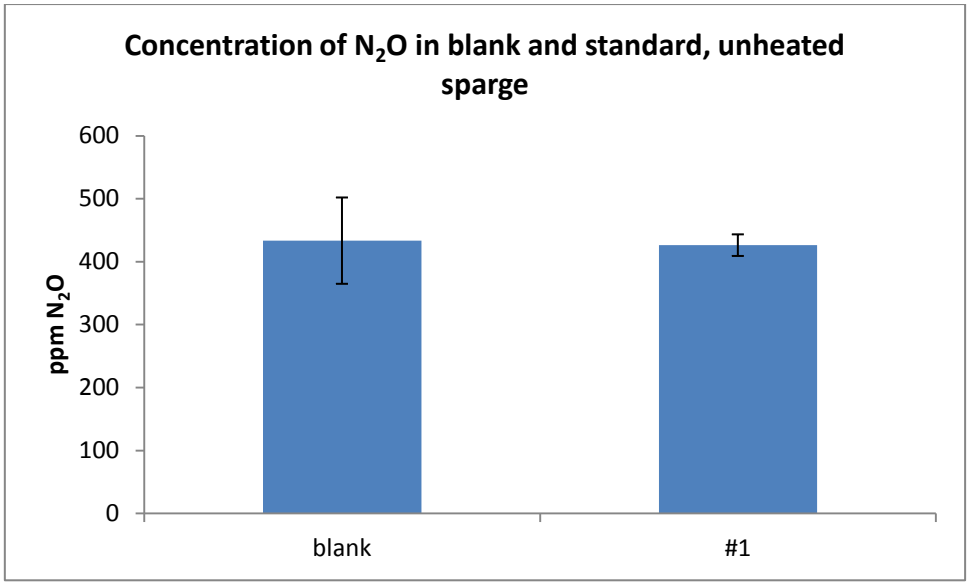


Fig. 3.7. N_2O concentrations of blank and standard, unheated sparge treatment.

The high yield of N_2O indicates a considerable amount of residual N-oxides had been denitrified during incubation with samples with no difference occurring in the N_2O yields of blank and standard.

(iii) Comparison of the δ^{15}N values of blank and standard, unheated sparge.

The δ^{15}N values in standard #1 showed a depletion in ^{15}N ($\delta^{15}N$ = -13.03) compared to the blanks ($\delta^{15}N$ = -12.48) indicating lighter ^{14}N nuclides occurring in standard #1 ($\delta^{15}N$ = 0.09) were being denitrified during the second incubation, but that the reaction was incomplete.

(iv) Comparison of N_2O yield, unheated sparge and heated sparge

Fig. 3.8 shows a sparging gas heated to 22 ^{o}C increased denitrification rates and the mean N_2O yield to 645 ppm in blanks and 665 ppm in standard #1 which were 200 ppm greater compared to N_2O yields from the unheated sparge condition (raw data, Appendix 5).

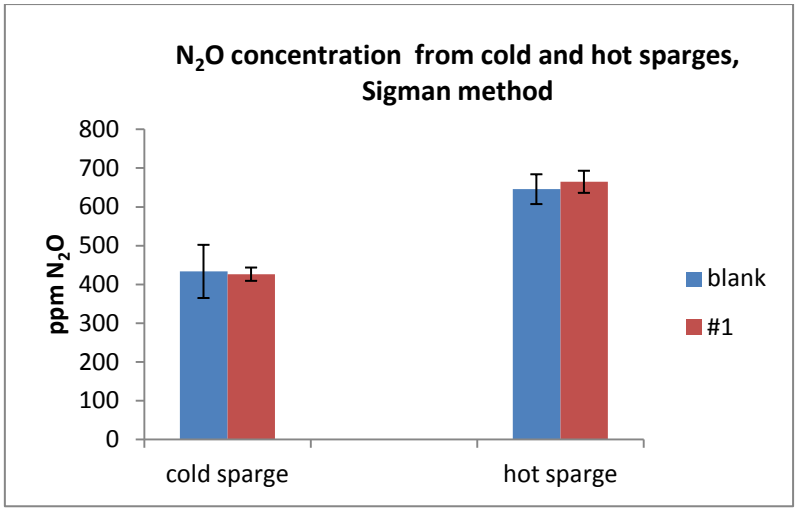


Figure 3.8. Comparison of N_2O (ppm) concentrations from an unheated and heated sparge, Sigman et al. (2001) method.

Key

Blank distilled water

Std #1 *($\delta^{15}N = 0.09$)* 1 ppm NO_3-N

Although temperature increased denitrification rates, the extremely high production of N_2O in both blanks and standards (500% recoveries) clearly shows there is an amount of nitrate getting through that is swamping the sample signal. The similarity in N_2O yield also indicates denitrification with a heated sparging gas remained incomplete. Results are the mean values of triplicate samples.

v) Comparison of the $\delta^{15}N$ values of blank and standard, heated sparge

The standard ($\delta^{15}N = 0.09$) of the heated sparge produced a depleted $\delta^{15}N$ value ($\delta^{15}N = -10.37$) compared the $\delta^{15}N$ value of blank #1 ($\delta^{15}N = -10.15$), indicating that the main source of nitrogen in the N_2O produced was not from the standard but rather from some other isotopically-depleted nitrogen oxide.

Purity plates of denitrifying cultures from the Sigman et. al., (2001) experiments produced colonies of normal appearance and good growth similar to colonies in Fig. 3.1, confirming that the culture was viable and uncontaminated.

3.5 The Reston method (Revesz & Coplen, 2007)

The Reston method used non amended media instead of supernatant to resuspend enriched cultures. This was expected to eliminate any carry–over of residual N–oxides occurring in the original growth media.

(i) N_2O concentrations of the Reston method

Fig. 3.9 shows mean N_2O yields of 136.3 ppm and 135.6 ppm N_2O in standards and 1–2 ppm blanks by an enriched 7 d culture resuspended in non amended media and sparged 2 h with heated gas. Results are the mean values of triplicate samples (raw data, Appendix 6).

The standard was diluted to to yield 100 ppm N_2O.

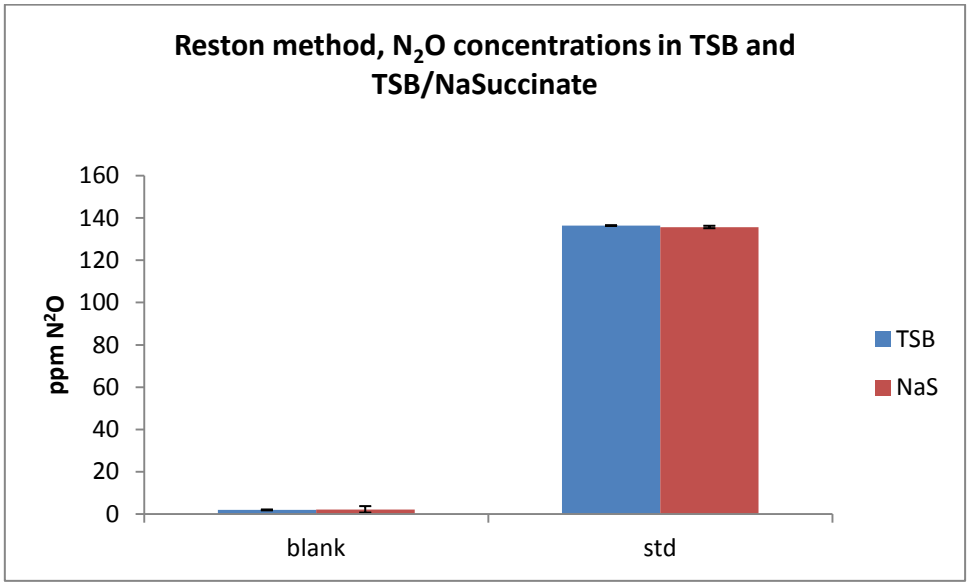


Figure 3.9. Concentration of N_2O (ppm), TSB or TSB/Na succinate media, Reston method.

Key

Blank	distilled water
Std #1 ($\delta^{15}N$ 0.092)	(1 ppm NO_3-N)

A similarity in N_2O yields from both TSB and TSB/Na succinate media also showed the the nutrients in TSB were sufficient to enable denitrification of the standard.

(ii) $\delta^{15}N$ values from the Reston method (Revesz & Coplen, 2007)

Resulting $\delta^{15}N$ values of both media from the Reston (Revesz & Coplen, 2007) method are shown in Fig. 3.10 below and indicate some fractionation of nitrogen isotopes had occurred.

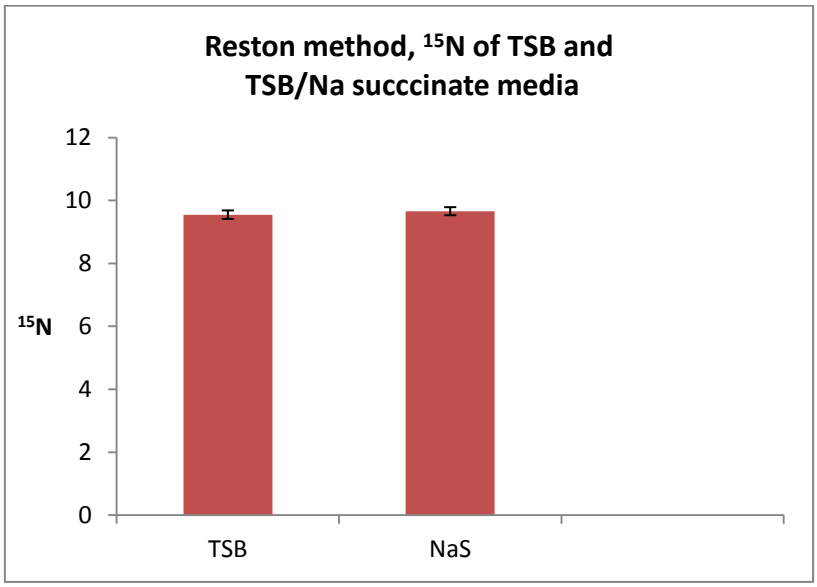


Figure 3.10. $\delta^{15}N$ values from TSB and Na succinate conditions, Reston method.

Key

TSB	NO_3-free broth
NaS	sodium succinate
Std	standard #1, $\delta^{15}N$ 0.09

The $\delta^{15}N$ results of TSB and TSB & Na succinate media show no significant difference in mean values of triplicates (student's t test , CI 95%, p=0.05, but these values, $\delta^{15}N$ 9.59, SD 0.13 (TSB) and $\delta^{15}N$ 9.65, SD 0.12 (TSB & Na succinate) are considerably enriched in ^{15}N when compared to the $\delta^{15}N$ -10.37 value of the previous Sigman et. al., (2001) experiment, heated sparge.

3.6. New method, observations and results

As results enriched in $\delta^{15}N$ may have originated from KNO_3 amendments in the culture medium, cultures were resuscitated in microaerobic conditions to produce a denitrifying inocula which was then amplified in unamended TSB.

(i) Resuscitating culture in microaerobic conditions

Fig. 3.11 shows the gross morphology of a "candle jar" culture.

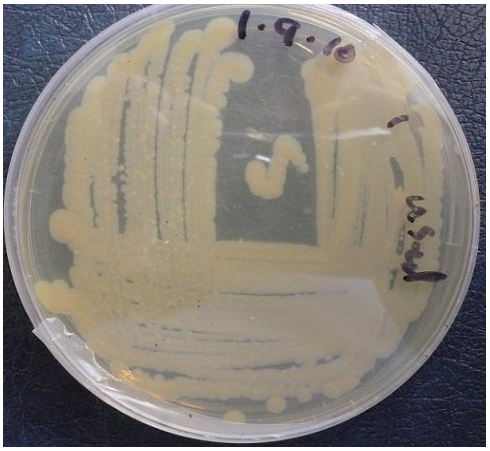


Figure 3.11. Gross morphology of an *O.anthropi* culture resuscitated in a candle jar over 48 h at 30 ^{o}C.

The candle jar culture differs from normal cultures (Fig. 3.1) and cold stressed cells (Fig. 3.7) by being thin, semi-translucent and merging.

(ii) NED trials, new method

Fig 3.12 shows the results after 1 ml aliquots of amplified "candle jar" culture were incubated in 0.014 mM $NaNO_2$ and tested with NED. LH tubes containing unconcentrated culture and $NaNO_2$ exhibited a lighter colouration compared to RH controls containing TSB and $NaNO_2$ showing a considerable reaction had taken place.

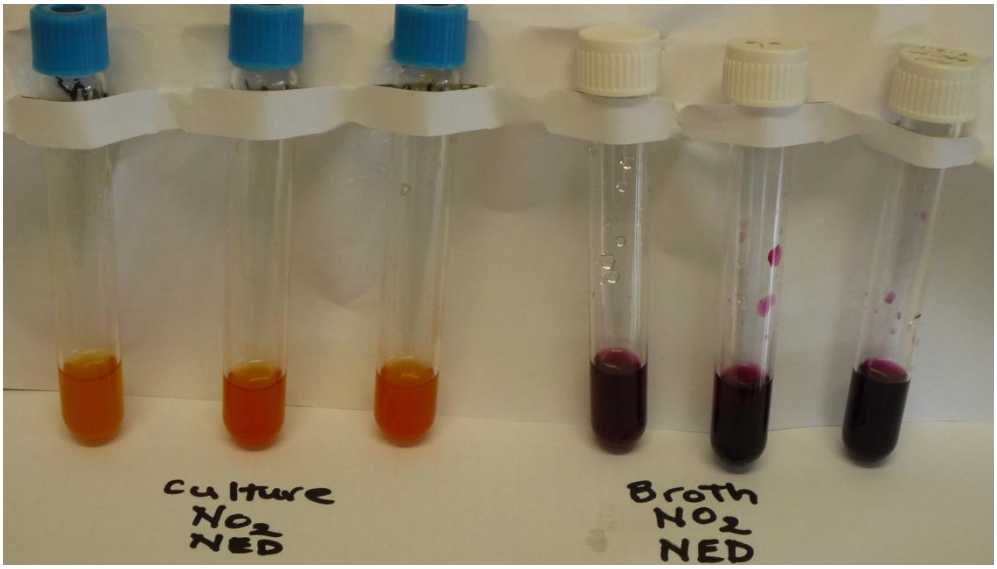


Figure 3.12. Results of NED trials showing new method culture had denitrified $NO2^-$.

Key:

LH tubes: 1 ml culture incubated with 1 ml 0.014 M $NaNO_2$ after application of the NED test.

RH tubes: 1 ml TSB & 1 ml 0.014 M $NaNO_2$ after application of the NED test.

The lighter colouration shows less NO_2^- exists in the medium containing culture.

(iii) N_2O concentrations in TSB and TSB / PO_4 , New method

Fig. 3.13 shows the recovery of N_2O from three standards of known $\delta^{15}N$ values after incubation with 1 ml aliquots of unconcentrated culture amplified from a "candle Jar" inoculum. All standards had been diluted to yield 100 ppm N_2O.

Concentrations of N_2O produced from standard #1 were above 100 ppm (TSB medium) while standard #2 (79.9 and 75.6 ppm) and standard #3 (58.6 and 55.2 ppm) were lower than the expected N_2O yield (raw data, Appendix 7).

Blanks produced concentrations of 20–22 ppm N_2O which were higher compared to blanks of the Reston method (see Fig. 3.19).

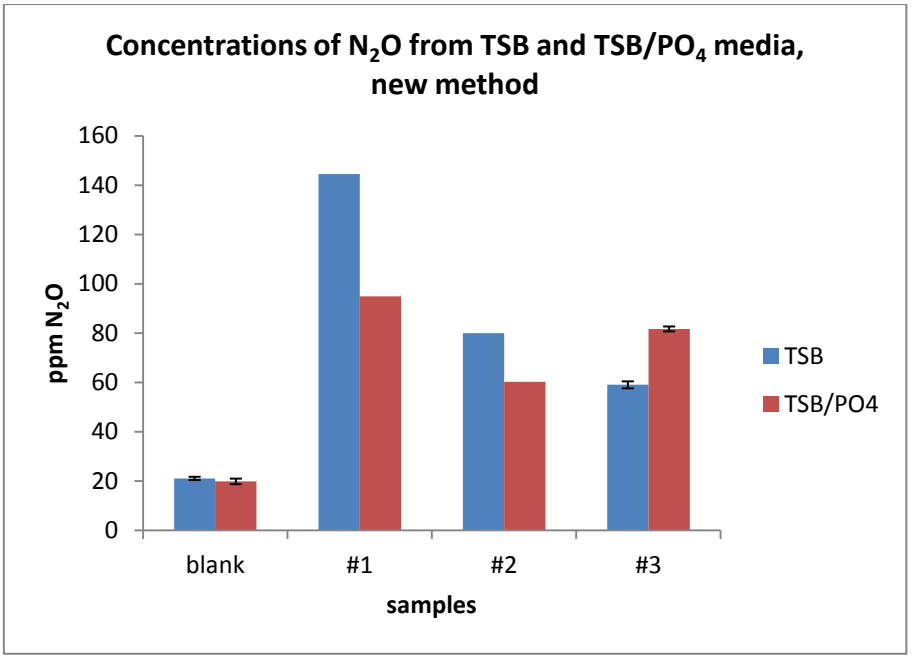


Fig. 3.13. New method, recovery of N_2O from three standards in two media. The expected recovery is 100 ppm.

Key

TSB NO_3-free TSB

PO_4 NO_3-free TSB and PO_4

Because the culture was homogenous and the standards were diluted to the same concentration, these results could be due to a technical error.

(iv) New method, $\delta^{15}N$ / ^{14}N ratio of three standards in TSB and TSB & PO_4

Results in Fig. 3.14 show a $\delta^{15}N$ increase in accordance with increasing $\delta^{15}N$ values of standards #1, #2 & #3 although an enrichment factor is evident in the both media.

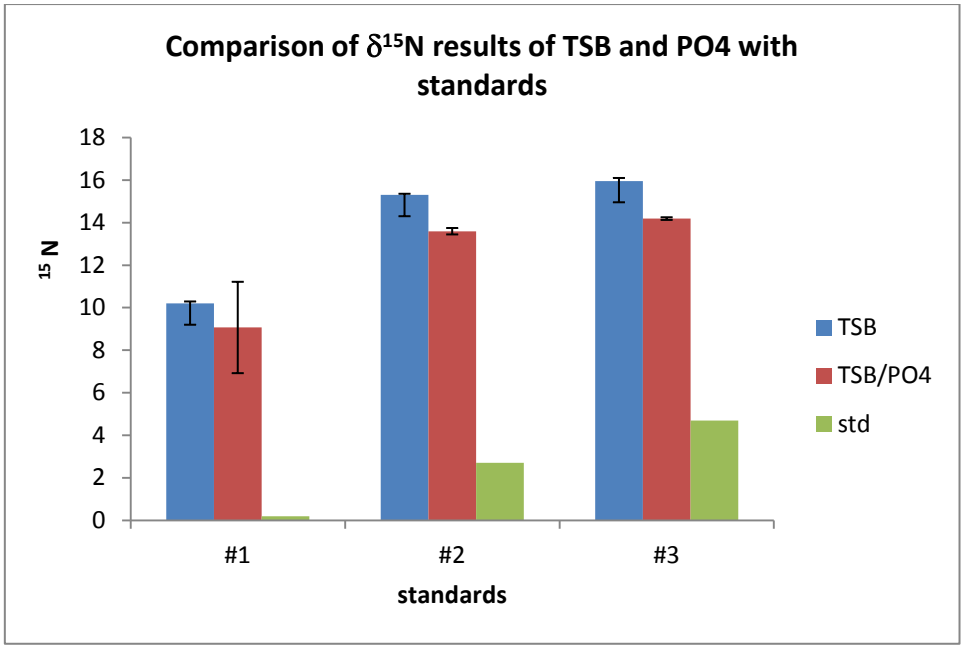


Fig. 3.14. $\delta^{15}N$ of samples compared with the known values of standards.

Key

TSB NO_3-free broth

PO_4 NO_3-free TSB and PO_4

Std #1 $\delta^{15}N$ 0.09

Std #2 $\delta^{15}N$ 2.7

Std #3 $\delta^{15}N$ 4.7

Because all values are enriched but increase in accordance with known values, results may be due to a technical error.

(v) Repeat of New method, TSB/PO_4 media only

Table 3.2 shows the concentrations of N_2O produced in the repeat experiment using TSB/PO_4 media only. The highlighted figures reveal a decrease in N_2O yields after tube 3 was removed to replace a blocked needle on the 1st manifold (raw data, Appendix 7a).

Table 3.1. ppm yield N_2O according to placement of tubes on two manifolds. A decrease in N_2O concentrations (bold figures) was observed after tube 3 was temporarily removed

Repeat of new method, ppm N_2O concentration, mean raw data.						
1st manifold						
Tubes	1	2	**3**	**4**	**5**	6
Gas >>>	blank	blank	**blank**	**Std #1**	**Std #1**	Std #1
ppm	20.2	20.8	**18.6**	**15.9**	**50.5**	94.9
2nd manifold						
Tubes	7	8	9	10	11	12
Gas >>>	Std #2	Std #2	Std #2	Std #3	Std #3	Std #3
ppm	55.6	65.2	60.0	80.5	83.3	81.3

Because sparging individual aliquots of culture could alter conditions and affect denitrification, the new method was adjusted to sparge an entire culture during amplification.

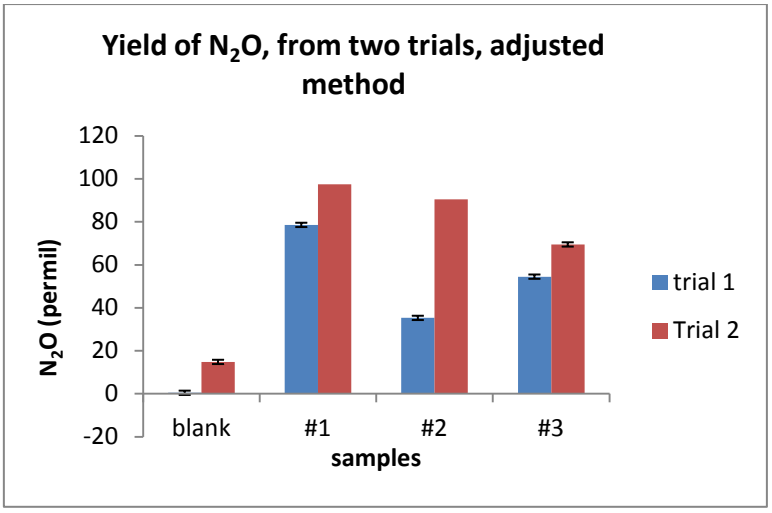
3.7 Results from the Final method

(i) N_2O concentrations, trial 1 & 2

Figure 3.15 shows permil concentrations of N_2O from two trials of the final method.

Trial 1 culture denitrified 78.6 ppm N_2O in standard #1, 35.3 ppm N_2O in standard #2 , and 54.5 ppm N_2O in standard #3.

Trial 2 culture denitrified 97.4 ppm N_2O in standard #1, 90.3 ppm N_2O in standard #2 , and 69.5 ppm N_2O in standard #3 (raw data, Appendix 8) .



.

Fig. 3.15. Ppm concentrations of N_2O from 2 trials, Final method.

Key Std #1 $\delta^{15}N$ 0.09

Std #2 $\delta^{15}N$ 2.7

Std #3 $\delta^{15}N$ 4.7

Blank distilled water

Standards were diluted to 1ppm NO_3-N to yield 100 ppm N_2O

The N_2O yield was unexpected as all standards had been diluted to the same concentration and each batch was inoculated with a homogenous culture. These results strongly indicate a technical problem unrelated to bacterial denitrification rates.

(ii) Corrected $\delta^{15}N$ values, trial 1

Results of $\delta^{15}N$ values for trial 1 are shown in Fig. 3.16 below

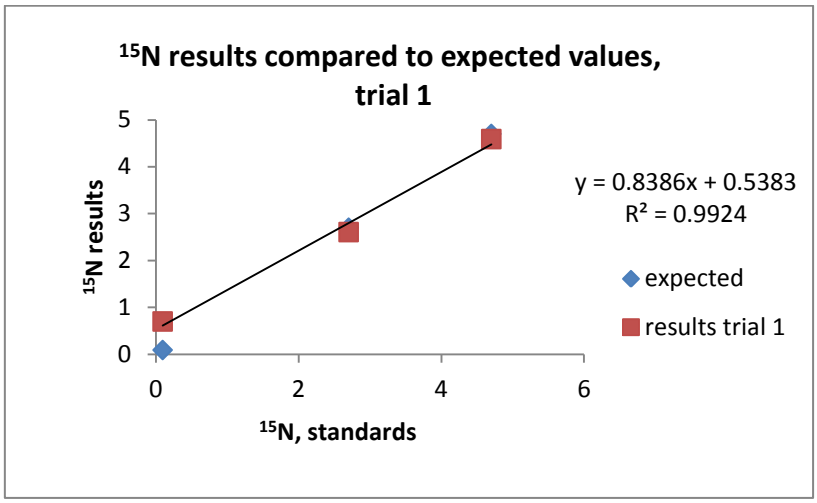


Fig. 3.16. Corrected $\delta^{15}N$ values of the final method, trial 1, SD 2.14. The $\delta^{15}N_{measured}$ are the results from the N_2O. The $\delta^{15}N_{standards}$ are the previously determined values of the NO_3 standards used as presented in the key to figure 3.14.

Samples were diluted to 1 ppm NO_3-N for a yield of 100 ppm N_2O

Because the $\delta^{15}N$ results were close to actual values of standards, the isotope ratios of O_2 were also examined and compared with known $\delta^{18}O$ values of standards #1, #2 & #3.

(iii) Corrected $\delta^{18}O$, values trial 1

Results of $\delta^{18}O$ values for trial 1 are shown in Fig. 3.17 below.

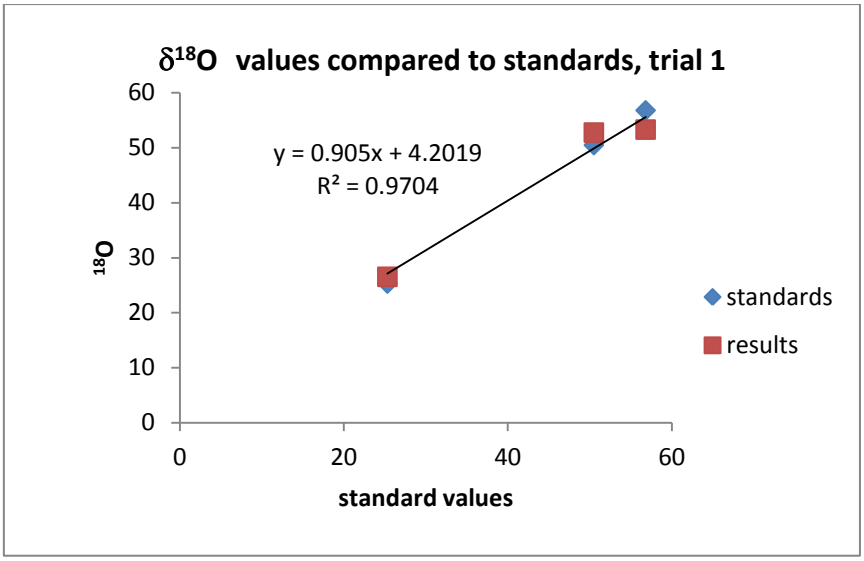


Fig. 3.17. Corrected $\delta^{18}O$ values of the final method, trial 1, SD 4.4. $\delta^{18}O_{measured}$ are the results from the N_2O. The $\delta^{18}O_{standard}$ are the previously determined values of the NO_3^- standards used.

Key

Std #1 $\delta^{18}O = 50.5$

Std #2 $\delta^{18}O = 56.8$

Std #3 $\delta^{18}O = 25.3$

The similarity between results and standards indicates that total denitrification occurred as N_2O produced is representative of the oxygen isotope composition of the NO_3^- sample. The erratic N_2O yields may therefore be the result of technical error.

(iv) Corrected $\delta^{15}N$, values trial 2

Results of $\delta^{15}N$ values for trial 2 are shown below in Fig. 3.18 (raw data Appendix 8a).

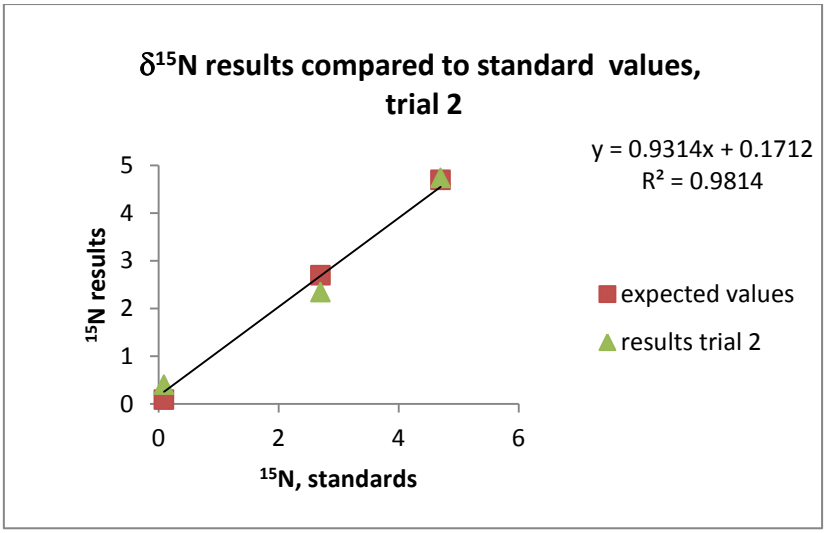


Fig. 3.18. Corrected $\delta^{15}N$ values of the final method, trial 1, SD 0.74. The $\delta^{15}N_{measured}$ are the results from the N_2O. The $\delta^{15}N_{standards}$ are the previously determined values of the NO_3 standards used as presented in the key to figure 3.14.

Samples were diluted to 1 ppm NO_3-N for a yield of 100 ppm N_2O

The good agreement between the $\delta^{15}N$ results and the $\delta^{15}N$ of the standards indicates that the N_2O produced is representative of the isotopic composition of the NO_3 sample despite erratic N_2O yields.

(v) Corrected $\delta^{18}O$, values trial 2, final method

The oxygen isotope composition of the NO_3^- sample was corrected and results of ^{18}O values for trial 2 are shown in Fig. 3.19

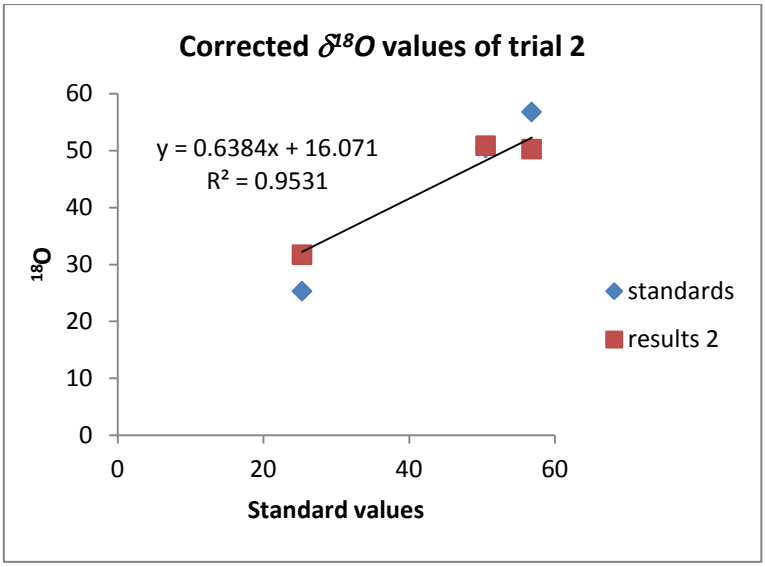


Fig. 3.19. Corrected $\delta^{18}O$ values of the final method, trial 2, SD 7.7. $\delta^{18}O_{measured}$ are the results from the N_2O. The $\delta^{18}O_{standards}$ are the previously determined values of the NO_3 standards used as presented in the key to figure 3.17.

The less accurate results in $\delta^{15}N$ and $\delta^{18}O$, trial 2 may be due to incomplete consumption of NO_3^- standards as culture had not reached starvation state.

3.8. Summary

Results of experiments verified the species of bacterium to be *O.anthropi* which required modifications to the Revesz (2007) and Sigman et.al. (2001) methods as these methods did not provide conditions for optimal denitrification when using this denitrifier.

This required:

- Altering temperatures
- Removing KNO_3 amendments
- Producing anaerobic inocula
- Using unconcentrated cultures
- Sparging entire cultures
- reducing incubation times

and resulted in a new approach when using the *O.anthropi* for δ^{15}N isotope ratio analysis that has produced δ^{15}N results which are in good agreement with the δ^{15}N values of standards with an added bonus of δ^{15}N results which are in good agreement with the δ^{15}N values of standards. These results are discussed in Chapter 4.

Chapter 4

Discussion

The previous chapters described a series of experiments undertaken to verify the species of denitrifying bacterium, confirm this organism's purported tolerance to salinity and establish culture protocols that produced accurate $\delta^{15}N$ analysis.

This chapter discusses the results of these experiments and concludes by recommending another modification to ensure reliable $\delta^{15}N$ results from samples of NO_3^- or NO_2^- when using this bacterium. A general overview of culture storage is outlined and a specific bacteriocide and cleaning protocol are briefly covered.

4.0. Overview of storage

Rapid freezing of microbes in supernatant is considered to be an effective form of preservation (Swift, 1921) as lattice type ice crystals form which are less disruptive to cell structure compared to pointed crystals arising from slow freezing (Swatek, 1967).

Cells frozen in supernatant also have advantages which are not available to cultures stored in glycerol of the Casciotti et. al. (2002) Sigman et. al. (2001) or Reston (Revesz,2007) methods as supernatant can provide resuscitating cells with cellular material for self reparation (Swatek, 1967) Contamination or damage from repeated thawing is also prevented as only one aliquot of culture is used per experiment.

All rehydrated culture for this project was therefore enriched and frozen in supernatant which proved to be a successful method of storage as thawed cultures always recovered.

4.1. Characterisation of the candidate bacteria

Species taxonomy was queried by (Hart, 1965) who found *C. nephridii* did not exhibit the morphological characteristics of a *Corynebacterium* species. Questions regarding *C.nephridii* ATCC 11425 were also raised by the writer of this thesis as the recommended nutrient of blood agar and an incubation temperature of 37^{o}C seemed unusual for a soil organism.

Bacterial characterisation undertaken by standard microbiological techniques (Reed, 1998), showed the gross morphology of pure cultures (see Fig. 3.1) and Gram negative cocci which did not conform with the Gram positive, irregularly shaped cells of a *Corynebacterium* species (Hart, 1965; Liebl, 2001) when cells were examined under a microscope (oil immersion).

DNA analysis of 16s rRNA genes was used to identify the candidate bacterium as these genes are highly conserved and can enable reliable differentiation from genus to subspecies (Clarridge, 2004).

Results from the DNA analysis of the current project determined a 99% homology with *O.anthropi,* confirming the candidate bacteria ATCC 1142, is not a *Corynebacterium* species (ref. appendix 11). These findings are supported by DSMZ, a supplier of bacterial cultures who stated *C.nephridii,* ATCC 11425 was a different species of bacterium *O.anthropi*, DSM No. 20150, and concluded that *C.nephridii* is an invalid name (ref. appendix 12 & 13.). Based on this evidence, the candidate bacterium used for this project was accepted as *O. anthropi.*

4.2. The growth curve

Work undertaken for this project relied on optical density (OD) to determine each growth phase, as it is an effective alternative for measuring cell densities compared to actual cell counts (Brock, 1994; Reed, 1998). OD measurements of triplicate inocula were averaged (ref. appendix 2) and graphed, showing distinct changes in density that marked the lag, log, stationary and decline phase of a typical growth curve (see Fig. 3.4). This enabled the correlation of a specific growth phase with N_2O yields at a given temperature and time.

4.3. Tolerance to salinity

As O.anthropi has been found to possess Nar (nitrate) and Nir (nitrite) reductase enzymes that are functional in 12 g/L^{-1} NaCl, (Kesseru et. al.,2002), the candidate bacterium was examined for tolerance to three different salinities which would confirm if the organism was suitable for δ^{15}N analysis of N-oxides in seawater.

Salinity experiments revealed unconcentrated cultures at an early log phase tolerated all three conditions by increasing in density during a 24 h incubation at 34 ^{o}C (see Fig. 3.5) and produced filamentous precipitates which were regarded as biofilm synthesis by viable

surface associated organisms (Dunne Jr, 2002), rather than plasmolysis from hyperosmotick shock (Joklik, 1968).

Nutrient rich medias of (b) and (c) showed a second increase in OD at 25 h which may be the result of exopolysaccharide synthesis (Costerton et.al., 1995; Dunne Jr. 2002), shedding of planktonic cells (Costerton et. al., 1994, 1995) or induction of VBNC (Faruque et. al., 2004).

In comparison, filtered seawater produced an OD_{600} of 0.026 that was only 21% and 37% of conditions (b) and (c) respectively which may be the result of higher salinity as 42.5 g/L NaCl inhibits growth by 50% (Kesseru et. al.,2002).

Alternatively, limited N in seawater may cause starvation stress (Dawson et. al., 1981; Wrangstadh et. al., 1986) that will drive cells to settle and form biofilms from nutrients concentrated near solid surfaces (Dunne Jr., 2002). This is thought to be the most likely explanation as salinity of the Kesseru et. al., (2002) experiment was greater than seawater.

Cells in seawater reached an of OD_{600} 0.026 which should be sufficient to denitrify µM concentrations NO_3^- that are likely to occur in seawater samples (Sigman et al., 2001) as low counts of denitrifying bacteria (100 per ml), in aquatic and marine environments have a high denitrification potential (Knowles, 1982). The OD was also 5% of an aerobic stationary phase which Mørkved et. al. (2007) found could denitrify 1-5 µg NO_3-N in 2-4 mL freshwater samples.

If the culture cannot denitrify all N-oxides at µM concentrations, δ^{15}N analysis of samples in seawater is still possible by adding a phosphate buffer or Na succinate, as *O.anthropi* was found to denitrify 25 mg/L NO_3-N in a salinity of 30 with these amendments (Kesseru et.al., (2002).

4.4. Two Bacterial methods for $\delta^{15}N$ analysis

Two known bacterial methods, the (Sigman et. al., 2001) method and the Reston method (Revesz 2007) were trialled for δ^{15}N analysis of NO_3^- standards using the candidate bacterium.

The first two experiments followed the (Sigman et. al., 2001) method and resulted in excessive N_2O concentrations, (see Fig 3.8) regardless of long incubation times and high cell titres. These N_2O concentrations, thought to originate from unconsumed NO_3^- remaining in supernatant (used to resuspend the culture), persisted after an unheated and heated sparge for reasons which are explained below.

In comparison, cultures resuspended in unamended media of the Reston (Revesz, 2007) method, caused a reduction in N_2O concentrations (see Fig. 3.9) but gave $\delta^{15}N$ values that were enriched by fractionation or contamination (see Fig. 3.10).

These findings initiated a series of alterations that led to the development of a new bacterial method which is described in 4.5 and 4.6 below.

(i) The Sigman et. al. (2001) method

Although the ATCC recommended temperature for *O.anthropi* is 25 ^oC, a temperature of 22^oC also produced colonies of normal appearance and was therefore combined with a conservative volume (two loops) of inocula to slow proliferation and prevent stresses of a decline phase during the 6 – 10 d incubation (Aertsen & Michiels, 2004; Braun, 1947; Costerton et. al., 1995; Dunne Jr, 2002; Pfeiffer & Bonnhoeffer, 2004; Swatek, 1967), but incubation was halted after 4 d as cells had declined to an OD_{600} of 0.518.

The NED test indicated little NO_2^- remained in the media, suggesting the 6 – 10 d incubation required for consumption of KNO_3 amendments (Christensen & Tiedje, 1988; Sigman et. al., 2001) was unnecessary, but resultant N_2O concentrations in both blank and standard were well above the projected values of zero and 100 ppm N_2O respectively (see fig 3.8) and indicate that residual NO_3^- amendments, which are undetected by NED tests, were carried over in the supernatant.

These residues should have been denitrified and resultant gases scoured out during the sparge, but results suggest denitrification was halted at this time, causing residues to remain and be denitrified with samples. N_2O concentrations also showed the reaction was incomplete as an expected difference of 100 ppm N_2O in standards did not occur.

Cell titre and nutrients were eliminated as causes which could affect denitrification as the culture produced over 400 ppm N_2O after sparging, but a possibility remained that a change in temperature during the sparging process had affected denitrification rates.

This appeared to be the most likely cause as the gas temperature was found to be 12^{o}C, the same temperature that had stressed resuscitating cells and inhibited growth (Costerton et. al., 1994; Dunne Jr, 2002; White-Zeigler, 2008) during a 24 h incubation (see Fig. 3.6). A repeat experiment was therefore undertaken with the sparging gas heated to 22 - 23 ^{o}C (see Fig. 2.2).

(ii) Sigman et. al. (2001) method, heated sparge

The repeat experiment produced a stationary phase culture of maximum viable cells (Swatek, 1967) after a 2 d incubation that showed no detectable levels of NO_2^- in the growth medium after being tested with NED.

Incubation was therefore halted to avoid problems associated with a decline phase (Aertsen & Michiels, 2004; Braun, 1947; J. W. Costerton, et. al., 1995; Dunne Jr, 2002; Pfeiffer & Bonhoeffer, 2004; Swatek, 1967) and enriched cells were sparged with a gas heated to 22^{o}C .

Results confirmed a heated sparging gas increased denitrification (see Fig. 3.8), but similar N_2O concentrations in blank and standard revealed that denitrification remained incomplete during sparging and the incubation with standards..

This can be explained by a negative feedback system (Zumft, 1997) induced by a maximum number of viable cells rapidly denitrifying a large quantity of residual NO_3^- in the supernatant, producing NO_2^- levels that activated biological feedback mechanisms to slow denitrification and prevent toxic, mutagenic NO from accumulating (Betlach & Tiedje, 1981; Zumft, 1997).

After sparging, the addition of blank or standard would dilute these concentrations of NO_2^- and allow further denitrification, producing increased but similar N_2O concentrations in blank and standard before another rise in NO_2^- levels reactivated the negative feedback system.

Based on these conclusions, the Sigman et al.,(2001) method was deemed unsuitable when using the candidate bacterium *O.anthropi*, as cells had reached stationary or decline phase before most KNO_3 amendments had been consumed. The method was therefore abandoned for the Reston (Revesz, 2007) method which resuspended enriched cultures in NO_3-free media (TSB) instead of supernatant to prevent a carry-over of residual N-oxides.

(iii) The Reston method (Revesz & Coplen, 2007)

The Reston method (Revesz, 2007) also specified 6 – 10 d incubation times which could lead to microbial changes in physiology, metabolism or death (Aertsen & Michiels, 2004; Kolter et. al., 1993; Monod 1949; Schimel et.al., 2007; Swatek, 1967) and affect denitrification. Such problems were reported by other laboratories (ref. Appendix 3) when following the Sigman et.al.,(2001); Casciotti et.al.,(2002) and Reston (Revesz, 2007) method to include:

high N_2O blanks,

1) $\delta^{15}N$ samples with no peak and
2) $\delta^{15}N$ samples with peaks that were too high
3) - a 25% drop in bacterial survival and sample output,
4) - toxicity causing bacteria to clump,
5) - failure of bacteria to resuscitate and
6) - samples with an acrid odour which test positively to NED

Casciotti et. al., (2002) proposed that failed cultures which had an acrid odour and abnormal cell colouration were due to:

1) a limited O_2 availability in the HS to media ratio which inhibited proliferation of culture
2) the volume and titre of an inoculum
3) increased HNO_2 concentrations and pH in a medium with low buffering capacity

4) growth on lower levels of NO_2^- which slowed induction rates of the NIr (nitrite reductase) enzyme (Casciotti et. al., 2001)

These aspects were considered when producing cultures for the current project with the following conclusions:

1) a HS to media ratio of 7:5ml provided sufficient ambient O_2 for an inocula to produce a viable culture at mid- log phase
2) the volume and cell density of an inoculum produced viable cultures providing growth was controlled by temperature and incubation times
3) an increase in pH should not be a problem as the candidate bacteria can denitrify in a pH of 8 (Kesseru et. al., 2002).
4) Low levels of NO_2^- are improbable as the candidate bacterium accumulates NO_2^- during microbial denitrification of NO_3^- (Kesseru et. al. 2002) that will increase not decrease NO_2^- and enhance, not retard induction of *Nir* enzymes (Zumft, 1997)

This suggests that uncontrolled growth could cause failed cultures as a high volume (5 ml) of starved cells can cause a burst of exponential growth (Kolter et. al., 1993) and rapidly denitrify NO_3^- amendments, producing toxic levels of NO before a negative feedback system was activated.

The Reston method was therefore trialled with three temperatures to control growth, cell density and subsequent nutrient availability during a 7 d incubation.

Five ml aliquots of inocula were incubated in 25^{o}C and 22^{o}C during longer incubations which prevented rapid growth, produced viable cultures, formed biofilms and gave N_2O yields that were only 1% of the expected yield (data not shown).

As temperatures below 22^{o}C could cause cold stress reactions (Costerton, 1981; Dunne Jr. 2002; Panoff et.al, 1998), a higher incubation temperature of 36^{o}C was selected which slowed proliferation to an OD_{600} of 0.177 over 7 d, showing that altered conditions can change growth of in vitro subcultures (Costerton et. al., 1981).

Enriched cultures resuspended in TSB and TSB & Na succinate (Kesseru et al.,2002) produced similar concentrations of 135 – 136 ppm N_2O (see Fig. 3.10), demonstrating that the TSB media contained sufficient nutrients to enable denitrification of more than 1 mM NO_3-N.

The greater than expected yield of 136 and a 135 ppm N_2O occurring in the standards (see Fig 3.9) may have originated from an unknown quantity of NO_3^- or NO_2^- existing in unamended media, but residual N-oxides may also have remained in the periplasmic space of cells (Zumft, 1997) or the interstitial spaces of concentrated bacteria and suggest that KNO_3 amendments, long incubation times and cell enrichment may be unnecessary.

The δ^{15}N values of the Reston (Revesz 2007) method were considerably enriched (see Fig. 3.10) compared to the results of the Sigman et. al. (2001) method, unheated and heated sparge [see 3.4 (iv) & (v)] and may be due to fractionation resulting from an altered microbial response to a higher temperature and longer incubation.

Alternatively, enriched δ^{15}N values could be caused by technical problems such as cross contamination or memory effects in the mass spectrometer (Meijer et al., 2000), as the Lincoln stable isotope facility works almost exclusively on δ^{15}N-enriched samples, where 99% of analyses are on samples enriched in $\delta^{15}N$ of between 5 atom % to 60 atom % (personal correspondence, Roger Cresswell).

A new method (discussed below) was therefore developed to eliminate potential δ^{15}N enrichment eventuating from unconsumed N-oxides or an altered microbial response to a higher temperature.

4.5. New method

The new method (see table 2,2) aimed to eliminate KNO_3 amendments and long incubation times by resuscitating cells on agar amended with NO_3^- in a candle jar (Jensen, 1977; Martin & Smith, 1974) to produce a denitrifying inoculum.

Ambient O_2 was combusted to CO_2 which denitrifiers cannot be utilize as an alternative electron acceptor (Brock et. al., 1994) thus creating conditions that forced resuscitating cells to respire using NO_3^- amendments in the agar.

An incubation temperature of 30 ^oC was selected as this temperature was found to drive optimal denitrification (Allen et. al., 1952; Anderson et. al., 1986; Baumann et. al., 1997; Kesseru et. al.,2002; Kim et. al.,2006; Lee et. al., 2002; Mahmood et. al., 2009; Saleh-Lakha et. al., 2005; Sung et. al., 2002).

Resulting colonies were thin, spreading and semi-translucent which indicated growth in near anaerobic conditions (see Fig. 3.11) when compared to the dense and mucoid colonies from aerobic conditions (see fig. 3.1).

A denitrifying colony was amplified in unamended TSB with limited O_2 to maintain microaerobic conditions, thus ensuring any NO_3^- or NO_2^- present in the media was consumed. This produced a denitrifying culture of starved cells in a "feast or famine" condition (Kolter et. al., 1993) which was tested with NED for viability and denitrification of NO_2^- [see (i) below].

(i) Viability of the New method culture

Based on the findings that *O. anthropi* is resistant to 40 mM levels of NO_2^- (Takaya & Takizawa, 2009) a new approach was taken to test an unenriched candle jar culture for denitrification of NO_2^-. This differed from the Casciotti et. al., (2001) and Reston (Revesz 2007) methods that used an NED test to identify unhealthy cultures for removal and disposal.

NED tests showed that a considerable reaction had taken place when a lag phase candle jar culture (OD_{600} 0.208) was incubated in high levels of NO_2^- (see Fig.3.12) confirming the candle jar method is a plausible option for analysis of NO_3^- at μM levels.

(ii) Production of N_2O, New method

Denitrification was enhanced by a temperature of 30 ^{o}C (Allen et. al., 1952; Anderson et. al., 1986; Baumann et. al., 1997; Kesseru et.al.,2002; Kim et. al.,2006; Lee et. al., 2002; Mahmood et. al., 2009; Saleh-Lakha et. al., 2005; Sung et. al., 2002) which was maintained during incubation and sparging to avoid disrupting the synchronized metabolic rhythms of temperature sensitive lag phase cells (Swatek, 1967).

Contrary to the findings of Merzouki et. al.,(1999) and Rust et. al.,(2000), the addition of PO_4 to TSB media did not enhance denitrification and cultures of both media produced erratic N_2O yields for each standard (see Fig. 3.13) and blanks produced 20-22ppm N_2O which were greater than the N_2O yields of the Reston method (see Fig.3.9).

These results were perplexing, as each sample had been denitrified by low numbers of homogenous, lag phase cells grown in TSB with no added NO_3^- amendments. A conclusion therefore reached that erratic N_2O yields may be caused by technical problems of cross contamination or memory effects (Meijer et. al., 2000).

(iii) δ^{15}N values

Both media produced enriched δ^{15}N values which increased in accordance with the δ^{15}N values of standards (see fig. 3.14). This indicated a total denitrification of standards had occurred and that the enrichment was most probably due technical problems in the mass spectrometer (Meijer, et. al., 2000).

(iv) Changes in N_2O concentrations during a repeat experiment

During sparging, (repeat experiment, New method) the needle of tube 3, manifold 1 became blocked, requiring the manifold to be returned to an upright position while the needle was replaced.

This interrupted the sparging process and caused a noticeable drop in N_2O concentrations of tubes downstream from tube 3, (see Table 3.1) which was thought to be the result of sensitive lag phase cultures (Swatek, 1967) responding to changes, as standards 2 & 3 of the uninterrupted sparge from manifold 2, showed no such drop (see Table 3.2).

A final modification eliminated the need to sparge individual aliquots of culture, thus avoiding any potential problems that might occur during this process.

4.6. Final method

In order to avoid potential changes which could affect sensitive lag phase cells, an entire culture was sparged during amplification. This eliminated the need to sparge individual samples, as one ml aliquots of culture could be injected directly into exetainers containing standards which had been preflushed with helium to maintain anaerobic conditions. Prewarming the samples to 30 ^{o}C and rapidly transferring the culture also prevented fluctuations in temperature.

(i) Production of N_2O, final method

Because mass balance dictates quantitive recoveries, the ideal for any analytical technique is to prepare a sample to obtain high recoveries of product. This is relevant in stable isotope analysis as a high recovery should accurately represent the isotopic composition of the sample being measured and can be confirmed by comparing the quantity of initial substrate with product yield. This was done for the present project using three NO_3^- standards whose δ^{15}N values had previously been determined by bulk stable isotope analysis.

Although a high recovery of product may achieve accurate isotopic composition, processes yielding low recoveries can still produce isotopically representative products as long as the fractionation is insignificant or very reproducible.

The final method of this project produced blanks of <1 ppm N_2O (see Fig. 3.15) which indicated total denitrification had occurred but recoveries of product N_2O were high and erratic).

Examination of δ^{15} N values however, showed enriched results which were consistent (see Appendix 8). Corrections were therefore undertaken by calculating the mean enrichment and deducting this value from all samples (see Appendix 8) with the following results shown below.

(ii) δ^{15}N of the final method

The N_2O produced by the final method gave corrected $\delta^{15}N$ and values which were in good agreement with the expected values (see Fig. 3.16) and were considered to represent a total denitrification of standards.

This indicates that the problems of erratic recoveries were of a technical rather than biological origin which was confirmed in a repeat experiment that produced $\delta^{15}N$ values of 84 – 109‰ in blanks and δ^{15}N values of 184 – 581‰ in standard #1 (see Appendix 9).

(iii) $\delta^{18}O$ of the final method

Results also confirm the new method is suitable for δ^{18}O analysis when using *O.anthropi* (see Fig. 3.17) compared to the Casciotti et. al., (2002) method where *C.nephridii* (a.k.a. *O.anthropi*) showed a 29.7 % O_2 exchange rate with H_2O, despite the bacterium possessing Cu enzymes which do not allow this reaction (Causey et. al., 2006; Metz et. al., 2003; Witzel, 2000; Zumft, 1997).

(iv) Observations, final method

Of note was the precipitation in blanks of trial 1 which differed from the filamentous precipitation observed in standards by being loose and grainy. This indicated cultures in the blank condition were experiencing starvation stress and was based on findings by Kolter et. al. (1993) who reported starved *E. Coli* cells exhibited size reduction and production of cell walls that caused aggregation or clumping.

The grainy precipitation was therefore regarded as a starvation response to total consumption of any NO_3^-, as cells had only reached early log stage in a medium which can otherwise support growth to stationary phase (ref 2.1 (iii), Reston method)

(v) Production of N_2O, final method repeat experiment

A repeat of the final method was undertaken to ensure results were reproducible but the N_2O concentrations (see appendix 8) gave a mean yield of 14 ppm N_2O in blanks and erratic N_2O yields in standards (refer Fig. 3.15) which again suggests problems were of a technical

rather than biological origin as the culture was homogenous and standards were diluted to 1 ppm NO_3-N.

(iv) δ^{15}N and δ^{18}O results, final method, repeat experiment

Although corrected δ^{15}N and δ^{18}O results of trial 2 (see Figs. 3.18 & 3.19) showed a good agreement with delta values of standards, the R^2 values show trial 1 (see Figs. 3.16 & 3.17) produced better results as well as <1 ppm N_2O concentrations in blanks (Fig. 3.15). This may be explained by the culture of trial 2 (OD_{600} of 0.153) producing less growth compared to the trial 1 culture (OD_{600} 0.419), indicating maximum growth and total starvation had not been reached. Should this occur in future experiments, an extra few hours of incubation will be required to ensure maximum growth.

(v) Technical problems and continuous flow mass spectrometers

The results from the final method produced unexplained N_2O concentrations which were attributed to technical problems, as all conceivable sources of N-oxides had been eliminated. Causes of these N_2O concentrations included memory effects from gas-wall interactions, low pump efficiency and valve leakages (Meijer, et al., 2000) or sequence design, (Berryman et. al., 2011.)

Low pump efficiency and carry over were clearly evident in a failed repeat experiment of the final method (see Appendix 9) as N_2O yields varied from 0.8 to 97.0 ppm while δ^{15}N values were unrelated to the N_2O concentrations with values varying between δ^{15}N = 581.8 to 61.4‰.

Variable N_2O concentrations may occasionally be due to a difference between atmospheric pressure and internal pressures of vials (Cambaliza et. al., 2009) which was apparent when excess pressure in a flushed vial caused the plunger of a syringe to be ejected during the current project.

4.7. Other observations

(i) Antifoam

Antifoam B Emulsion (Sigma A5757) is a silicone-type antifoam which can remain on glassware after washing. This caused translucent colonies on agars and was rectified when the manufacturer's recommendations of a post wash rinse in bleach or alcohol were followed.

(ii) Bacteriocides

Original methods used 10N NaOH to lyse bacteria and immobilize CO_2 as it has the same molecular weight as N_2O, but was replaced with Trigene after the accident involving an overpressurised exetainer described in 4.6 (v) above. This did not affect results as N_2O is separated by automatic cryofreezing /trapping and CO_2 is scrubbed out with Carbsorb. (Personal communication, Roger Cresswell, Lincoln University). Trigene therefore provided a safe microbe specific alternative.

4.8. Conclusions

(i) Factors affecting the bacterial method

The effects of a) temperature, b) incubation times, c) cell density and d) the use of KNO_3 amendments were found to impact on microbial growth and denitrification rates of the candidate bacterium and consequently affect δ^{15}N results when following the protocols of existing bacterial methods.

a) Colder temperatures of 12 ^{o}C stressed cells, attenuated growth and caused biofilm formation [see 3.4 (i)] as well as impacting on denitrification rates [see 3.4 (ii)] In this situation, incubation times were irrelevant as cold stress caused bacterial responses which reduced denitrification rates (J. Costerton, et. al., 1994; J. W. Costerton, et. al., 1995; Hall-Stoodley et. al., 2004).

The sparging gas temperature was also thought to affect denitrification by slowing metabolism and stimulating a negative feedback mechanism.

Ambient room temperatures of 22 – 25 ^{O}C produced optimal growth in the Sigman et. al., (2001) trials, but cultures reached a stationary phase after 2 days, declined after 4 days and formed biofilms. Temperatures were therefore manipulated to slow growth, increase incubation times and avoid biofilm formation.

A temperature of 36 ^{O}C slowed growth rates and producing a low titre of culture which did not form any biofilm during a 7 d incubation [Reston method, (Revesz, 2001)], but produced δ^{15}N results that showed some isotopic fractionation may have occurred.

In contrast, an incubation temperature of 30 ^{O}C enabled optimal denitrification by a low titre of cells which could readily produce 100ppm N_2O over 24 h.

b) Incubation times produce lag, log, stationary and decline phases at a given time and temperature. A short incubation produced lag phase cells which were sensitive to changes in the environment [Ref. 4.5 (iv)] while longer incubations produced declining cultures (Fig. 3.4) or biofilms that inhibited denitrification (see 4.4 (iii) above).

Incubation times were problematic, as the original methods required long incubations with KNO_3 amendments which resulted in biofilms, declining cultures and could cause mutations as pH alters, nutrients decrease, redox potentials are lowered and adaptive dynamics eventuate (Braun, 1947; Kolter et. al., 1993).

c) Cell density could explain the failed cultures reported by Casciotti et. al. (2002) as a large volume and high titre of starving cells can cause an explosion of exponential growth, (Kolter et. al., 1993) producing toxic levels of NO that resulted in failed cultures which tested positively to NED.

A high cell density ensured sufficient viable cells were available for total denitrification but required long incubations with KNO_3^- amendments to produce a denitrifying culture. A high cell density also caused cells to settle and form biofilms that affected denitrification rates.

Although cell density could be controlled by temperature and length of incubation to prevent biofilm formation, it also resulted in incomplete consumption of KNO_3 amendments which then contaminated results.

d) The use of KNO_3 amendments proved to be the most challenging aspect of this project as it was required to ensure a maximum of denitrifying cells, but required long incubation times that caused cultures to decline or form biofilms before total denitrification had occurred. Incomplete denitrification of KNO_3 amendments then caused contamination of results which could, in part, explain erratic results experienced by other laboratories (refer to Appendix 3).

The situation was eventually solved by producing a denitrifying candle jar culture which enabled the elimination of KNO_3 amendments that subsequently reduced incubation times. This produced a low titre of denitrifying culture in media uncontaminated with residual N-oxides which was capable capable of denitrifying the standard concentrations required for the project providing anaerobic conditions and a temperature 30 ^{o}C were constant.

(ii) Further improvements

The new method can be further simplified by one final step which removes the use of a syringe to transfer aliquots of culture from the culture flask to exetainers containing samples and ensures constant conditions.

This involves building a cap for the culture flask with a Leur valve inserted to provide an inlet point for gas and an exit point for culture or gas. Gas pressure in the culture flask is controlled by the Leur valve tap which can be opened for gas to exit into the atmosphere or closed to increase pressure and force culture out. A diagram for this system is shown in Fig. 4.1 below.

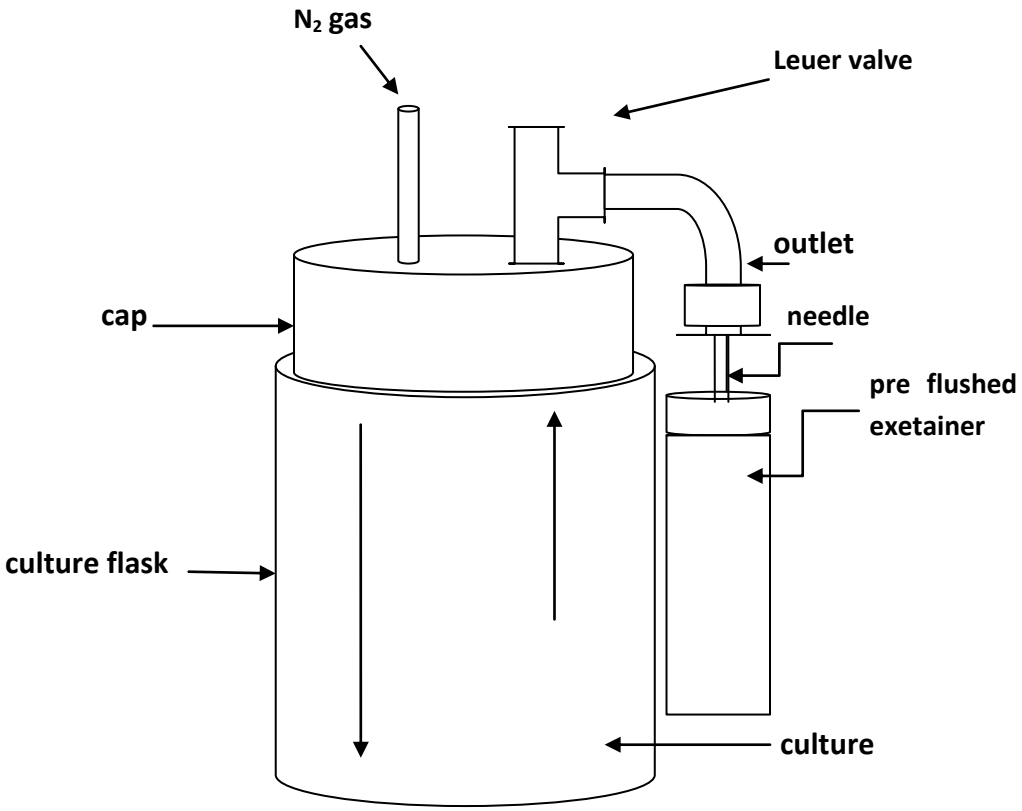


Figure 4.1. Diagram showing how cultures can be transferred directly into the sample

The Leuer valve allows the culture to flow through an attached needle into a prepared exetainer when the gas pressure is increased. After transfer, the tap is opened to release gas pressure, the flow of culture stops and the exetainer is replaced with a new one. Providing the flow of culture is rapid, this method has the potential to anlyse up tp 99 samples in a short time.

4.9. Future directions

1) Direct transfer of culture to sample can be trialled using the arrangement described above. If successful, the number of samples able to be processed in one day can be evaluated.

2) The new method can be trialled for analysis of NO_3^- and NO_2^- in seawater. Lag phase cultures of the new method are expected to give successful results based on the growth and tolerance observed by lag phase cultures in undiluted seawater (ref Chapter 2, iii) and although enyme synthesis is inhibited, function of existing enzymes is enhanced by salinity (Causey et. al., 2006).

3) The new method can be trialled for $\delta^{15}N$ ratios in polluted water as *O.anthropi* is recognised as an organism that can tolerate heavy metals and xenobiotic substances (Causey et. al., 2006, Kesseru et. al., 2002).

4*)* Analysis of $\delta^{15}N$ ratios of N-oxides in seawater and aquatic samples preserved with $HgCl_2$ (Bartholomay & Williams, 1996; Kattner, 1999) may also be trialled using the new method as *O.anthropi* possesses Mer A genes which code for enzymes that reduce mercuric ions to elemental, volatile mercury (Lal & Lal, 2002) denitrifying 1.5 g/L^{-1} NO_3 –N in the presence of 1 mM Hg (Kesseru et.al., 2002).

Future directions have been made possible by work undertaken for this project which established that low titres of lag phase culture could denitrify and tolerate salinity. This was achieved by producing a growth curve of the candidate bacteria which provided a means of comparing cell density, phase of growth and collating this with resulting N_2O concentrations. Salinity tolerance was also established by observing increased density in three saline concenrtations.

The growth curve also revealed existing methods for $\delta^{15}N$ analysis were not suitable when using the candidate bacteria, based on $\delta^{15}N$ results which were produced by a high titre of culture but contaminated by incomplete denitrification of KNO_3 amendments or fractionated by cellular responses of a low titre of cells to high temperature.

Subsequent manipulations of existing methods have produced a promising new method for $\delta^{15}N$ analysis which is uncomplicated, faster and achieves accurate isotopic compostion for nitrogen as well as oxygen.

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Appendix

1 Materials

2 Collated data for growth curve

3 Collaborated information from seven laboratories

4 Cold sparge results

5 Heated sparge results

6 Results of Reston method at 36 ^{o}C

7 Results of new method, TSB media

7a Results of new method, TSB / PO_4 media

8 Results of final method, sparged culture

8a Results of final method repeat, sparged culture

8 Failed Results, showing carry–over, final method

10 Calculations for Standards

11 DNA results

12 DSMZ Nomenclature of candidate bacterium

13 CABRI nomenclature of candidate bacterium

Appendix 1

Materials

(i) Media

DNA extraction:

DNeasy tissue Kit (250), (Qiagen, Germany)

QIA PCR Purification Kit (Qiagen GmbH, Hilden, Germany)

10mM TE Buffer (10 mM Tris HCL, Bio Rad),

1 mM EDTA, (BOH Chemicals, Poole, England)

QIA PCR Purification Kit (Qiagen GmbH, Hilden, Germany)

Agarose gel

Culture media

Tryptic soy agar (TSA : Difco ™ TSA agar)

Tryptic soy media (TSB : Difco ™ TSB broth)

KNO_3 (AF 307228 Univar, Auckland, NZ)

$(NH_4)_2SO_4$, Analr, BDH Chemicals Ltd. Poole, England

Sodium succinate dibasic hexahydrate (Sigma-Aldrich, St Louis, USA

Antifoam B emulsion (Sigma-Aldrich, St Louis, USA

$NaNO_3$ (Univar, Mt Wellington, Auckland, NZ)

NaCl

NED tests

$NaNO_2$ (Univar) Mt Wellington, Auckland NZ

Greiss reagents: sulphanilamide (Prolabo, Paris, France) & N-(1-Napthyl) ethylene diamine dihydrochloride (NED), Sigma-Aldrich, St. Louis, USA

$\delta^{15}N$ analysis

Nitrogen gas, Std SM3

KNO_3 #1: (ex laboratory)	1ppm KNO_3-N,	$\delta\varepsilon^{15}N$ = 0.09‰
$NaNO_3$ #2 USGS 35	1ppm $NaNO_3$-N,	$\delta\varepsilon^{15}N$ = 2.7‰
KNO_3 #3 IAEA	1ppm KNO_3-N,	$\delta\varepsilon^{15}N$ = 4.7‰

where $\delta\varepsilon^{15}N$ represents the isotopic ratio in standard delta notation.

(ii) Equipment

Pharmacia Biotech Novaspec II spectrometer set at OD_{600} nm.

Semco temperature controlled water bath and shaker.

Hotplate : Heidolph MR 2002.

Polycarbonate tubes (LBS 1209 12 ml screw top PC)

Eppendorf tubes, 1 ml, 1.5 ml

Centrifuge tubes, 20 ml

30 ml glass universals and screwcaps

0.45 μm filters (Membrane-Solutions)

MSE minor centrifuge

SIGMA 1 – 15 centrifuge (Sigma Laboratory Centrifuge, Osterode amd Harx, Germany)

Beckman 212 M/E centrifuge, (Beckman coulter Inc. California, USA)

Gene Amp PCR system 9600 (Perkin-Elmer corporation, Norwalk, USA) thermocycler

Glass exetainers, 12 ml, and VC 301 blue caps & butyl septa.

Venting needles, 25G BD PrecisionGlide™

Sparging needles,6G BD PrecisionGlide™

Terumo spinal Needles, 0.9 x 90 mm

Aperture caps, GL45, Cat no. 2922 710 (Technical Glass products Ltd. Dunedin, NZ)

Silicone septa, GL45, Cat no. 2924 610 (Technical Glass products Ltd. Dunedin, NZ)

PDZ Europa TGII /20-20 mass spectrometer

An airtight tin and tealight candles

Appendix 2

Collated data for growth curve

hour	Mean 1	Mean 2	Mean 3	Mean 4	Mean5	Mean 6	Absorbance
0	0.058	0.055333	0.058	0.056	0.058667	0.054	0.05666667
2	-0.00633	-0.00633	-0.004	0.001	-0.003	-0.00433	-0.00388667
4	0.002333	0.01	0.011	0.016	0.011667		0.0102
6	0.011	0.032667	0.034	0.035333			0.02825
8	0.042	0.063	0.0603				0.055111
10	0.085	0.098767					0.0918835
12	0.118667						0.118667
14						0.343333	0.343333
15						0.393333	0.393333
16					0.487	0.468333	0.4776665
17					0.559333	0.555333	0.557333
18				0.469667	0.658	0.651333	0.593
19				0.647667	0.665667	0.686	0.66644467
20			0.672667	0.666	0.671	0.675333	0.67125
21			0.666333	0.662333	0.675333	0.695	0.67474975
22		0.685	0.666333	0.659333	0.682333	0.689667	0.6765332
23		0.674333	0.670333	0.66	0.672	0.693	0.6739332
24	0.668667	0.676667	0.656333	0.654333	0.666	0.691667	0.6689445
25	0.663333	0.67	0.656	0.647667	0.652333		0.6578666
26	0.648	0.658	0.645333	0.644333	0.646		0.6483332
27	0.642	0.645667	0.630333	0.628333			0.63658325
28	0.632333	0.638667	0.628667	0.622333			0.6305
29	0.627667	0.631333	0.618667				0.625889
30	0.616667	0.625333	0.617				0.61966667
31	0.610333	0.616					0.6131665
32	0.603667	0.614					0.6088335
33	0.595667						0.595667
34	0.600						0.600

Mean OD of (triplicate) inocula after OD of TSB has been deducted.

Appendix 3

Collaborated information from seven laboratories

On Tue, 1 Feb 2011 04:57:09 -0800, "A. Schauer" <aschauer@U.WASHINGTON.EDU>
wrote:
> Hello,
>
> Remember 9 months ago when I inquired about the specifics of every
> ones bacterial denitrifier methodologies? And remember I said I would
> compile all replies into a table? You have been on the edge of your
> seat, I know. Better late than never? Rest assured my tardiness is
> excused for feeble insufficient reasons. Here is the table:
>
> http://depts.washington.edu/isolab/index.php?menu=7&id=3
>
> Its best viewed with a high resolution monitor laying flat under a
> dissecting scope. I have also provided links to the original replies
> expunged of identifying text. The bottom line, after this effort,
> seems to be that this method is, of course, a bit like sour dough. We
> do some things the same, a lot of things different, and are bound by
> what the bugs will tolerate. Thank you all for your patience.
>
> andy
>
> Andrew Schauer
> Earth and Space Sciences
> University of Washington
> Seattle, WA 98195
>
> 206.543.6327
> aschauer@uw.edu
> http://depts.washington.edu/isolab/
>
> This message was sent from my telegraph.

Bacterial Denitrifier Harvesting Strategy Survey

Below is a table of various strategies used by anonymous laboratories to harvest *Pseudomonas chlororaphis* or *Pseudomonas aureofaciens* bacterial colonies for the purpose of denitrification of nitrate for $\delta^{15}N$ and/or $\delta^{18}O$ determination. See the original inquiry below the table. To see each laboratories response (scrubbed of any identifying information), click on the Lab Number column heading.

	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7
1. Freezer Stock Visit	-	n/a	-	weekly	Once per 2-6 months	never	regularly
2. Create Freezer Stock	-	n/a	-	as needed, use stock tube 3x then discard	not yet	never	regularly
3. Plates before Inoculation	-	n/a	-	2 - 3	1	n/a	3
4. Plate growth conditions	-	n/a	ambient temperature	Ambient temperature, dark	Ambient temperature and light	n/a	21 *C, dark
5. Colony from Plates	-	n/a	single	single to multiple	couple	n/a	single
6. Starter Culture	-	n/a	yes	yes	yes	yes	yes
7. Media Recipe Different?	no	1.5 L, 45g TSB, 7.34g KH2PO4, 1.70g KNO3,0.0812 g NH4Cl2	-	no antifoam until harvest	no	no	60 g TSB, 10g K2HPO4, 2g KNO3, 2g (NH4)SO 4 in 2.0 L hydro pure water, no antifoam

8. Media bottle size	4/5 media, 1/5 headspac e	130 mL with 100 mL media	500 mL, 100 mL headspace	500 mL	250 mL, crimp cap	125 mL serum vials	250 mL bottle, 202 mL media
9. Number of bottles	-	10-12 vials for 100 samples	-	1 per 25 samples	8 per 40 samples	6	4 per 40 samples
10. Media growth conditions	-	dark, 25 *C	away from direct sun	ambient temperature , light uncontrolled	ambient temperatur e and light	ambient temperatur e and light	21 *C, dark
11. Autoclave settings	-	1 hr, 121 *C	15 min, 121 *C, 1.5 hour cycle	121 *C, 30 minutes	liquid cycle	122 *C, 20 minutes	125 *C, 20 minutes
12. Shaker type	Shaker, unknown type	no shaking	reciprical	orbital	orbital	manual	reciproca l
13. Pellet appearanc e	-	always pink	-	occasional black flecks and variable opacity	light pink, uniform size	consistent size and amount	yes
14. Success	90%	near 100%	-	near 100%	near 100%	100%	50-60%

The original inquiry is below:

Hi all,

I am hoping to extract as much information from you as possible regarding the exact conditions imposed on your P. aureofaciens from freezer stock to sample injection. The end result will be a table on the wiki and our web page summarizing the growth and prep conditions. The immediate need and motivation for this particular email is our survival rate has fallen off to ~25% and thus our sample throughput.

The most succinct response would be we treat our bacteria identically to Sigman 2001 or identically to Casciotti 2002. Please however, take a moment to respond to each point, recognizing the details I am asking for are not necessarily in the Sigma or Casciotti papers.

1) How often to you visit your freezer stock?
2) How often do you replenish your freezer with new stock?
3) How many plates do you make before inoculation?
4) What conditions do you grow your plates in (e.g. dark vs light, temperature).
5) When you grab colonies from the plate, do you grab single colonies or a smear?
6) Do you use the starter culture as in Sigman 2001?
7) Does your growth media recipe differ from Sigman 2001 and if so, how?
8) What bottle type / size do you use to grow P. aureofaciens?
9) How many of the bottles in #8 do you use for a single harvest?
10) What conditions are the bottle(s) in when the bacteria are growing (e.g. dark vs light, temperature)?
11) What are the autoclave settings for the growth media (temperature, duration, etc)?
12) Do you use an orbital shaker or a reciprocal shaker or manual shaking?
13) Can you see qualitative differences in pellets?
14) How well does your method work (e.g. 50% success, 100% success, etc)?

If you feel I have missed some detail that you think is particularly important, please add those items. If I receive enough of a response, I will create a table and keep labs anonymous. Reply to me directly or to the list as you see fit. Thank you for your time.

andy

Denitrifier_Lab1_Response.txt

Bottle = 4/5 medium 1/5 headspace. Something to do with oxygen

sensitivity during accumulation of nitrogen oxides.

There should be absolutely NO oxygen leaking into the bottle during

growth on the shaker. Otherwise the cells get killed off from the

formation of free radicals (presumably, reactions with NO).

Our success rate is ~90%. Clumped cells are usually indicative of

toxicity, which we think is indirectly mediated by oxygen-NOx chemistry.

Make sue you have no contaminating strain in the stock cultures.

Denitrifier_Lab2_Response.txt

The following is the real situation in our lab starting from 3 years ago...

>1) How often to you visit your freezer stock?
>2) How often do you replenish your freezer with new stock?
>3) How many plates do you make before inoculation?
>4) What conditions do you grow your plates in (e.g. dark vs light, temperature).
>5) When you grab colonies from the plate, do you grab single colonies or a smear?
>6) Do you use the starter culture as in Sigman 2001?

Honestly speaking, we don't have any facility to keep denitrifier correctly. I mean, we don't have any clean benches
to innoculate denitrifer in the microbe-free environment and we don't have any good deep-freezer
to stock the denitrifer strain. So when I moved to (ANONYMOUS UNIVERSITY), I left my denitrifer stock in (ANONYMOUS UNIVERSITY), and I started to innoculate denitrifier liquid-to-liquid
without checking the contamination with plate methods. Moreover we have not replenish the denitrifer from the
frozen stock since 2007.

I had planed to visit our freezer stock in (ANONYMOUS UNIVERSITY) when we found the accumulation of nitrite in our growth media,
but in these 3 years, we only have one occation (one bottle from 14 bottles in this case) with the NO2 accumulated.

>7) Does your growth media recipe differ from Sigman 2001 and if so, how?

We usually make 1500ml growth media with

* 45g TSB
* 7.34g KH2PO4
* 1.70g KNO3
* 0.0812g NH4Cl2

A while ago, we tried to reduce NO3 to reduce N2O blank, but we came back to this original recipe.
To reduce the N2O blank in the media, we just wash the denitrifier with new media (without nitrate).

>8) What bottle type / size do you use to grow P. aureofaciens?

We put 100ml of growth media into 100ml vial
Every week, we prepare 14 vials from 1500ml media.

We keep the ratio -- 100ml liquid in 130ml vial. We don't know how low the DO is when the growth media is 6-10days old.
But as far as we experience, this ratio (headspace vs liquid volume) works well for 3 years.

>9) How many of the bottles in #8 do you use for a single harvest?

We usually prepare 14 vials (1400ml in total). Normally we use 10-12 vials for 100samples.
From one vial, we split the media into 2 centrifuge tube (40ml), and we use the 20ml left for NO2 concentration measurements.

>10) What conditions are the bottle(s) in when the bacteria are growing (e.g. dark vs light, temperature)?

We put the vials in the incubator (dark, 25C). My collegue told me tha incubation with low temperature can induce the
accumulation of NO2, but we are not sure, actually...

>11) What are the autoclave settings for the growth media (temperature, duration, etc)?

1hr, 121C. The media should be in "nice dark brown". The autoclaved media should be used (inoculated) asap. When we used
relatively old media (1 day after autoclaving), the growth was bad. So I usually autoclave the media in the morning, and inoculate
the media in the afternoon in the same single day.

>12) Do you use an orbital shaker or a reciprocal shaker or manual shaking?

I feel that shaking is not good -- growth was not good when we shaked the vials, so we just let the vials sit for 6-10 days.
Actually we don't shake the vials until just before the centrifuging...

>13) Can you see qualitative differences in pellets?

Among our 14 vials in each batch, the color of pellets are always pink, and the amount of pellets does not differ so much,,,

>14) How well does your method work (e.g. 50% success, 100% success, etc)?

So far almost 100% success. Even when I taught undergrad students who have never experienced microbial stuff and isotope anlaysis,
the regression curve with USGS32, 34, 35 and IAEA was good.

But, just today, our post-doc got the bad data -- d15N of USGS 32 was 150 permill. Normally d15N of USGS 32 has been measured as
165-179 permill, so something bad happens...Woops,,,,
Denitrifier_

Lab3_Response.txt

Hi there, As I am using a different bacteria I cannot give specific instructions
for P. aurofaciens but have used this growing method for P. Chlororaphis, a subspecies of P aurofaciens.

1) I keep all of original which has been rehydrated and centrifuged, in 1 ml
eppendorfs in -80C freezer. They are kept in the original supernatant (but glycerine is apparently a good cryogenic agent). I hand thaw and inoculate 100
ul into 8 ml lots of unamended TSB and grow to mid log stage, then centrifuge
and resuspend in 1 ml supernatant. I refreeze in a domestic freezer(-4 to -12C)

and use these cultures.

2) For plates I thaw by hand, streak, (cool the loop in agar first) incubate 24h
or longer if necessary at temps recommended by supplier. It would pay to grow at
different temps around recommended (is it 26C?) to get an idea of how the colonies appear, large mucoid colonies being probably too old. The aim is to get
vegetative cells before the stationary phase.

3)For inoculum I lift a small colonies and inoculate into 8 ml lots of TSB, grow
to mid log and use one loopful to inoculate into 400 ml broth, (100 ml headspace) with NO3 and antifoam. I then centrifuge and store the remaining inocula in the domestic freezer. I repeat (3)until the cells grow less well,
then start at 1) again.

5) I grow the 400 ml culture to mid - late log phase. For all broth cultures I
use a reciprocal shaker, it ensures all cells are exposed to all the nutrients.

4) Light/dark does not seem to be important but cultures are kept away from direct sunlight.

5) For a harvest I decant 10 ml lots (one 10 ml lot = one sample) and centrifuge
and resuspend in one ml. 6) Autoclaving is 121 C for 15 mins, but the
autoclave cycle takes about 1.5h from start to finish.

I have focused on the suppliers recommendations for growth as I found the Casciotti/Sigman methods outlined too unspecific regarding temperatures and incubation times.

If your survival rate has fallen off, most cells may be too old, try to revive
by growing a culture in broth for a longer time to allow the younger cells to regenerate a population.

Denitrifier_Lab4_Response.txt

1) We revisit our freezer stock each time we begin the procedure (roughly 1x/week).

2) We replenish our freezer stock as needed. Generally, we freeze 7-12 individual vials of P. aureofaciens at once, and access each vial at most 3 times before discarding. As a result, we tend to replenish our freezer stock
every 4-7 months.

3) We make 2 to 3 plates before inoculation.

4) We grow our plates at room temperature in the dark.

5) We aim to grab single colonies, but often end up grabbing multiple colonies from a single area.

6) Yes - We grow starter cultures of P. aureofaciens in 5 mL vials on an orbital shaker. The time for growing our starter culture varies: between 6-30 hours.

7) We do not add antifoaming agent to our growth media. We only add antifoam
on sample prep day, after centrifuging and resuspending the bacteria.

8) We use 500 mL Pyrex bottles.

9) We use 1-500 mL bottle for every 25 vials needed. Since we prepare vials in batches of ~50 or ~100, that amounts to either 2 or 4 bottles per harvest.

10) Bottles are kept at room temperature, in varying conditions of light/dark.

11) We autoclave our growth media at 121 deg C for 30 minutes (up to 50 minutes for large batches).

12) An orbital shaker.

13) Occasionally we see slight qualitative differences in pellets with respect to size, color (presence of black 'flecks'), and opacity. If a pellet is >50% translucent, we consider the culture unsuccessful and start over. However, for the most part our pellets are consistent in appearance.

14) With regard to bacteria viability through harvest, our success rate is near 100%. However, we sometimes encounter other issues, such as high method
blanks. We recently increased flushing time from 2 to 4 hours in order to reduce our method blanks.

Denitrifier_Lab5_Response.txt

1. Once every few months. Right now it's been about 6 months and the bacteria
are still going strong. We are about to have some extended down time on our
machine, so I will probably start a new culture when we get up and running again.
2. We use such a small amount to make the cultures (and do it so infrequently)
that we have not had to replenish the freezer stock yet (since we started about
2 years ago).

3. One plate

4. We put them in the hood and grow them at room temperature.

5. We usually grab a couple single colonies.

6. Yes, we follow Sigman.

7. I think the media is exactly the same. One thing we do that I don't know if
they do is before we inoculate the bacteria into the broth bottles, we sterilize
the caps with rubbing alcohol and a lighter.

8. 250 mL clear glass bottle with 20 mm crimp cap

9. We use 8 and mix them all together before harvesting, but I think we really
only need 6 or 7 (for a run of 40 samples).

10. Light (and dark at night), on a shaker table, room temperature

11. I can't find my autoclave manual right now, but it's a liquids setting that
was pre-programmed, and it gets pretty hot (the bottles are hot to the touch
when it opens). It runs for about 20 minutes, but it takes about 30 minutes to
get to the right temperature and pressure and then it takes about an hour to
cool enough for the door to open.

12. Orbital shaker

13. No, they are all light pink and about the same size.

14. We had a lot of problems getting this method started up. One of the best
pieces of advice is to test the bacteria with nitrite test strips prior to
harvesting, to ensure that the bacteria have "eaten" all of the nitrite in the
broth. Once we started doing that, we have not had many problems with the
bacteria. Once in a while you will get a sample with no peak, but I would say
that is rare and can possibly be attributed to other factors (e.g. leaky bottle,
auto-sampler needle clog, etc.). I don't think we've ever had a problem with
the bacteria dying off.

Denitrifier_Lab6_Response.txt

Our original starter culture of P. aureofaciens was taken (ANONYMOUS LAB)
in ~2005. We also purchased our own
stock which is stored in the (ANONYMOUS DEPARTMENT) (in glycerine? In 2008
or 2009, we tried to regrow from the freezer stock, but they didn't
work (the technician used a different broth mix). We didn't try again,
but instead continue to innoculate from vials which have their origin
from the (SAME ANONYMOUS LAB).

In answer to your questions:

1) Never
2) Never
3) n/a
4) n/a

5) n/a
6) we innoculate from starter cultures (from 100 ml serum vials to fresh vials of broth).
7) we follow Signman exactly
8) 125 ml Serum vials (VWR #16171-385)
9) 6
10) room temperature on the lab bench, no special precautions regarding light
11) 122 oC for 20 minutes
12) manual shaking
13) sometimes but usually they are consistent size and amount
14) so far 100% success. We've not had a bad batch yet
Denitrifier

_Lab7_Response.txt

*1) How often to you visit your freezer stock? *

*
*We normally start each cultivation cycle with a freezer stock of bacteria. We open a freezer stock sample, take 5 ?l out with a sterile pipette and transfer it on a plate, mark the tube as used, freeze it again and use it again in the next cycle until it is finshed.

2) How often do you replenish your freezer with new stock?

* *

In the beginning we replenished our freezer with a new stock each time we started a new cycle. Meanwhile (they became far too many) we do that every fourth or fifth time.

*3) How many plates do you make before inoculation? *

*
*We make new plates every 14 days and store them in a fridge. They last for about two 2 cycles.

We use three plates (#1, #2, #3) and each 2 plates (if one is no good...) before inoculation.

*4) What conditions do you grow your plates in (e.g. dark vs light, temperature)? *

We cultivate our plates in the dark in an inoculation cupboard at a constant temperature of 21?.

5) When you grab colonies from the plate, do you grab single colonies or a smear?

We grab a single colony which means a single well defined dot.

*6) Do you use the starter culture as in Sigman 2001? *

Yes. After reviving bacteria cultures on #1 and transferring to #2 and #3 we transfer a single colony to a tube with 5ml nutrient broth (1,6 g TSB in 200 ml hydro pure water). It grows overnight on the shaker in the inoculation cupboard.

*7) Does your growth media recipe differ from Sigman 2001 and if so, how? *

Our medium is compound of 60 g TSB, 10 g K_2 HPO_4 , 2 g KNO_3 and 2 g (NH_4)SO_4 in 2,0 l hydro pure water. We do not ad antifoaming agent.

*8) What bottle type / size do you use to grow P. aureofaciens? *

We started to incubate /P. aureofaciens/ in 200 ml (nominal volume, true volume 250 ml) bottles with about 166 ml of medium. Bacteria seemed to grow well and never smelled bad. However, isotope data showed an unsatisfied high standard deviation and it seemed that bacteria did not work properly so that measured values were way too high for the IAEA N3 and USGS 34 standards. Now we increased the medium volume up to 202ml (but we are still using the same bottles) to raise the medium to air supernatant ratio in the bottles. Bacteria "work" perfectly now. Standard deviation of international reference material is within 0.20/00 for ?15N and 0.5 for ?18O. However, now we do have the problem, that almost every other day the bacteria are not working well and we obtain way to high peaks in the MS. It seems that the bacteria do not reduce the nitrate from the medium but it is hard to tell from the smell which flask is good and which one is not. We did several times the nitrite test to check whether there is still nitrite in the medium bottles, but still if it is negative we might get the "high peak phenomenon"....

*9) How many of the bottles in #8 do you use for a single harvest? *

We use four bottles a day for 40 samples.

*10) What conditions are the bottle(s) in when the bacteria are growing (e.g. dark vs light, temperature)? *

Bottles are in the same incubator cupboard as the bacteria plates: in the dark and with constant temperature of 21?C.

*11) What are the autoclave settings for the growth media (temperature, duration, etc)? *

We do autoclave the medium (first and second) at 125?C for 20 min (small table autoclave) and the medium bottles for inoculation at 121?C for 30 min in a big lab autoclave.

*12) Do you use an orbital shaker or a reciprocal shaker or manual shaking? *

It's a reciprocal shaker. We increased the shaking to ~180 rpm so that media get really foamy.

*13) Can you see qualitative differences in pellets? *

Yes. When we had really smelly (stabbing) medium in the bottles after the incubation the pellets were rather small or they did not really become a pellet but remained sticky on the walls of the tube. However, we do have pellet which look perfectly but still peaks in the MS is much too high (see above) and isotope measurement is then nonsense....

*14) How well does your method work (e.g. 50% success, 100% success, etc)? *

50-60%

We had a lot of problems in the beginning and we did not know where and what to change. E.g. not only that our standard deviation was too high, but we did not really "hit" the accepted value for the international standards such as IAEA N3 and USGS 34.

However, we changed our N_2 O bottle since we did not know if it was rather empty. That helped a lot to measure accepted values and to stabilize the ratios for the standards. The most important thing we changed was the media volume from 166 to 202 ml. And we increased the shaking form 160 to 180 rpm.

Appendix 4

Cold sparge results

	$[N_2]$	$^{15}N_2$			$[N_2O]$	$\delta^{15}N$ vs AIR	$\delta^{18}O$ vs V-SMOW
	%	Atom%			ppm_V	‰	‰
Bl 1	120	0.36378			439	37.30	25.95
Bl 2	124	0.36377			502	37.79	23.48
Bl 3	118	0.36383			365	37.46	25.55
Std 1	120	0.36374			442	36.48	25.56
Std 2	119	0.36373			429	37.20	24.78
Std 3	119	0.36376			408	37.22	25.69

QC Check							
QC					97.3	58.47	51.85
					95.7	58.15	51.12
					98.4	58.57	51.71
				Average	97.1	58.39	51.56
				Std Dev	1.4	0.22	0.39
				Actual value	96.0	58.30	52.10

	Mean ppm N2O	15N mean
supernatant		
Blank	435.3	37.5
Std	426.3	36.9

Appendix 5

Heated sparge results

	$[N_2]$	$^{15}N_2$			$[N_2O]$	$\delta^{15}N$ vs AIR	$\delta^{18}O$ vs V-SMOW
	%	Atom%			ppm_V	‰	‰
Bl 4	126	0.36505			625	39.72	23.08
Bl 5	127	0.36508			622	39.93	20.96
Bl 6	124	0.37000			690	39.89	22.91
Std 1	125	0.36508			665	39.43	22.55
Std 2	125	0.36504			693	39.82	22.02
Std 3	125	0.36504			636	39.63	22.95
QC Check							
QC					96.9	58.54	51.41
					101.1	58.28	52.23
			Average		99.0	58.41	51.82
			Std Dev		3.0	0.18	0.58
			Actual value		96.0	58.30	52.10

	Mean ppm N2O	15N mean
supernatant		
Blank	645	39.8
Std	664	39.6

Appendix 6

Results of Reston method at 36 ^{o}C

	$[N_2]$	$^{15}N_2$			$[N_2O]$	$\delta^{15}N$ vs AIR	$\delta^{18}O$ vs V-SMOW
	%	Atom%			ppm_V	‰	‰
1B	129.6	0.36543	TSB		1.89	85.36	80.38
2B	129.9	0.36545	TSB		2.17	82.96	60.89
3B	116.7	0.36545	TSB		1.77	88.17	65.97
4S	125.4	0.36546	TSB		138	59.41	84.65
5S	128.4	0.36548	TSB		136	59.55	85.28
6S	120.1	0.36548	TSB		135	59.68	82.53
7B	124.0	0.36550	NaSucc		2.30	74.87	65.50
8B	123.0	0.36547	NaSucc		2.17	77.67	59.35
9B	127.7	0.36538	NaSucc		2.17	74.63	70.12
10S	123.4	0.36538	NaSucc		135	59.51	92.23
11S	119.5	0.36541	NaSucc		136	59.71	94.14
12S	123.7	0.36539	NaSucc		136	59.75	95.37

QC Check								
				Average	44.8	58.46	52.11	n
				Std Dev	0.2	0.03	0.29	2
				Actual value	45.0	58.30	52.10	

TSB	Mean ppm N2O	15N mean
Blank	1.94	
Std	136.33	59.5
Na succinate		
Blank	2.21	
Std	135.6	59.6

Appendix 7

Results of new method, TSB media

TSB	$[N_2]$	$^{15}N_2$			$[N_2O]$	$\delta^{15}N_{AIR}$	$\delta^{18}O_{V\text{-}SMOW}$
	%	Atom%			ppm_V	‰	‰
13	108.0	0.36536		blank	21.5	19.39	55.77
14	115.1	0.36536		blank	21.4	18.55	54.72
15	117.7	0.36539		blank	20.3	19.15	55.13
16	109.8	0.36537		#1	143.4	10.11	75.36
17	104.8	0.36536		#1	144.0	10.20	74.48
18	115.6	0.36537		#1	146.1	10.29	75.13
22	115.3	0.36537		#2	59.3	15.27	58.06
23	115.4	0.36536		#2	58.4	15.37	58.51
24	118.9	0.36539		#2	59.4	15.27	57.71
19	111.8	0.36538		#3	78.4	15.80	72.2
20	112.9	0.36538		#3	80.1	16.09	71.6
21	111.0	0.36536		#3	81.3	15.98	73.37
QC Check							
			Average		45.0	58.26	51.86
			Std Dev		0.9	0.14	0.63
			Actual value		45.0	58.30	52.10

TSB	15N R	stdev
sample	mean	
# 1	10.2	0.09421
# 2	15.3	0.05419
# 3	15.96	0.143235

Appendix 7a

Results of new method, TSB / PO_4 media

Highlighted figures show effects after temporary removal of tube 3

TSB/PO4	$[N_2]$	$^{15}N_2$			$[N_2O]$	$\delta^{15}N_{AIR}$	$\delta^{18}O_{V\text{-}SMOW}$
	%	Atom%			ppm_V	‰	
1	107.0	0.36539		b	20.2	16.82	56.53
2	106.3	0.36540		b	20.8	15.35	54.18
3	106.9	0.36539		b	18.6	15.53	55.16
4	108.2	0.36543		1	15.9	10.78	103.59
5	111.4	0.36545		1	50.5	6.66	81.15
6	112.4	0.36543		1	94.9	9.77	73.88
10	109.3	0.36537		3	55.6	13.76	57.10
11	111.0	0.36537		3	65.2	13.57	56.76
12	101.8	0.36535		3	60.0	13.46	57.30
7	109.6	0.36539		2	80.5	14.14	70.46
8	114.7	0.36539		2	83.3	14.26	71.41
9	112.9	0.36538		2	81.3	14.17	70.42
QC Check							
			Average		45.2	58.34	52.45
			Std Dev		0.7	0.19	0.41
			Actual value		45.0	58.30	52.10

TSB/PO4	15N R	
sample	mean	stdev
# 1	11.89	2.147347
# 2	13.92	0.151767
# 3	14.19	0.06245

Appendix 8

Results of final method, sparged culture

		$[N_2O]$	$\delta^{15}N_{AIR}$	$\delta^{18}O_{V\text{-}SMOW}$	
		ppm_V	‰	‰	
B		0.59	43.64	65.63	
B		0.44	0.00	0.00	
B		0.39	0.00	0.00	
1		75.4	56.70	92.85	
1		82.3	58.15	94.05	
1		78.1	58.61	95.44	
2		34.9	62.37	99.01	
2		33.2	61.49	97.84	
2		37.9	55.41	87.10	
3		56.3	62.76	68.37	
3		53.5	60.29	65.24	
3		53.7	63.41	70.01	
QC Check	Average	50.3	58.30	51.99	n
	Std Dev	1.0	0.07	0.18	3
	Actual value	50.0	58.30	52.10	

^{15}N stds : #1 = 0.09 #2 = 2.7 #3 = 4.7

	15N R			E		15N
sample	mean			(R-Std)		R-D
# 1	57.82					0.40
# 2	59.76		Mean D	57.41		2.34
# 3	62.15		SD	2.15		4.74

^{18}O stds: #1 = 50.5 #2 = 56.81 #3 = 25.32

	18O R			E		18O
sample	mean			(R-Std)		R-D
# 1	94.11					52.78
# 2	94.65		Mean D	41.34		53.31
# 3	67.87		SD	4.1		26.54

Appendix 8a

Results of repeat, final method, sparged culture

samples		$[N_2O]$	$\delta^{15}N_{AIR}$	$\delta^{18}O_{V\text{-}SMOW}$	
		ppm_V	‰	‰	
B		14.0	72.3	57.90	
B		14.5	72.96	57.80	
B		15.9	73.03	59.14	
1		97.4	62.23	79.51	
1		2.79	66.24	107.85	Invalid
1		27.9	66.07	101.94	invalid
2		91.7	64.59	82.91	
2		91.7	64.65	82.94	
2		88.1	63.17	70.86	
3		63.5	66.92	60.09	
3		72.7	65.26	57.79	
3		72.3	66.18	63.15	
QC Check	Average	48.8	51.87	n	
	Std Dev	3.0	0.41	3	
	Actual value	50.0	52.10		

^{15}N stds : #1 = 0.09 #2 = 2.7 #3 = 4.7

	15N R			E		15N
sample				(R-Std)		R-D
# 1	62.23					0.80
# 2	64.14		Mean D	61.43		2.71
# 3	66.12		SD	0.73		4.69

^{18}O stds: #1 = 50.5 #2 = 56.81 #3 = 25.32

	18O R			E		18O
sample				(R-Std)		R-D
# 1	79.51					50.89
# 2	78.9		Mean D	28.62		50.28
# 3	60.34		SD	8.51		31.72

Appendix 9

Failed Results, showing carry –over, final method

		$[N_2O]$	$\delta^{15}N_{AIR}$	$\delta^{18}O_{V\text{-}SMOW}$
		ppm_V	‰	‰
B		14.8	97.11	89.63
B		23.2	84.20	74.38
B		13.3	109.21	102.33
1		0.8	581.82	656.80
1		3.31	166.24	201.61
1		2.8	184.77	216.81
2		92.9	61.87	82.57
2		89.7	62.42	83.72
2		97.0	61.74	82.50
3		73.2	64.29	63.47
3		69.3	65.14	64.71
3		79.0	63.56	61.57

QC Check	Average	51.0	58.59	52.18	n
	Std Dev	0.9	0.31	0.84	3

Actual value	50.0	58.30	52.10

Appendix 10

Calculations for Stds:

Total mass of std #1 (in house KNO_3) 20.3 ug/ml (NO3-N) x 1 ml = 20.3 *ug*,

(analysed by Citilab.)

Molar quantity: 20.3 ug/14 *ug* *u*mol = 1.45 *u*Mol

Volume N_2O ($Vm = RT_o/P_o = 0.02241\ m^3\ mol/^{-1}$) ½ x 1.45 umol x 22.4 ul/umol = 16.24 ul

expected ppm v N_2O in 9 ml of gas: 16.24ul/9000 ul = 1804 ppm N_2O ul/L^{-1}

Standard diluted to produce 90 ppm N_2O 1804/20 = 90 ppm N_2O ul/L^{-1}

Other standards:

#2) USGS .35 $NaNO_3$-N, $\delta 15N$ = 2.7 Na = 23, O_3= 48, N = 14. Fm = 85

#3) IAEA KNO_3-N , $\delta 15N$ = 4.7 k = 39, N = 14, O_3= 48. Fm, 101

Preparation of standard solution from NO3- salts of stds #2 & #3

1 ppm N x FM of KNO_3/N. 1mg/L N x 101/14 = 7.2 mg/L

1 ppm N x FM of $NaNO_3$/N 1mg/L N x 85/14 = 4.14 mg/L

Ppm: g/M^3 mg/L or *ug*/ml

(1g/L KNO_3 = 10 mMol (10^{-3}) = 10/1000 = 0.01 M/L (0.01 M x1000 = 10 mM)

Fm = formula mass,

Gas constant R = 8.3144 $Jmol^{-1}\ K^{-1.}$

277.15K

$V/n = RT_o/P_o = 0.02241\ m^3\ mol/^{-1}$ (molar vol of gas = 22 L/M)

Appendix 11

NCBI results of BLAST

BLASTN 2.2.14 [May-07-2006]

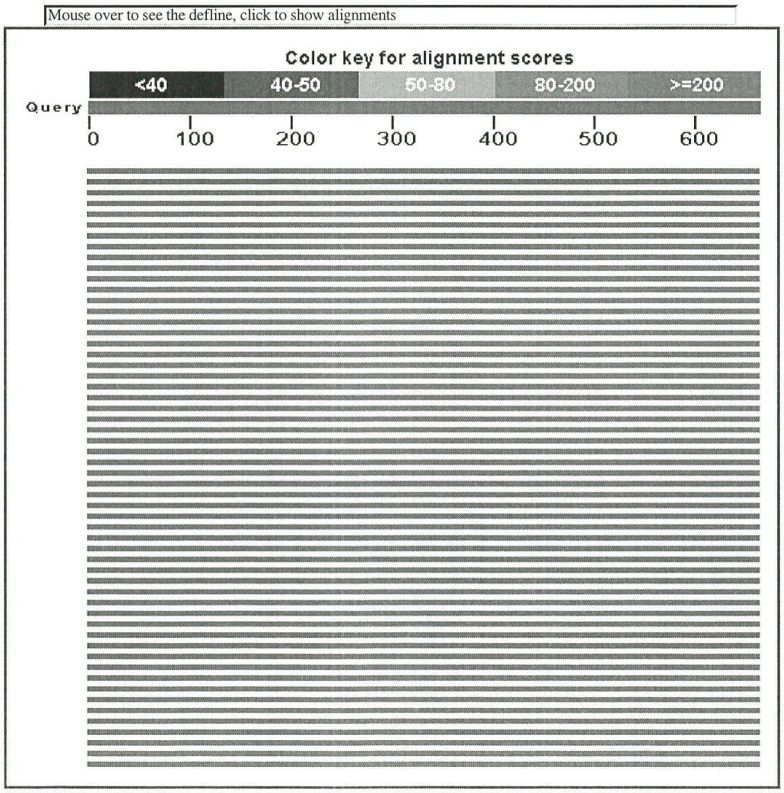
Reference:
Altschul, Stephen F., Thomas L. Madden, Alejandro A. Schäffer, Jinghui Zhang, Zheng Zhang, Webb Miller, and David J. Lipman (1997), "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs", Nucleic Acids Res. 25:3389-3402.

RID: 1158273973-21288-97340357679.BLASTQ1
Database: All GenBank+EMBL+DDBJ+PDB sequences (but no EST, STS, GSS, environmental samples or phase 0, 1 or 2 HTGS sequences)
4,377,236 sequences; 17,904,826,729 total letters

If you have any problems or questions with the results of this search please refer to the **BLAST FAQs**
Taxonomy reports

Query=
Length=666

Distribution of 102 Blast Hits on the Query Sequence



Distance tree of results NEW

http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi

Sequences producing significant alignments:		Score (Bits)	E Value
gi|54873569|gb|AY776289.1|	Ochrobactrum anthropi 16S ribosomal R	1307	0.0
gi|53801274|gb|AY623625.1|	Ochrobactrum sp. TD 16S ribosomal RNA	1307	0.0
gi|52421783|gb|AY730720.1|	Ochrobactrum anthropi 16S ribosomal R	1307	0.0
gi|77696189|gb|DQ211904.1|	Ochrobactrum sp. LJ-D 16S ribosomal R	1307	0.0
gi|110184979|gb|DQ815113.1|	Uncultured bacterium clone aab50c...	1307	0.0
gi|110184894|gb|DQ815028.1|	Uncultured bacterium clone aab49a...	1307	0.0
gi|76886547|gb|DQ205311.1|	Ochrobactrum anthropi strain GB 16...	1307	0.0
gi|46242271|gb|AY513495.1|	Ochrobactrum anthropi strain 19990...	1307	0.0
gi|46242270|gb|AY513494.1|	Ochrobactrum anthropi strain 20000...	1307	0.0
gi|46242269|gb|AY513493.1|	Ochrobactrum anthropi strain 20020...	1307	0.0
gi|50511761|gb|AY662685.1|	Ochrobactrum sp. AS12 16S ribosomal R	1307	0.0
gi|50429255|gb|AY661464.1|	Ochrobactrum sp. B2 16S ribosomal RNA	1307	0.0
gi|30408111|gb|AY274164.1|	Uncultured bacterium clone D8 16S ...	1307	0.0
gi|62465859|gb|AY972430.1|	Ochrobactrum tritici strain S55 16...	1307	0.0
gi|62465782|gb|AY972353.1|	Ochrobactrum tritici strain S108 1...	1307	0.0
gi|62465764|gb|AY972335.1|	Ochrobactrum tritici strain R90 16...	1307	0.0
gi|62465762|gb|AY972333.1|	Ochrobactrum tritici strain R88 16...	1307	0.0
gi|62465653|gb|AY972224.1|	Ochrobactrum tritici strain P64 16...	1307	0.0
gi|62465651|gb|AY972222.1|	Ochrobactrum tritici strain P62 16...	1307	0.0
gi|62465612|gb|AY972183.1|	Ochrobactrum tritici strain P26 16...	1307	0.0
gi|62465595|gb|AY972166.1|	Ochrobactrum tritici strain P10 16...	1307	0.0
gi|50539487|emb|AJ784809.1|	Ochrobactrum anthropi partial 16S rR	1307	0.0
gi|94421559|gb|DQ468351.1|	Ochrobactrum anthropi 16S ribosomal R	1307	0.0
gi|33188081|gb|AY331580.1|	Ochrobactrum sp. mp-6 16S ribosomal R	1307	0.0
gi|33188080|gb|AY331579.1|	Ochrobactrum sp. mp-5 16S ribosomal R	1307	0.0
gi|91805178|gb|DQ466569.1|	Ochrobactrum sp. B16SA 16S ribosomal	1307	0.0
gi|59939803|gb|AY785314.1|	Ochrobactrum anthropi strain STM 2...	1307	0.0
gi|89474858|gb|DQ417342.1|	Ochrobactrum anthropi strain WZR 1...	1307	0.0
gi|89276980|gb|DQ403854.1|	Biphenthrin-degrading bacterium LB...	1307	0.0
gi|29540609|gb|AF526521.2|	Ochrobactrum anthropi isolate ADV1...	1307	0.0
gi|29540608|gb|AF526520.2|	Ochrobactrum anthropi isolate ADV1...	1307	0.0
gi|68146511|emb|AJ867292.1|	Ochrobactrum anthropi 16S rRNA ge...	1307	0.0
gi|68146510|emb|AJ867291.1|	Ochrobactrum anthropi 16S rRNA ge...	1307	0.0
gi|68146509|emb|AJ867290.1|	Ochrobactrum anthropi 16S rRNA ge...	1307	0.0
gi|68146508|emb|AJ867289.1|	Ochrobactrum anthropi 16S rRNA ge...	1307	0.0
gi|56368475|emb|AJ242580.3|	Ochrobactrum anthropi 16S rRNA ge...	1307	0.0
gi|56368470|emb|AJ242578.2|	Ochrobactrum anthropi 16S rRNA ge...	1307	0.0
gi|56368469|emb|AJ242577.2|	Ochrobactrum anthropi 16S rRNA ge...	1307	0.0
gi|56368468|emb|AJ242576.2|	Ochrobactrum anthropi 16S rRNA ge...	1307	0.0
gi|7242709|emb|AJ276036.1|OAN276036	Ochrobactrum anthropi partia	1307	0.0
gi|2832588|emb|AJ002812.1|OSPAJ2812	Ochrobactrum sp. 16S rRNA ge	1307	0.0
gi|26324205|gb|AY162056.1|	Alpha proteobacterium PII_GH1.2.A1...	1307	0.0
gi|26324193|gb|AY162044.1|	Alpha proteobacterium PI_GH2.1.C6 ...	1307	0.0
gi|97954835|emb|AM231057.1|	Ochrobactrum sp. R-24286 partial 16S	1307	0.0
gi|77415821|emb|AM084005.1|	Ochrobactrum sp. R-24638 16S rRNA ge	1307	0.0
gi|77415857|emb|AM084042.1|	Ochrobactrum sp. R-24618 16S rRNA ge	1307	0.0
gi|90017418|emb|AM114406.1|	Ochrobactrum anthropi partial 16S rR	1307	0.0
gi|90017413|emb|AM114401.1|	Ochrobactrum anthropi partial 16S rR	1307	0.0
gi|90017412|emb|AM114400.1|	Ochrobactrum anthropi partial 16S rR	1307	0.0
gi|90017411|emb|AM114399.1|	Ochrobactrum anthropi partial 16S rR	1307	0.0
gi|90017410|emb|AM114398.1|	Ochrobactrum anthropi partial 16S...	1307	0.0
gi|303715|dbj|D12794.1|OAN16SRRNA	Ochrobactrum anthropi gene for	1307	0.0
gi|55469852|gb|AY457038.2|	Ochrobactrum lupini 16S ribosomal RNA	1307	0.0
gi|59939797|gb|AY917134.1|	Ochrobactrum anthropi isolate CYP2...	1307	0.0
gi|83627103|emb|AM113857.1|	Ochrobactrum anthropi partial 16S rR	1307	0.0
gi|85062638|gb|DQ342340.1|	Ochrobactrum sp. 11a 16S ribosomal RN	1307	0.0
gi|85002009|gb|DQ337583.1|	Ochrobactrum sp. B2 BBTR46 16S rib...	1307	0.0
gi|2735221|gb|U88441.1|OAU88441	Ochrobactrum anthropi 16S riboso	1307	0.0
gi|82754253|gb|DQ288889.1|	Ochrobactrum sp. J10 16S ribosomal RN	1307	0.0
gi|9965645|gb|AF229884.1|	Ochrobactrum sp. 3CB5 16S ribosomal RN	1307	0.0
gi|9965644|gb|AF229883.1|	Ochrobactrum sp. 3CB4 16S ribosomal RN	1307	0.0
gi|9965636|gb|AF229875.1|	Ochrobactrum sp. 4FB9 16S ribosomal RN	1307	0.0
gi|9965626|gb|AF229865.1|	Ochrobactrum sp. 2FB10 16S ribosomal R	1305	0.0
gi|38155011|gb|AY322487.1|	Uncultured bacterium clone Malan A...	1303	0.0
gi|77696188|gb|DQ211903.1|	Ochrobactrum sp. LJ-A 16S ribosomal R	1301	0.0
gi|62465731|gb|AY972302.1|	Ochrobactrum tritici strain R59 16...	1301	0.0
gi|97954826|emb|AM231054.1|	Ochrobactrum sp. R-24343 partial 16S	1301	0.0

gi|113196062|gb|DQ884346.1| Ochrobactrum sp. JS-4 16S ribosomal	1299	0.0
gi|110184819|gb|DQ814953.1| Uncultured bacterium clone aab55h...	1299	0.0
gi|30408100|gb|AY274153.1| Uncultured bacterium clone D31 16S...	1299	0.0
gi|30408098|gb|AY274151.1| Uncultured bacterium clone D22 16S...	1299	0.0
gi|29825856|gb|AF337885.2| Uncultured gold mine bacterium D2 ...	1299	0.0
gi|29825839|gb|AF337864.2| Uncultured gold mine bacterium D11...	1299	0.0
gi|7406681|emb|AJ249458.1|OSP249458 Ochrobactrum sp. partial 16S	1299	0.0
gi|61658679|gb|AY948236.1| Ochrobactrum sp. HPC 1033 16S ribo...	1299	0.0
gi|2735222|gb|U88442.1|OAU88442 Ochrobactrum anthropi 16S riboso	1299	0.0
gi|22023918|gb|AF526524.1| Ochrobactrum anthropi isolate CLF2...	1297	0.0
gi|22023916|gb|AF526522.1| Ochrobactrum anthropi isolate CLF1...	1297	0.0
gi|29540610|gb|AF526523.2| Ochrobactrum anthropi isolate CLF1...	1297	0.0
gi|22023920|gb|AF526526.1| Ochrobactrum anthropi isolate ADV2...	1295	0.0
gi|22023919|gb|AF526525.1| Ochrobactrum anthropi isolate Nime...	1295	0.0
gi|29825836|gb|AF337861.2| Uncultured gold mine bacterium D6 ...	1295	0.0
gi|62465760|gb|AY972331.1| Ochrobactrum tritici strain R86 16...	1293	0.0
gi|97954837|emb|AM231058.1| Ochrobactrum sp. R-24448 partial 16S	1293	0.0
gi|97954823|emb|AM231053.1| Ochrobactrum sp. R-24291 partial 16S	1293	0.0
gi|77415820|emb|AM084004.1| Ochrobactrum sp. R-24653 16S rRNA ge	1293	0.0
gi|38155012|gb|AY322488.1| Uncultured bacterium clone Malan A...	1293	0.0
gi|38155008|gb|AY322484.1| Uncultured bacterium clone Malan A...	1293	0.0
gi|110185140|gb|DQ815274.1| Uncultured bacterium clone aab52e...	1291	0.0
gi|110184931|gb|DQ815065.1| Uncultured bacterium clone aab49e...	1291	0.0
gi|62465778|gb|AY972349.1| Ochrobactrum tritici strain S104 1...	1291	0.0
gi|2735223|gb|U88443.1|OAU88443 Ochrobactrum anthropi 16S riboso	1291	0.0
gi|29540607|gb|AF526519.2| Ochrobactrum anthropi isolate ADV1...	1287	0.0
gi|10732841|gb|AF309080.1|AF309080 Ochrobactrum sp. MB2 16S ribo	1287	0.0
gi|54112366|gb|AE014292.2| Brucella suis 1330 chromosome II, com	1283	0.0
gi|54112365|gb|AE014291.4| Brucella suis 1330 chromosome I, comp	1283	0.0
gi|46949176|gb|AY594216.1| Brucella melitensis biovar Neotoma...	1283	0.0
gi|46949175|gb|AY594215.1| Brucella melitensis biovar Meliten...	1283	0.0
gi|62959456|gb|AY945867.1| Uncultured bacterium clone SS-37 1...	1283	0.0
gi|89258608|gb|DQ409215.1| Ochrobactrum sp. 3b 16S ribosomal RNA	1279	0.0

Alignments

Get selected sequences | Select all | Deselect all | Distance tree of results

>☐ gi|54873569|gb|AY776289.1| Ochrobactrum anthropi 16S ribosomal RNA gene, partial sequence
Length=1476

Score = 1307 bits (656), Expect = 0.0
Identities = 661/666 (99%), Gaps = 0/666 (0%)
Strand=Plus/Minus

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Query  1    GTATCTAATCCTGTTTGCTCCCCACGCTTTCGCACCTCAGCGTCAGTAATGGTCCAGTGA  60
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Sbjct  729  GTATCTAATCCTGTTTGCTCCCCACGCTTTCGCACCTCAGCGTCAGTAATGGTCCAGTGA  670

Query  61   GCCGCCTTCGCCACTGGTGTTCCTCCGAATATCTACGAATTTCACCTCTACACTCGGAAT  120
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Sbjct  669  GCCGCCTTCGCCACTGGTGTTCCTCCGAATATCTACGAATTTCACCTCTACACTCGGAAT  610

Query  121  TCCACTCACCTCTACCATACTCAAGACTTCCAGTATCAAAGGCAGTTCCGGGGTTGAGCC  180
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Sbjct  609  TCCACTCACCTCTACCATACTCAAGACTTCCAGTATCAAAGGCAGTTCCGGGGTTGAGCC  550

Query  181  CCGGGATTTCACCCCTGACTTAAAAGTCCGCCTACGTGCGCTTTACGCCCAGTAAATCCG  240
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Sbjct  549  CCGGGATTTCACCCCTGACTTAAAAGTCCGCCTACGTGCGCTTTACGCCCAGTAAATCCG  490

Query  241  AACAACGCTAGCCCCCTTCGTATTACCGCGGCTGCTGGCACGAAGTTAGCCGGGGCTTCT  300
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Sbjct  489  AACAACGCTAGCCCCCTTCGTATTACCGCGGCTGCTGGCACGAAGTTAGCCGGGGCTTCT  430

Query  301  TCTCCGGTTACCGTCATTATCTTCACCGGTGAAAGAGCTTTACAACCCTAGGGCCTTCAT  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  429  TCTCCGGTTACCGTCATTATCTTCACCGGTGAAAGAGCTTTACAACCCTAGGGCCTTCAT  370
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Query  361  CACTCACGCGGCATGGCTGGATCAGGCTTGCGCCCATTGTCCAATATTCCCCACTGCTGC  420
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Sbjct  369  CACTCACGCGGCATGGCTGGATCAGGCTTGCGCCCATTGTCCAATATTCCCCACTGCTGC  310

Query  421  CTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCTGATCATCCTCTCAGACCA  480
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Sbjct  309  CTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCTGATCATCCTCTCAGACCA  250

Query  481  GCTATGGATCGTCGCCTTGGTGAGCCTTTACCTCACCAACTAGCTAATCCAACGCGGGCC  540
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Sbjct  249  GCTATGGATCGTCGCCTTGGTGAGCCTTTACCTCACCAACTAGCTAATCCAACGCGGGCC  190

Query  541  GATCCTTTGCCGATAAATCTTTCCCCCGAAGGGCACATACGGTATTAGCACAAGTTTCCC  600
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Sbjct  189  GATCCTTTGCCGATAAATCTTTCCCCCGAAGGGCACATACGGTATTAGCACAAGTTTCCC  130

Query  601  TGAGTTATTCCGTAGCAAAAGGTACGWTCCCACGCSTTACTYACCCGTMTRCCGCTCCCC  660
            |||||||||||||||||||||||||| |||||||| ||||| |||||| | |||||||||
Sbjct  129  TGAGTTATTCCGTAGCAAAAGGTACGTTCCCACGCGTTACTCACCCGTCTGCCGCTCCCC  70

Query  661  TTGCGG  666
            ||||||
Sbjct  69   TTGCGG  64
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>☐ gi|53801274|gb|AY623625.1| Ochrobactrum sp. TD 16S ribosomal RNA gene, partial sequence
Length=1449

Score = 1307 bits (656), Expect = 0.0
Identities = 661/666 (99%), Gaps = 0/666 (0%)
Strand=Plus/Plus

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Sbjct  720   GTATCTAATCCTGTTTGCTCCCCACGCTTTCGCACCTCAGCGTCAGTAATGGTCCAGTGA  779

Query  61    GCCGCCTTCGCCACTGGTGTTCCTCCGAATATCTACGAATTTCACCTCTACACTCGGAAT  120
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Sbjct  780   GCCGCCTTCGCCACTGGTGTTCCTCCGAATATCTACGAATTTCACCTCTACACTCGGAAT  839

Query  121   TCCACTCACCTCTACCATACTCAAGACTTCCAGTATCAAAGGCAGTTCCGGGGTTGAGCC  180
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Sbjct  840   TCCACTCACCTCTACCATACTCAAGACTTCCAGTATCAAAGGCAGTTCCGGGGTTGAGCC  899

Query  181   CCGGGATTTCACCCCTGACTTAAAAGTCCGCCTACGTGCGCTTTACGCCCAGTAAATCCG  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  900   CCGGGATTTCACCCCTGACTTAAAAGTCCGCCTACGTGCGCTTTACGCCCAGTAAATCCG  959

Query  241   AACAACGCTAGCCCCCTTCGTATTACCGCGGCTGCTGGCACGAAGTTAGCCGGGGCTTCT  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  960   AACAACGCTAGCCCCCTTCGTATTACCGCGGCTGCTGGCACGAAGTTAGCCGGGGCTTCT  1019

Query  301   TCTCCGGTTACCGTCATTATCTTCACCGGTGAAAGAGCTTTACAACCCTAGGGCCTTCAT  360
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Sbjct  1020  TCTCCGGTTACCGTCATTATCTTCACCGGTGAAAGAGCTTTACAACCCTAGGGCCTTCAT  1079

Query  361   CACTCACGCGGCATGGCTGGATCAGGCTTGCGCCCATTGTCCAATATTCCCCACTGCTGC  420
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Sbjct  1080  CACTCACGCGGCATGGCTGGATCAGGCTTGCGCCCATTGTCCAATATTCCCCACTGCTGC  1139

Query  421   CTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCTGATCATCCTCTCAGACCA  480
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1140  CTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCTGATCATCCTCTCAGACCA  1199

Query  481   GCTATGGATCGTCGCCTTGGTGAGCCTTTACCTCACCAACTAGCTAATCCAACGCGGGCC  540
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Sbjct  1200  GCTATGGATCGTCGCCTTGGTGAGCCTTTACCTCACCAACTAGCTAATCCAACGCGGGCC  1259

Query  541   GATCCTTTGCCGATAAATCTTTCCCCCGAAGGGCACATACGGTATTAGCACAAGTTTCCC  600
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Sbjct  1260  GATCCTTTGCCGATAAATCTTTCCCCCGAAGGGCACATACGGTATTAGCACAAGTTTCCC  1319
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Query  601   TGAGTTATTCCGTAGCAAAAGGTACGWTCCCACGCSTTACTYACCCGTMTRCCGCTCCCC  660
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Sbjct  1320  TGAGTTATTCCGTAGCAAAAGGTACGTTCCCACGCGTTACTCACCCGTCTGCCGCTCCCC  1379

Query  661   TTGCGG  666
              ||||||
Sbjct  1380  TTGCGG  1385
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>☐ gi|52421783|gb|AY730720.1| Ochrobactrum anthropi 16S ribosomal RNA gene, partial sequence
Length=1310

Score = 1307 bits (656), Expect = 0.0
Identities = 661/666 (99%), Gaps = 0/666 (0%)
Strand=Plus/Minus

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Sbjct  713   GTATCTAATCCTGTTTGCTCCCCACGCTTTCGCACCTCAGCGTCAGTAATGGTCCAGTGA  654

Query  61    GCCGCCTTCGCCACTGGTGTTCCTCCGAATATCTACGAATTTCACCTCTACACTCGGAAT  120
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  653   GCCGCCTTCGCCACTGGTGTTCCTCCGAATATCTACGAATTTCACCTCTACACTCGGAAT  594

Query  121   TCCACTCACCTCTACCATACTCAAGACTTCCAGTATCAAAGGCAGTTCCGGGGTTGAGCC  180
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Sbjct  593   TCCACTCACCTCTACCATACTCAAGACTTCCAGTATCAAAGGCAGTTCCGGGGTTGAGCC  534

Query  181   CCGGGATTTCACCCCTGACTTAAAAGTCCGCCTACGTGCGCTTTACGCCCAGTAAATCCG  240
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Sbjct  533   CCGGGATTTCACCCCTGACTTAAAAGTCCGCCTACGTGCGCTTTACGCCCAGTAAATCCG  474

Query  241   AACAACGCTAGCCCCCTTCGTATTACCGCGGCTGCTGGCACGAAGTTAGCCGGGGCTTCT  300
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Sbjct  473   AACAACGCTAGCCCCCTTCGTATTACCGCGGCTGCTGGCACGAAGTTAGCCGGGGCTTCT  414

Query  301   TCTCCGGTTACCGTCATTATCTTCACCGGTGAAAGAGCTTTACAACCCTAGGGCCTTCAT  360
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Sbjct  413   TCTCCGGTTACCGTCATTATCTTCACCGGTGAAAGAGCTTTACAACCCTAGGGCCTTCAT  354

Query  361   CACTCACGCGGCATGGCTGGATCAGGCTTGCGCCCATTGTCCAATATTCCCCACTGCTGC  420
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Sbjct  353   CACTCACGCGGCATGGCTGGATCAGGCTTGCGCCCATTGTCCAATATTCCCCACTGCTGC  294

Query  421   CTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCTGATCATCCTCTCAGACCA  480
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Sbjct  293   CTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCTGATCATCCTCTCAGACCA  234

Query  481   GCTATGGATCGTCGCCTTGGTGAGCCTTTACCTCACCAACTAGCTAATCCAACGCGGGCC  540
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Sbjct  233   GCTATGGATCGTCGCCTTGGTGAGCCTTTACCTCACCAACTAGCTAATCCAACGCGGGCC  174

Query  541   GATCCTTTGCCGATAAATCTTTCCCCCGAAGGGCACATACGGTATTAGCACAAGTTTCCC  600
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Sbjct  173   GATCCTTTGCCGATAAATCTTTCCCCCGAAGGGCACATACGGTATTAGCACAAGTTTCCC  114

Query  601   TGAGTTATTCCGTAGCAAAAGGTACGWTCCCACGCSTTACTYACCCGTMTRCCGCTCCCC  660
              ||||||||||||||||||||||||||| |||||||| ||||| |||||| | |||||||||
Sbjct  113   TGAGTTATTCCGTAGCAAAAGGTACGTTCCCACGCGTTACTCACCCGTCTGCCGCTCCCC  54

Query  661   TTGCGG  666
              ||||||
Sbjct  53    TTGCGG  48
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>☐ gi|77696189|gb|DQ211904.1| Ochrobactrum sp. LJ-D 16S ribosomal RNA gene, partial sequence
Length=1342

Score = 1307 bits (656), Expect = 0.0
Identities = 661/666 (99%), Gaps = 0/666 (0%)
Strand=Plus/Minus

Appendix 12

From DSMZ catalogue

Name:

Ochrobactrum anthropi Holmes et al. 1988

DSM No.:

20150

Other collection no.
or WDCM no.:

ATCC 11425

Isolated from:

urine of leech (*Hirudo*)

History:

<- ATCC <- E.V. Morse <- R.S. Breed <- Büsing (*Corynebacterium nephridii*)

Cultivation conditions:

Medium 53 , 30°C

Complete DSMZ Media List

Summary and
additional information:

<- ATCC <- E.V. Morse <- R.S. Breed <- Büsing (*Corynebacterium nephridii*). Until 2001 preserved as "*Corynebacterium nephridii*". Urine of leech (*Hirudo*) (377). Murein: A31 (346). Contains ubiquinone (1306). (Medium 53, 30°C)

Literature:

346, 377, 1306

Risk group:

2 (classification according to German TRBA)

Restrictions:

Act dealing with the prevention & control of infectious diseases in man (Infektionsschutzgesetz), Category A1

Supplied as:

- vacuum dried culture
- actively growing culture available on request at an extra charge
- DNA

Appendix 13

This entry contains an HTML link to the reference database for synonyms of this catalogue. If there is no synonym data available, these links will be empty.

CABRI:DSMZ_BACT

Strain_number	DSM 20150
Other_collection_numbers	ATCC 11425
Name	Ochrobactrum anthropi, Holmes et al. 1988 VP
Infrasubspecific_names	-
Organism_type	Bacteria
Restrictions	Risk group 2 (A)
Status	-
History	<- ATCC <- E.V. Morse <- R.S. Breed <- Buesing (Corynebacterium nephridii)
Other_names	Corynebacterium nephridii, INVALID NAME
Isolated_from	urine of leech (Hirudo)
Literature	DSM ref.no. 346; DSM ref.no. 377; DSM ref.no. 1306
Conditions_for_growth	Medium 53, 30C
Form_of_supply	Dried
Further_information	murein: A31 (346); contains ubiquinone (1306)