

A study of a denitrifying alphaproteobacterium from the Arabian Sea and its N-cycling potential in sub-oxic microzones within the upper water column

Emmalina Burd

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Supervised by: Mike Wyman^{1a}, Clare Bird¹, Sabine Matallana-Surget¹, Tom Preston²
and Nick Owens³

¹ Biological & Environmental Sciences, School of Natural Sciences, University of
Stirling, FK9 4LA, UK

² Scottish Universities Environmental Research Centre (SUERC), University of
Glasgow, East Kilbride, UK

³ Scottish Association for Marine Science (SAMS), Oban, UK

^a Retiree



Statement of originality

I hereby confirm that this PhD thesis is an original piece of work conducted independently by the undersigned and all work contained herein has not been submitted for another degree.

All research material has been duly acknowledged and cited.

Signature of candidate:

Emmalina Burd

Date: 11th December 2020

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General Abstract

Deoxygenation, the progressive loss of dissolved oxygen, is accelerating under climate change and is a major threat to the World Ocean. Oxygen is not only essential for the health of the oceans, but diminishing concentrations have profound economic and socio-economic consequences that impact on human society and well-being. Low oxygen waters (O_2 , <30% saturation) represent ~10% of the ocean volume presently and have expanded by a fifth in the last 50 years. Knowledge of the organisms driving nutrient cycling and biological productivity in these regions is key to predicting and potentially mitigating the effects of deoxygenation. This thesis provides a comprehensive study of a model denitrifying alphaproteobacterium, an isolate from the Arabian Sea; the most intense of the three major Oxygen Minimum Zones. *Labrenzia* sp. strain 5N and its close relatives are present within both oxic and suboxic waters of the Arabian Sea. It is free-living but in surface waters it is also found in association with the major N_2 -fixer *Trichodesmium*. By a combination of growth experiments, biochemical profiling, genomics, proteomics and N-tracing, it was established that this bacterium has the metabolic potential for a number of environmentally and climatically important processes. As well as denitrifying in the absence of oxygen, this bacterium is capable of coupled nitrification and aerobic denitrification. Simultaneous respiration of oxygen and nitrogen oxides by organisms like *Labrenzia* sp. strain 5N suggests that denitrification may be more widespread in the oceans than is accounted for by current ecosystem models. This is of concern because N is an essential element for all marine life. Any increase in the losses of N across the air/sea boundary as deoxygenation progresses will lead to an accelerating decline in ocean productivity. Of further importance, strain 5N produces environmentally significant amounts of the potent greenhouse gas nitrous oxide as the primary end product of aerobic denitrification. As well as its warming potential, nitrous oxide is the most significant trace gas contributing to stratospheric ozone depletion following the withdrawal of chlorofluorocarbons under the Montreal Protocol. Further investigations of the significance of these oxygen-sensitive metabolic processes, and establishing the scale to which they might drive ecosystem change under future oceanic conditions, is essential for climate change mitigation.

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Chapter 1:

General Introduction

1.1 Nitrogen in marine systems

1.1.1 Marine Nitrogen Cycling, an overview

Nitrogen (N) is the most abundant element in the biosphere, constituting ~78% of the Earth's atmosphere. It is essential for all life on Earth and is indispensable for processes such as nucleotide and amino acid synthesis. As a result, the bioavailability and distribution of different forms of nitrogen have crucial effects on ocean productivity (Carpenter and Capone, 2008). Nitr is one of the essential nutrients required to support primary production by phytoplankton and its availability has a knock-on effect on the entire marine ecosystem. Although nitrogen is abundant in the atmosphere, the inability of most organisms to use nitrogen in this gaseous form (N_2) means nitrogen is considered to be a limiting nutrient in the marine environment (Moore *et al.*, 2013). For nitrogen fixers, however, the atmosphere essentially supplies an unlimited source of nitrogen that they can convert to bioavailable forms, thus lessening potential limitation. For this reason it is argued that phosphorus is the ultimate limiting nutrient in the oceans because it must be obtained from other non-marine sources (Tyrrell, 1999; Moore *et al.*, 2013).

In the oceans, nitrogen is present in many forms, including inorganic nitrate (NO_3^-), nitrite (NO_2^-), ammonium (NH_4^+), dissolved gaseous nitrogen (N_2) and nitrous oxide (N_2O). Nitrogen is interconverted between these different states as it is assimilated into cells and transformed during growth and metabolic processes (Tyrrell, 1999). The balance between the concentrations of each of these forms of nitrogen within the oceans is mediated by a number of biochemical reactions that together form the basis of the nitrogen cycle. The primary reactions of the nitrogen cycle include nitrogen fixation, the uptake of bioavailable forms of nitrogen by bacterioplankton and phytoplankton, nitrification, denitrification, mineralization and the Anammox pathway (Figure 1.1). The marine nitrogen cycle depends on both oxidation and reduction reactions to convert nitrogen between its different redox states. Combined, these reactions control the bioavailability of nitrogen and, as a result, ocean productivity.

Microbial activity plays a pivotal role in the nitrogen cycle, making microorganisms indispensable in generating the available nutrients required by all life forms (Tyrrell, 1999).

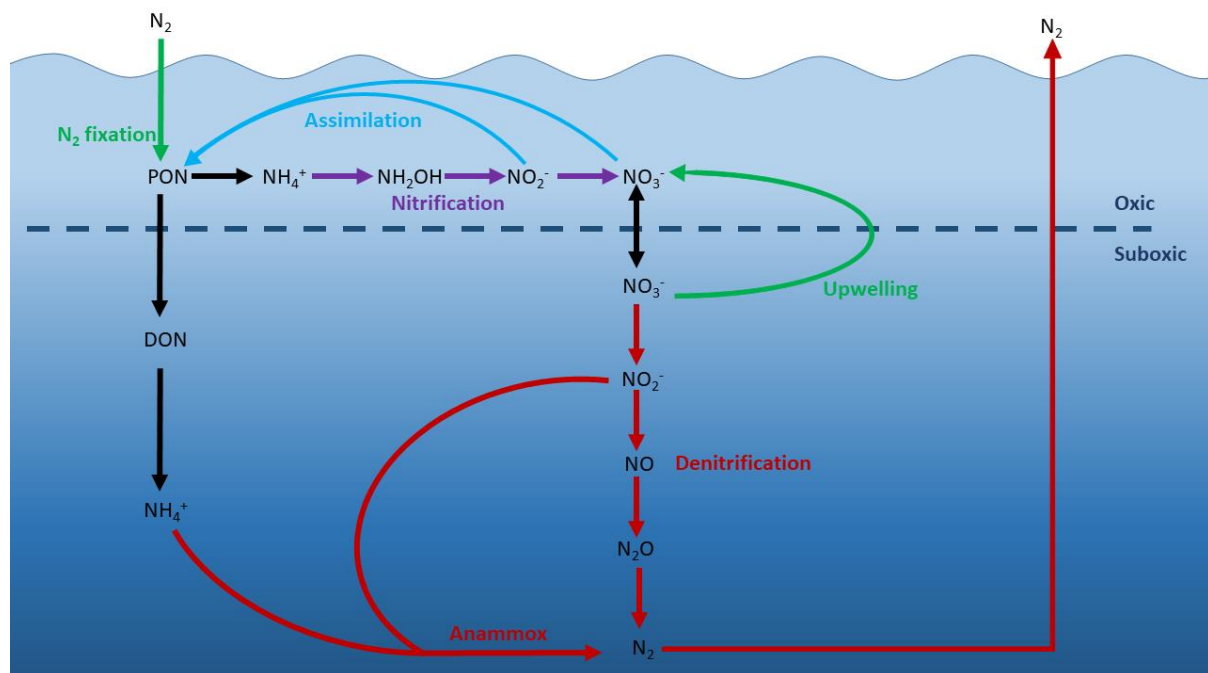


Figure 1.1. An overview of nitrogen cycling in Oxygen Minimum Zones (OMZs). Processes resulting in nitrogen gains are shown in green and those resulting in nitrogen losses from the oceans are shown in red. PON, particulate organic nitrogen; DON, dissolved organic nitrogen.

1.1.2 Nitrogen Fixation

Although the most common form of nitrogen on earth is dinitrogen gas (N₂), most organisms are incapable of utilizing nitrogen in this stable form. For most cells, gaseous nitrogen must first be converted into other bioavailable forms through a process known as nitrogen fixation. Nitrogen fixation can occur naturally in lightning strikes, industrially through the burning of fossil fuels or fertilizer production via the Haber-Bosch process, and biologically by nitrogen fixing organisms (Smil, 2001; Falkowski, 1997). Marine biological nitrogen fixation generates an estimated 140 TgN.yr⁻¹ and the supply of this newly-fixed N to the oceans supports marine productivity (Deutsch *et al.*, 2007; Gruber, 2008). Relatively few bacteria and archaea, collectively known as diazotrophs, can use nitrogen gas directly. Diazotrophs have been found to inhabit a diversity of marine environments including the open ocean

(including pelagic and deeper waters), coral reefs, coastal zones, estuaries and the seabed sediment (LaRoche and Breitbarth, 2005). Diazotrophs may also live with larger organisms, such as within the digestive tract of zooplankton (Braun *et al.*, 1999) or in association with various eukaryotic phytoplankton (Scavotto *et al.*, 2015). During the process of nitrogen fixation, diazotrophs reduce atmospheric nitrogen to ammonium that is more readily useable by other organisms. Diazotrophs are therefore major players in N cycling and their activity regulates nutrient availability within marine systems. Hence, knowledge of marine diazotrophs, and their interactions with other organisms, greatly enhances understanding of ocean productivity and is essential for predicting the impact of anthropogenic perturbations of the nitrogen cycle (Galloway *et al.*, 2004; Carpenter and Capone, 2008).

The ammonium (NH_4^+) produced by nitrogen fixation is an ideal form of nitrogen for many organisms because it contains nitrogen already in the correct oxidation state needed for its incorporation into amino acids. The strong triple bond in N_2 needs to be broken in order for the cells to utilize the nitrogen atoms in biomolecule synthesis (Peters *et al.*, 1995), however, and most organisms do not contain the cellular machinery needed to complete this energetically expensive process. Nitrogen was first termed an 'azote' (meaning 'lifeless') by Lavoisier (1790), due to its high stability, which illustrates how inert this element is and highlights the high energetic costs involved in nitrogen fixation (Reitzer, 2003). The chemical reaction of nitrogen fixation, through which dinitrogen is reduced into ammonia, uses 8 hydrogen atoms (H^+) as well as hydrolysing over 16 molecules of ATP (Figure 1.2).

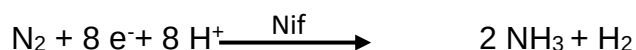


Figure 1.2. Biological nitrogen fixation equation. Nif, nitrogenase.

The nitrogenase enzyme complex, encoded by *nif* genes, catalyzes this reaction (Figure 1.2). This complex constitutes 2 protein subunits: an iron (Fe) dinitrogenase reductase enzyme bound to either a molybdenum-iron protein (MoFe) or a Vanadium-iron protein (VFe) (Hu *et al.*, 2012). The *nif* genes encode the nitrogenase complex as well as numerous additional genes responsible for synthesis and regulating activity of the proteins involved in nitrogen fixation. As a result of the high

iron requirement of nitrogenase, N₂-fixers thrive best in iron rich waters because they need ~100-fold higher iron concentrations for growth than non-diazotrophs (Berman-Frank *et al.*, 2001). Wind-borne sources bring a considerable amount of iron to some parts of the oceans and diazotroph distribution reflects this requirement. Nitrogen-fixers are generally scarce in areas remote regions such as the South Pacific Ocean (Falkowski, 1997).

Nitrogenase is highly sensitive to oxygen. The Fe-S cluster within the active site of the enzyme is readily inactivated if oxidized (Martin *et al.*, 2001). The oxygen sensitivity of nitrogenase is particularly problematic for nitrogen-fixing cyanobacteria because they are photoautotrophs and generate oxygen as a by-product of photosynthesis. Diazotrophic cyanobacteria have evolved various mechanisms to circumvent this potential conflict through spatial and/or temporal segregation of nitrogenase activity and photosynthesis (Newton, 2007). Some cyanobacteria, such as *Anabaena cylindrica*, physically compartmentalize nitrogenase within specialised nitrogen-fixing cells known as heterocysts, that have thick hydrophobic cell walls that exclude oxygen (Fay 1992; Fay *et al.*, 1968). In other cyanobacteria nitrogen fixation and photosynthesis are separated temporally with photosynthesis taking place during the daylight hours and nitrogen fixation confined to the night (Stal and Krumbein, 1987). In general, cyanobacteria also maintain high cellular respiration rates that helps to keep their cytoplasmic oxygen levels low, protecting nitrogenase (Poole and Hill, 1997; LaRoche and Breitbarth, 2005; Großkopf and LaRoche, 2012; Inomura *et al.*, 2017).

Interestingly, the photosynthetic cyanobacterium *Trichodesmium* overcomes nitrogenase oxygen sensitivity despite nitrogen fixation being confined to the daytime alongside photosynthesis (Küpper *et al.*, 2008). The non-heterocystous *Trichodesmium* achieves this by combining both spatial and temporal segregation of nitrogen fixation and photosynthesis. *Trichodesmium* confines nitrogen fixation to cells called 'diazocytes', presenting a similar compartmentalization function to heterocytes (Fredriksson and Bergman, 1997). Additionally, although nitrogen fixation takes place during the daytime, nitrogenase activity itself is concentrated around mid-day, whilst photosynthesis is suspended. This provides a period of time during which conditions are more favourable for nitrogen fixation (Berman-Frank *et al.*, 2001) and also helps to protect the photosynthetic machinery from oxidative damage at high light.

1.1.3 Nitrification

Through the process of nitrification, remineralised nitrogen in the form of ammonia is oxidised to inorganic nitrate by organisms called nitrifiers. Nitrification takes place in a series of steps starting with the conversion of ammonia to nitrite using membrane associated enzymes and CO₂ as the carbon source in autotrophs (Figure 1.3). The nitrite formed is then further oxidised to nitrate by a second group of organisms although recent work has shown that some nitrifiers are able to oxidise ammonia fully to nitrate, a process called comammox (Daims *et al.*, 2015; Van Kessel *et al.*, 2015). Through these reactions, autotrophic nitrifiers generate the energy they need to power other cellular and metabolic processes (Mancinelli, 1996). Nitrification connects the processes of N₂-fixation and ammonification to denitrification through the conversion of ammonium to nitrate, the substrate for denitrification.



Figure 1.3. Nitrification reactions. 1, Ammonia oxidation to hydroxylamine; 2, Hydroxylamine oxidation to nitrite; 3, Nitrite oxidation to nitrate.

Ammonia-oxidizing archaea (AOA) and bacteria (AOB) are capable of oxidizing NH₃ to hydroxylamine (NH₂OH) using the enzyme ammonia monooxygenase (AmoABC). Hydroxylamine is then further oxidised to nitrite by the enzyme hydroxylamine oxidoreductase in AOB and presumably via a similar process in AOA although the oxidase responsible has not yet been identified (Schleper *et al.*, 2005; Vajjala *et al.*, 2013). The following step, the oxidation of nitrite to nitrate, catalysed by nitrite oxidoreductase, is performed by nitrite-oxidizing bacteria such as those belonging to the *Nitrobacter* genus. Until recently, the only organisms thought to be capable of the initial ammonia oxidation step were bacteria. However the discovery of AOA dramatically changed understanding of nitrification in the marine environment (Konneke *et al.*, 2005; Schleper *et al.*, 2005), because these previously undiscovered

microorganisms may exceed AOB in terms of numbers and activity (Wuchter *et al.*, 2006).

As the nitrification steps are oxidative, this process only occurs aerobically. In general, nitrification is at its peak in the lower reaches of the euphotic zone and gradually decreases with depth (Ward, 2008). Nitrification was traditionally considered to be a light-sensitive process but more recent studies have challenged this idea because AOA are also active in the surface ocean (Church *et al.*, 2010; Pedneault *et al.*, 2014; Smith *et al.*, 2014). At depths where light intensity is 5-10% of its surface value, nitrification is at its maximum (Lipschultz *et al.*, 1990). At this depth, the rates of ammonium uptake by phytoplankton are low owing to light limitation, enabling AOB to access ammonia with reduced competition from the primary producers (Smith *et al.*, 2014). The only habitat where the rates of nitrification in deep waters are of a similar magnitude to those in the lower euphotic zone are hydrothermal vents, which are characterised by high ammonium levels (Lam *et al.*, 2004).

1.1.4 Denitrification

Another major step in the nitrogen cycle is the process of denitrification. Through this metabolic pathway, denitrifiers carry out nitrate reduction as a form of anaerobic respiration. Denitrification returns nitrogen gas to the atmosphere, replenishing that removed by nitrogen fixation and completing the nitrogen cycle. When oxygen concentrations in the environment are limiting, denitrifying bacteria have the ability to switch from aerobic respiration to nitrate respiration. This flexibility in the choice of terminal electron acceptor means denitrifiers can adapt to changing environmental conditions by altering their respiratory machinery. Denitrification is a metabolic process through which nitrate (NO_3^-) is reduced sequentially to nitrite (NO_2^-), nitric oxide (NO), nitrous oxide (N_2O) and finally to dinitrogen gas (N_2) (Figure 1.4). These reactions are catalysed by the action of four reductases that are either membrane bound or located in the periplasm (Goering, 1985). The detection and quantification of the expression of the genes encoding these enzymes has been an important step in the identification of denitrifying organisms and their distribution (Bothe *et al.*, 2000; Pajares and Ramos, 2019). The complete denitrification reaction, from NO_3^- to N_2 , consumes 10 electrons that are transferred between components of an electron transport chain (Figure 1.4).

Simultaneously, the proton motive force generated through these reactions is used to drive ATP synthesis in the cell.

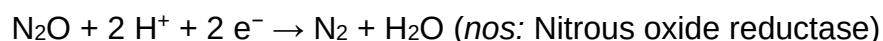
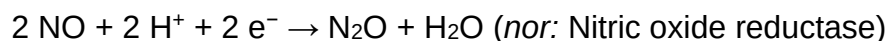
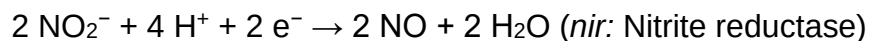
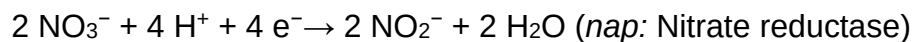


Figure 1.4. Reactions of the sequential denitrification pathway. Enzymes catalyzing each step are given in brackets.

In the marine environment, these reactions are carried out predominantly by bacteria, but also by various Archaea, Fungi, and members of the Rhizaria including foraminifera (Knowles, 1996; Zumft and Körner, 1997; Piña-Ochoa *et al.* 2010). Some microbes, including *Escherichia coli*, are capable only of incomplete respiratory nitrate reduction because they synthesize the nitrate reductase enzyme complex, encoded by the *narGHJ* or *napABC* operons, but not other components of the denitrification machinery (Kandeler *et al.*, 2006). Therefore, in these nitrate-respiring bacteria the pathway culminates with the production of nitrite. Bacteria, and particularly members of the Proteobacteria, are the major marine denitrifiers in pelagic waters (Zumft and Körner, 1997) while Foraminifera make a significant contribution to this process also in marine sediments (Piña-Ochoa *et al.*, 2010).

Denitrification reduces oxidised nitrogenous compounds and is most frequently associated with areas of low oxygen concentration. Nitrous oxide reductase (NosZ), responsible for the final conversion of N_2O to N_2 , is an oxygen sensitive enzyme. So, denitrification is likely to be incomplete when oxygen is present and terminate with the production of the greenhouse gas, N_2O (Davies *et al.*, 1989; Ji *et al.*, 2018). Denitrification is most widespread in areas of the ocean where oxygen levels are below $2\mu\text{M}$ (Knowles, 1996; Zumft and Körner, 1997). In these suboxic conditions ($\text{O}_2 < 1\%$ saturation), denitrifiers can use nitrate as an electron acceptor in place of oxygen. This gives them a competitive advantage over other organisms living in these oxygen-poor environments.

Classical denitrification was thought to be confined solely to strictly anaerobic environments because of the sensitivity of the denitrification enzymes (and *NosZ*, in particular) to oxygen. For this reason, denitrification in oxic surface waters has been generally overlooked although recent studies have provided evidence for active denitrification associated with particles and faecal pellets in these waters (Bianchi *et al.*, 2018). Molecular methods targeting *nosZ* have identified potential areas of active denitrification in the oxygenated surface layers of the Arabian Sea, Southern Indian Ocean, and Eastern Tropical Pacific (Wyman *et al.*, 2013; Raes *et al.*, 2016; Sun *et al.*, 2017). Numbers of *nosZ* copies detected varied from hundreds per millilitre (Wyman *et al.*, 2013; Sun *et al.*, 2017) to an average of $1.9 \times 10^5 \text{ mL}^{-1}$ in the Southern Indian Ocean. Whilst the composition of the two *nosZ* communities was distinct, both Wyman *et al.* (2013) and Sun *et al.* (2017) recorded active expression of *nosZ* in surface oxic waters as well as at depth within the OMZs of the Arabian Sea and Eastern Tropical Pacific respectively.

1.1.5 Dissimilatory nitrate reduction to ammonium

Dissimilatory nitrate reduction to ammonium (DNRA) is an anaerobic respiratory process via which microbes use nitrate as the electron acceptor, reducing it to ammonium via nitrite. As DNRA and denitrification both commence with the anaerobic reduction of nitrate to nitrite, the two processes often compete in low oxygen regions such as OMZs (Tiedje *et al.*, 1982; Kraft *et al.*, 2014). It is therefore unsurprising that some organisms inhabiting these waters appear capable of both denitrification and DNRA (Lam and Kuypers, 2010). The C:N ratio in the environment is reported to influence which process dominates (Rütting *et al.*, 2011; Kraft *et al.*, 2014). In general, when nitrate is limiting in the environment, bacteria carrying out DNRA have the competitive advantage over denitrifiers (Kraft *et al.*, 2014; van den Berg *et al.*, 2016). Unlike denitrification DNRA does not result in losses of N from oceanic systems. Rather, DNRA replenishes the supply of bioavailable ammonium that might be assimilated by other organisms or oxidised by nitrifiers.

1.1.6 Anaerobic ammonia oxidation: Anammox

Nitrifiers are obligate aerobes and so ammonium can accumulate in oxygen-depleted waters unable to support the nitrification process. Ammonium is toxic to most marine organisms but under such conditions an anaerobic process converting ammonium and nitrite to dinitrogen gas occurs. First described by van de Graaf *et al.* (1995), the pathway responsible for this process is known as anaerobic ammonia oxidation, or Anammox. Anammox is considered an alternative type of denitrification because it also ultimately removes nitrogen from the environment whilst providing anammox bacteria with a supply of electrons. All anammox bacteria are autotrophs belonging to the bacterial phylum *Planctomycetes*. Five anammox candidate genera have been described to date (Jetten *et al.*, 2010). First discovered in wastewater treatment systems, these organisms are now known to inhabit a variety of anoxic marine habitats including the Black Sea, tropical OMZs, productive estuaries and marine sediments (Thamdrup and Dalsgaard, 2002; Kuypers *et al.*, 2003; Trimmer *et al.*, 2005; Thamdrup *et al.*, 2006).

1.2 Hypoxia in the Ocean

1.2.1 Marine hypoxic and suboxic waters in a changing world

Marine dissolved oxygen (DO) concentrations are decreasing, primarily due to climate change and increased nutrient inputs into coastal regions. Average sea surface temperatures have risen by 0.11°C per decade since the 1970's (<https://www.ncdc.noaa.gov/cag/>, NOAA database accessed October 2020) and are predicted to continue to rise rapidly (IPCC, 2014). Ultimately, warmer surface waters (in which O₂ is less soluble) and the more frequent and intense stratification of the surface layers lead to ocean deoxygenation. Deeper waters cut off by stratification will continue to consume O₂, despite reduced replenishment of nutrients by mixing from the upper mixed layer, thus further depleting O₂ reserves.

Suboxic (O₂, <1% saturation) and hypoxic (O₂, <30% saturation) waters combined represent ~10% of total oceanic area (Canfield *et al.*, 2010). Hypoxic regions are found in both open waters and coastal/estuarine environments (Figure 1.5). Coastal and estuarine regions are particularly susceptible to hypoxia owing to increasing eutrophication from agricultural runoff and sewage discharges (Diaz and Rosenberg, 2008). These zones may also rely on O₂ replenishment from offshore waters, that

could themselves be suffering the effects of deoxygenation, thus exacerbating the impact on these shallow waters. In the open ocean, oxygen minimum zones (OMZs) extend between 60-200m and 1000m in depth. The major OMZs are located in the Pacific Ocean (the Northeastern subarctic Pacific, the eastern tropical North Pacific [ETNP] and the eastern tropical South Pacific [ETSP]), the Atlantic Ocean (Northwest-African upwelling and the Benguela upwelling), the Indian Ocean (Bay of Bengal and the Arabian Sea) and the Black and Baltic Seas. Hypoxic zones have already and, in line with future climate predictions, will continue to expand with ocean surface water warming (Diaz and Rosenberg, 2008; Keeling *et al.*, 2010). Over the past ~60 years, OMZs have grown by 4.5 million km² (Stramma *et al.*, 2010) and the upper boundaries of existing hypoxic zones are also closer to the surface (Whitney *et al.*, 2007).

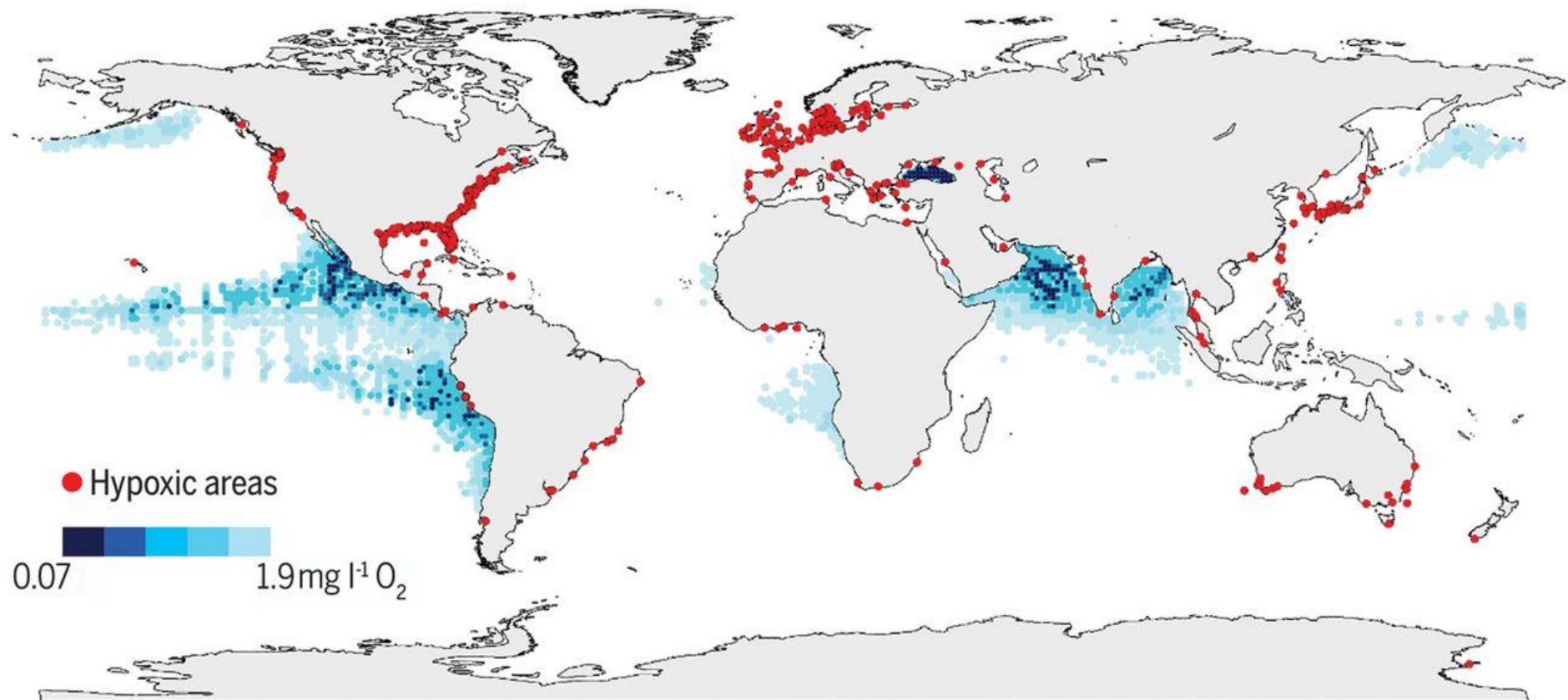


Figure 1.5. Global map of coastal hypoxic zones ($O_2 < 2 \text{ mg.L}^{-1}$, red dots) and open ocean oxygen minimum zones at 300m depth (blue regions). Map from Breitburg *et al.*, 2018, originally created from data provided by R. Diaz, updated by members of the GO₂NE network, and downloaded from the World Ocean Atlas 2009.

1.2.2 Global implications of the expansion of low oxygen waters

The consequences of deoxygenation of the oceans and the expansion of low oxygen waters are manifold leading to changes to global nutrient cycles, nutrient availability, productivity and greenhouse gas production/consumption (Figure 1.6). Deoxygenation has major impacts on marine biogeochemical cycling both directly, by influencing C, P and N cycles and indirectly, by changing community compositions (Levin *et al.*, 2009). In addition, rates of N₂-fixation, photosynthesis and respiration are linked to oxygen availability and in turn influence the drawdown of atmospheric CO₂. As dissolved oxygen (DO) levels decrease, P is released from sediments via chemical processes and fuels the growth of N₂-fixing cyanobacteria which, in turn, has impacts on N cycling, as is the case in the Baltic Sea (Conley *et al.*, 2009). When O₂-independent metabolic processes start to dominate, N losses through denitrification and rates of production and consumption of the greenhouse gas N₂O will rise. A switch towards anaerobic respiration alters the availability of micronutrients throughout the food chain. Aerobic life is reliant on the availability of O₂ and therefore a drop in DO to hypoxic levels can be incredibly stressful, and even fatal, for marine organisms (Keeling *et al.*, 2010). Fish and Crustacea are particularly susceptible to low oxygen and as a result, hypoxia disrupts existing marine ecosystems and food webs (Vaquer-Sunyer and Duarte, 2010). Communities are likely to shift towards increased microbial activity as fewer carnivores are able to inhabit these regions (Sperling *et al.*, 2013) and, in addition, as temperatures increase further fishes have even higher O₂ requirements (Nilsson *et al.*, 2010). These changes in community composition have knock-on effects on aquaculture and global food security (Global Ocean Oxygen Network, 2018). As such, knowledge of the processes and organisms that dominate low oxygen zones is key to predicting and potentially mitigating the effects of the expansion of hypoxic regions.

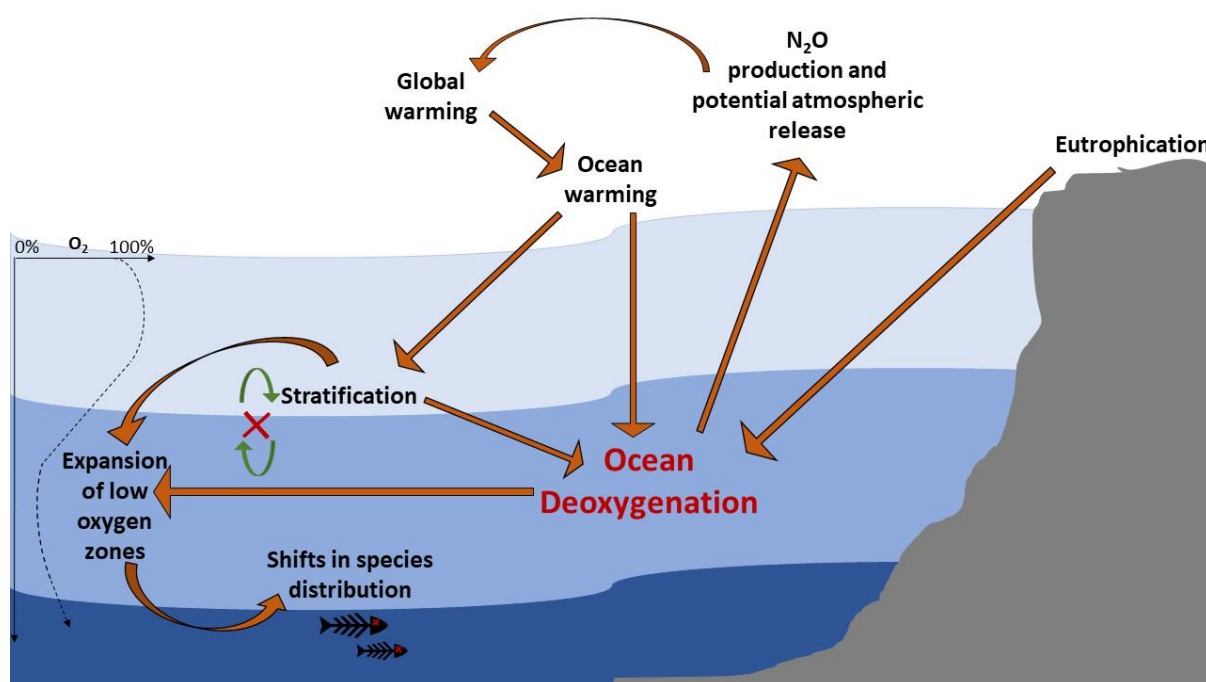


Figure 1.6. Potential causes, processes and consequences of ocean deoxygenation.

1.3 *Trichodesmium*: A key marine, nitrogen-fixing cyanobacterium

1.3.1 The influence of *Trichodesmium* on the marine nitrogen cycle

Knowledge of the numbers and geographic range of active diazotrophs in the oceans has expended considerably in recent years. These now include unicellular cyanobacteria (Moisander *et al.*, 2010; Harding *et al.*, 2018) as well as the more familiar filamentous *Trichodesmium* and diatom-associated *Richelia* spp. plus a wide range of taxonomically diverse heterotrophic bacteria (Farnelid *et al.*, 2011). Early studies investigating nitrogen fixation in pelagic systems concentrated on the diazotrophic, cyanobacterium, *Trichodesmium*, a major player in nitrogen cycling (Saino, 1977). *Trichodesmium*, known to generations of sailors as ‘sea sawdust’, was first described by Ehrenberg in 1830 who observed extensive blooms in the Red Sea (Ehrenberg, 1830). *Trichodesmium* is a Gram-negative cyanobacterium that differs from most other filamentous nitrogen fixers in that it lacks heterocysts. The cells contain abundant gas vacuoles that enable *Trichodesmium* to regulate its depth within the water column (Walsby, 1978; Villareal and Carpenter, 2003). The individual filaments can be up to one hundred cells in length and frequently aggregate (Figure

1.7) to form fusiform or spherical colonies known as ‘tufts’ or ‘puffs’ respectively (Janson *et al.*, 1995; Finzi-Hart *et al.*, 2009).

Global nitrogen fixation by *Trichodesmium* was initially underestimated at around 5 Tg N per annum (Capone and Carpenter, 1982), but more accurate recent estimates now place it between 60 and 80 Tg N annually (Carpenter and Capone, 2008). Based on these rates, *Trichodesmium* alone is thought to contribute to ~50% of total marine nitrogen fixation (Karl *et al.*, 2002; Fowler *et al.*, 2013), fixing ~30mg N/m²/day globally. It provides tropical and sub-tropical oceanic surface waters with more nitrogen than is replenished via natural upwelling (Carpenter and Romans, 1991). In these waters *Trichodesmium* shapes a number of biogeochemical processes with the potential to drawdown carbon dioxide in N-poor conditions that do not readily support the growth of other phytoplankton.



Figure 1.7. *Trichodesmium* colonies in fusiform (‘tuft’) arrangement (Source: AL Baker, University of New Hampshire).

1.3.2 *Trichodesmium* microcommunities

Marine organisms are often exposed to unfavourable conditions in nutrient poor waters, and must develop successful strategies to survive. Some take advantage of microenvironments such as marine snow (Silver *et al.*, 1978), phytodetritus (Biddanda and Pomeroy, 1988) and metazoan faecal products (González and Biddanda, 1990) that provide “hotspots” to overcome oligotrophic conditions. This strategy provides a substrate for attachment, a protective microenvironment and, potentially, a supply of nutrients from other associated organisms. *Trichodesmium* colonies provide such a habitat for a range of other organisms, including abundant attached bacteria, diatoms, protozoa and the harpacticoid copepod, *Macrosetella gracilis* (O’Neil JM and Roman, 1992; Selner, 1992; Nausch, 1996; Sheridan *et al.*, 2002). Associated organisms benefit further from living within a protected environment with *Trichodesmium* because apart from *M. gracilis*, it has few known grazers and provides a stable substrate (O’Neil and Roman, 1992).

Equally, epibionts benefit from their association with the buoyant *Trichodesmium* to maintain a presence in the upper photic zone, from which they might be excluded when attached to sinking particles. Heterotrophs associating with the colonies also benefit from a supply of nutrients such as combined nitrogen and fixed carbon from their host (Capone *et al.*, 1994; Glibert and Bronk, 1994). *Trichodesmium* releases a substantial portion of newly fixed nitrogen (up to 80%) as ammonium and amino acids, providing a rich source of nitrogen to organisms living with the colonies or in the surrounding seawater (Mulholland *et al.*, 2006). Using colonies collected in the Sargasso Sea, Sheridan *et al.* (2002) concluded that 85% of *Trichodesmium* colonies harboured other organisms, the most widespread being bacteria, dinoflagellates and amoebae. The density of associated species within the colonies was two to five orders of magnitude greater than that in the surrounding water, suggesting that these associations are beneficial to epibionts. Differences between the colonization profile of puffs and tufts were also apparent; a higher proportion of ‘puff’ type colonies harboured associates and these microcommunities were also more species rich.

Bacterial associates are the most abundant epibiotic organisms found with *Trichodesmium* and have been detected at concentrations between 8×10^8 and 2.6×10^{11} cells per ml of colony volume (Figure 1.8) (Paerl, 1982; Sheridan *et al.*, 2002). Recent 16S rRNA metagenomic sequencing studies of samples from the North

Atlantic and North Pacific Oceans determined *Trichodesmium* spp. sequences only accounted for 66.7% of those retrieved from the colonies while the remaining 31.8% belonged to heterotrophic bacteria and 1.5% to other organisms (Rouco *et al.*, 2016). These estimates are certainly conservative because of the polyploidy of *Trichodesmium* which may harbour up to 100 genome copies in natural populations and >600 in culture (Sargent *et al.*, 2016). Hmelo *et al.* (2012) investigated the taxonomic affiliations of the epibiotic bacteria in *Trichodesmium* colonies collected from the Sargasso Sea. Differences between the microcommunities of puff and tuft colonies were again confirmed; in puff-type colonies Flavobacteria, Chloroflexi, Gammaproteobacteria and Alphaproteobacteria dominated, whereas the tuft-type colonies were primarily host to members of the Bacteroidetes, Cyanobacteria, Alphaproteobacteria and Sphingobacteria.

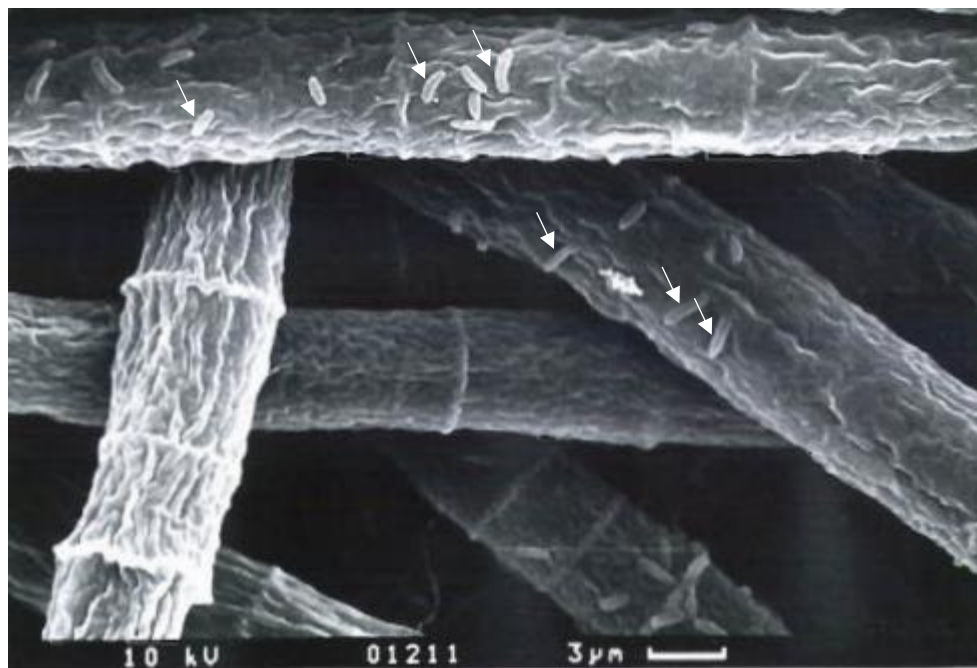


Figure 1.8. Bacteria associated with *Trichodesmium* trichomes. The arrows highlight individual attached rod-shaped bacteria. The cross walls of cells forming part of the *Trichodesmium* filaments are clearly visible. Image from Nausch (1996), credited to R. Bahlo, Baltic Sea Research Institute.

1.3.3 Functional role of *Trichodesmium* colony associates

While recent studies have increased knowledge of the taxonomic composition of *Trichodesmium*-associated communities, understanding of the biogeochemical significance of these associations remains limited. Characterization of the metabolic capabilities of these microcommunities is an essential first step in assessing the importance of individual members, the extent of their interactions with *Trichodesmium*, and ultimately to reveal the functional role of these associates. It is clear that the composition of the associations is far from random (Rouco *et al.*, 2016) although what factors determine the specific combinations of epibionts are yet to be determined. It is widely acknowledged that establishment and maintenance of axenic *Trichodesmium* cultures is highly problematic, suggesting that interactions with the epibiont community may be essential for maintaining viability (Waterbury, 1991).

Trichodesmium spp. in the North Pacific and North Atlantic experience different environmental stressors (Chappell *et al.*, 2012), and therefore it follows that their epibionts would do so also. It seems likely that epibiont composition varies markedly with location and colony form because of the unique conditions encountered in each environment. The presence and abundance of a particular associate probably depends on its metabolic capacities as well as the specific physical, biological and physiological challenges encountered within the colony microenvironment. Recent studies by Rao *et al.* (2015) reveal important behavioural changes in organisms living in association with *Trichodesmium*. The *Trichodesmium* epibiont *Silicibacter* sp. strain TrichCH4B forms biofilms in anaerobic cultures in response to the signalling molecule, nitric oxide (Rao *et al.*, 2015). It produces NO and the activity of the enzyme responsible, nitric oxide synthase, is enhanced markedly in the presence of spent growth medium from the *Trichodesmium* host. Rao *et al.* (2015) demonstrated that *Trichodesmium* secretes an extracellular heat- and protease-sensitive signalling compound that stimulates nitric oxide synthase activity and promotes colonization by *Silicibacter* sp. strain TrichCH4B.

Natural populations of *Trichodesmium* are frequently iron-stressed (Berman-Frank *et al.*, 2001; Rubin *et al.*, 2011; Chappell *et al.*, 2012) and it has been suggested that bacterial associates could play an important role in iron acquisition within the colonies (Roe *et al.* 2012). The latter authors demonstrated that two bacterial strains isolated from *Trichodesmium* colonies were capable of iron uptake from a variety of sources

that *Trichodesmium* itself cannot access. *Trichodesmium* is only capable of utilizing dissolved forms of iron but its associated bacteria produce siderophores (Roe *et al.*, 2012) that promote the dissolution of Fe-rich dust particles actively captured by the host (Rubin *et al.*, 2011; Basu *et al.*, 2019). These findings underline the vital importance of *Trichodesmium*'s epibionts in nutrient cycling within the colony microenvironment and underscore the need to characterise their functional significance in greater detail

1.3.4 Active denitrification within *Trichodesmium* colonies?

Intriguingly, in light of the distribution of *Trichodesmium* within the upper mixed water column (Bergman *et al.*, 2013), there is evidence that oxygen-sensitive processes like denitrification might occur within the colony environment. Wyman *et al.* (2013) reported that *nosZ* sequences amplified from a widespread, active, denitrifying alphaproteobacterium related to *Labrenzia* spp. were highly enriched within *Trichodesmium* colonies collected from the Arabian Sea. 16S rRNA clone libraries prepared from *Trichodesmium* from the Sargasso Sea retrieved alphaproteobacterial sequences from the epibiotic community very closely related to *Labrenzia* and the related denitrifier, *Nisaea denitrificans* (Hmelo *et al.*, 2012; see Wyman *et al.*, 2013). Elevated concentrations of nitrous oxide and very high numbers of alphaproteobacterial *nosZ* gene copies were recorded in the oxygenated surface waters of the South-Eastern Indian Ocean; an area that frequently supports intense, extensive blooms of *Trichodesmium* (Raes *et al.*, 2016). Gradoville *et al.* (2017) further demonstrated the genetic capacity for denitrification within the colony microbiome of *Trichodesmium* populations from the North Pacific Subtropical Gyre. These authors found denitrification genes routinely among the metagenomes of all colony forms sampled but not in the surrounding seawater. Further, they reported that >9% of the *Trichodesmium* epibiotic community associated with the “tuft” colony morphotype were related to *Nisaea* spp. (known denitrifiers) whereas, by contrast, 16S rRNA phylotypes from this alphaproteobacterium were rarer in radial “puffs”. Coates and Wyman (2017) extended earlier work (Wyman *et al.*, 2013) and confirmed that *Trichodesmium* colonies indeed harbour an entire community of denitrifying alphaproteobacteria. Furthermore, they showed that the *nosZ* genes from this community were being

actively expressed *in situ* in *Trichodesmium* populations sampled from a range of ocean provinces.

Klawonn *et al.* (2020) using stable isotopes (^{15}N), however, found denitrification rates were insignificant within *Trichodesmium* colonies from the North Pacific Subtropical Gyre. They sampled small ($\sim 0.5\text{--}1\text{ mm}$) “puff” type colonies in which the lowest oxygen concentrations measured were at $\sim 60\%$ saturation. However the same laboratory has shown previously that larger, more mature *Trichodesmium* colonies can develop much lower internal O_2 tensions (Eichner *et al.*, 2018). To date, therefore, it is uncertain whether the genetic potential of the denitrifiers associated with *Trichodesmium* contributes to nitrogen-cycling within the colony microenvironment.

To help resolve this uncertainty it is essential to establish exactly under what environmental conditions the denitrification machinery of the epibiotic denitrifiers might be expressed and active. Fortunately, model organisms representative of the *Trichodesmium*-associated denitrifying community are available. These are *Silicibacter* sp. TrichCH4B which harbours *nirK* and *nor*, but not *napA* or *nosZ* (Coates and Wyman 2017), *Nisaea denitrificans* (*napA*, *nirK*, *nor*, *nosZ*) and the *Labrenzia*-like alphaproteobacteria (*napA*, *nirS*, *nor*, *nosZ*) isolated by Wyman *et al.* (2013). The present study investigated the latter model denitrifiers to address outstanding questions about the genetic and biochemical/physiological potential of these organisms and how these might be realised within the *Trichodesmium* consortium. The next chapter explores the characteristics of these organisms and their nearest close relatives from the genus *Labrenzia*.

1.4 Project aims

This thesis constitutes a multidisciplinary study of a denitrifying alphaproteobacterium isolated from the suboxic waters of the Arabian Sea; strain 5N (Wyman *et al.*, 2013). Strain 5N is representative of a clade of denitrifying organisms known to associate with *Trichodesmium* (Coates and Wyman, 2017). These organisms are of biogeochemical and environmental interest because of their potential to denitrify in oxic and suboxic waters and within the *Trichodesmium* microbiome. Although previous studies have shown that denitrification might occur within *Trichodesmium* colonies, little is known about the denitrifiers themselves or their metabolic potential. In the

context of climate change, their capacity for the production/consumption of nitrous oxide in oxic waters is of concern. This project aims to characterize strain 5N in depth, using a combination of growth experiments, biochemical profiling and genomics. Proteomics and N-tracing approaches are then used to study its adaptation to anaerobiosis and N-cycling capacity. The feasibility of developing a genetic manipulation system for further studies in this model organism is then assessed. Laboratory studies with this model organism will provide new knowledge on the conditions supporting potential denitrification within *Trichodesmium* colonies and on the wider biogeochemical significance for marine nitrogen cycling.

Chapter 2:

Characterisation of marine alphaproteobacterium strain 5N and proposed classification within the genus *Labrenzia*

2.1 Abstract

The marine alphaproteobacterium, strain 5N, was isolated from suboxic waters of the northwest Arabian Sea. Strain 5N is of particular biogeochemical interest because it is closely related to a clade of denitrifiers associated with the colonial cyanobacterium, *Trichodesmium*. *Trichodesmium* is a globally important diazotroph that is estimated to contribute around 50% of the annual input of biologically fixed nitrogen to marine systems. The characterisation of strain 5N was undertaken to provide a first insight into the functional potential of the community of *Trichodesmium*-associated denitrifiers. A combination of phylogenetic, physiological and biochemical analyses was employed to identify the isolate taxonomically. 16S rRNA gene sequence analysis revealed the isolate is most closely affiliated with the genus *Labrenzia*, sharing the highest DNA sequence similarities with strains *Labrenzia aggregata* RMAR6-6 (100% identity) and *Labrenzia* sp. CP4 (100%). The high DNA–DNA hybridization values of 83.4–86.6% determined for these three strains, when compared to 33.4–38.5% between these strains and the highest scoring type strain, *Labrenzia aggregata* IAM12624, support their assignment to the same novel species. Strain 5N is a facultative denitrifier that utilizes a wide range of organic carbon sources to support growth aerobically but is incapable of fermentation. Growth rate is optimal at a pH of 8–8.5, a salinity of 2.5–3.5% (w/v), and at a temperature of 33°C. The DNA G+C content is 59 mol% while the fatty acids comprise C_{16:1} ω7c, C_{16:0}, 18:1 ω7c, C_{18:0}, 11-methyl C_{18:1} ω7c, C_{20:1} ω7c, C_{20:0} and the hydroxy fatty acids C_{14:0} 3-OH (ester linked) and C_{18:0} 3-OH. Polar lipids consist of phosphatidylglycerol, diphosphatidylglycerol, two glycolipids, phospholipid and two aminolipids. Based on phylogenetic analysis, *in silico* DNA–DNA hybridization estimates, and its phenotypic traits, it was determined that strain 5N belongs to a novel species within the genus *Labrenzia*, for which the provisional name *Labrenzia* sp. strain 5N is proposed. Furthermore it is suggested that

L. aggregata RMAR6-6 and *Labrenzia* sp. CP4, the closest relatives of strain 5N, should be reassigned to this same species group also.

2.2 Introduction

Labrenzia: Classification of a diverse genus comprising metabolically versatile, environmentally important species. Members of the *Labrenzia* genus have undergone a number of taxonomic reassignments in recent years as the sophistication of bacterial classification studies and techniques has progressed. First, two marine strains of the genus *Agrobacterium* originally classified as *A. stellulatum* were renamed *Stappia aggregata* and *Stappia stelulatta* to create the newly named *Stappia* genus (Uchino *et al.*, 1998). More recently, further characterisations of *Stappia* and related genera lead to the establishment of a new genus, *Labrenzia*. Three *Stappia* species were transferred to this genus and renamed *Labrenzia aggregata*, *Labrenzia alba* and *Labrenzia marina* (Biebl *et al.*, 2007). Traditionally classified as members of the Rhodobacterales, recent genome-wide phylogenies now place *Labrenzia* within the Rhizobiales (Hördt *et al.*, 2020).

Currently, the genus *Labrenzia* comprises six type species; *Labrenzia aggregata*, *L. alba*, *L. alexandrii*, *L. marina*, *L. salina* and *L. suaedae* (Biebl *et al.*, 2007; Bibi *et al.*, 2014; Camacho *et al.*, 2016). *Labrenzia* spp. are aerobic, marine alphaproteobacteria that have been isolated from benthic, planktonic or particle-associated habitats from coastal and open waters. *Labrenzia* spp. are Gram-negative, motile cells displaying white/cream coloured colonies on agar plates that may develop a deeper pinkish-brown colour when mature. They are chemoheterotrophs incapable of fermentation, though nitrate respiration is a trait found commonly among members of the genus. *Labrenzia* spp. are halophilic, with a preferred salinity between 1 and 10% NaCl (w/v). A number of strains have also been shown to be capable of the biosynthesis and degradation of dimethylsulfoniopropionate (DMSP), a precursor of the climatically important gas dimethyl sulfide (DMS) implicated in the promotion of cloud formation (Andrew R J Curson *et al.*, 2017a).

Characterisation of alphaproteobacterium strain 5N. The marine alphaproteobacterium strain 5N was isolated by enrichment culture of seawater

sampled from sub-oxic depths in the northwest Arabian Sea at a depth of around 120m and approximately 30 miles from shore (24.19N 58.17E) (Figure 2.1) (Wyman *et al.*, 2013). The strain was selected as a model for this study because it clusters with a major clade of epibiotic denitrifiers associated with the diazotrophic cyanobacterium, *Trichodesmium* (Coates and Wyman, 2017). The aim of the present study was to classify strain 5N using a polyphasic approach based on its phenotype, biochemistry, and phylogenetic comparisons with closely related species. The detailed characterisation of a representative of the *Trichodesmium*-associated denitrifying community will provide deeper knowledge of the physiological capacity of these epibionts.

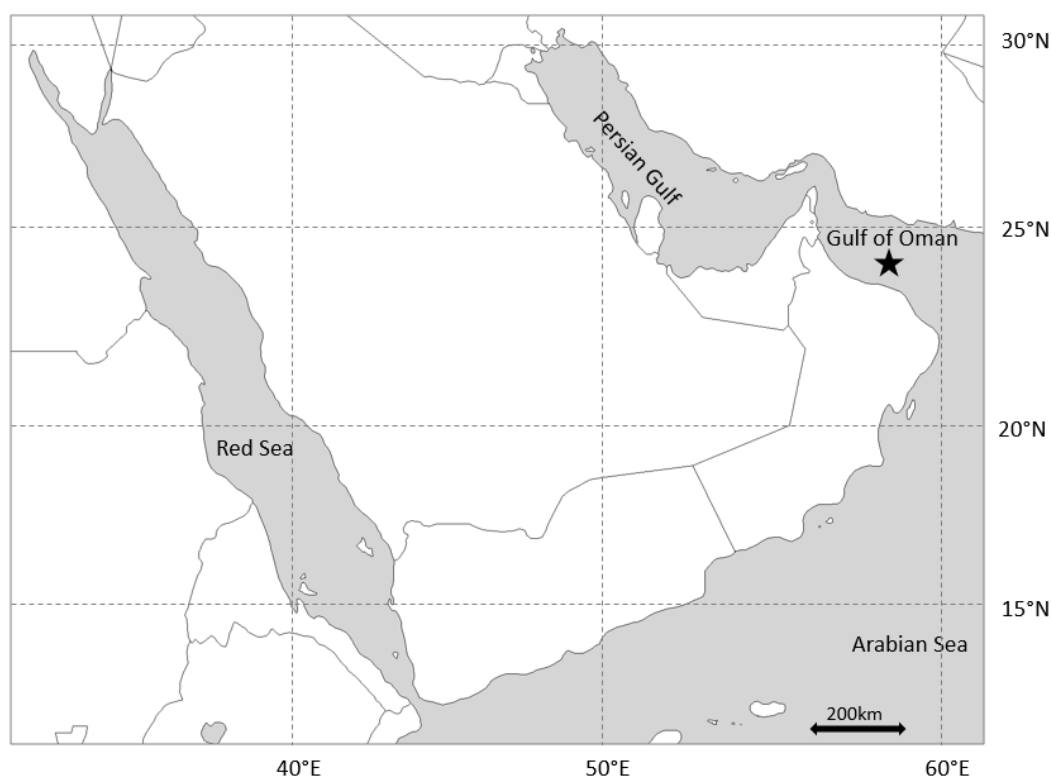


Figure 2.1. Geographical location of where seawater samples were previously collected for use in this study (24.19N 58.17E) (Wyman *et al.*, 2013).

A polyphasic approach to bacterial taxonomy. Bacterial taxonomy involves the identification, classification and nomenclature of a bacterium to establish its rightful place within the Prokaryotic kingdom (Cowan, 1965; Young *et al.*, 1992). The correct

classification of bacteria to species level is essential for all scientific applications including research, medicine and industry. Traditionally, bacterial taxonomic studies were reliant on classical microbiological methods for determining morphology, biochemistry and growth requirements. Bacterial classification is now based on a combination of phenotypic and genotypic characteristics. Since the development of the polymerase chain reaction (PCR) in the 1980's, phylogenies are frequently inferred using the 16S rRNA gene DNA sequence. However, this approach is reliant on a single conserved gene and has known limitations in resolution for the classification of Alphaproteobacteria (Hördt *et al.*, 2020). More recently, more precise techniques based on Multilocus Sequence Analyses (MLSA) were introduced to improve classification precision (Glaeser and Kämpfer, 2015). These days, the ease of availability of whole genome sequencing data enables more in-depth comparisons based on entire genomes as opposed to just a few hundred nucleotides. Although phenotypic characteristics and 16S rRNA gene sequencing still play an essential role in bacterial identification, modern phylogenomic methods have revealed many misclassifications of strains based solely on these traits (Mateo-Estrada *et al.*, 2019). As a result, a polyphasic approach is now considered necessary to confidently determine bacterial taxonomy (Prakash *et al.*, 2007; Tindall *et al.*, 2010; Bisen *et al.*, 2012). Where possible the more traditional phenotypic methods of taxonomic classification should be supplemented with genomic analyses, such as 16S rRNA gene phylogenies, DNA-DNA hybridisation and genome signatures, to improve confidence in identifications.

Phenotypic characteristics. Historically, examination of phenotypic characteristics was the only way to classify an unknown organism. These early studies focussed predominantly on bacteria, often facultative anaerobic heterotrophs, that could be easily cultivated within the laboratory at the time. The use of morphological characteristics also has a long history in the identification of bacteria. Observations of basic features including cell shape, size and motility contribute to the initial characterisation of an organism and in drawing comparisons between isolates. The Gram stain was first described in the 1880's and though since modified, the technique still remains a commonly used method of phenotypic classification based on bacterial cell wall characteristics (Gram and Friedlaender, 1884; Hucker, 1921; Steinbach and

Shetty, 2001). The ability of an organism to grow under different physical conditions, such as pH, temperature and oxygen concentration, are also useful traits by which to separate them into functional types (e.g. aerobe/anaerobe; acidophile/alkaliphile; mesophile/thermophile, etc.). The ability of strains to utilise different carbon and/or energy sources for growth can be accessed via metabolic tests. Traditionally carried in a series of individual test incubations, API test strips (Biomérieux) and similar small-scale systems are widely used today to assist in the identification of bacteria. Indicative biochemical and carbohydrate utilisation tests are selected based on characters such as Gram reaction and positive results are recorded by a colorimetric or other diagnostic change following incubation. Analyses show good correlation between API results and conventional, experimental methods at a larger scale (Lampe and Van der Reijden, 1984; Dance *et al.*, 1989; Lowe *et al.*, 2002).

Chemotaxonomic markers including fatty acids, respiratory quinones, lipids and polyamines are also useful analytical traits since their profiles differ between organisms and therefore aid identification (Gokdemir and Aras, 2019). The analysis of cellular fatty acid composition examines the types and relative amounts of fatty acids found within the cell membrane. Fatty acids are carboxyl acids with an aliphatic chain attached and they vary in terms of their saturation, branching, length, and number of carbon atoms. The profile of cell membrane polar lipids, commonly phospholipids, is also a useful tool for distinguishing between species as they often vary slightly between members of a genus (Gokdemir and Aras, 2019). A combination of gross (e.g. colony structure and colour) and microscopic morphology, metabolic capacity, growth requirements, chemotaxonomic markers and biochemistry provides an in-depth overview of the phenology of a strain. For all phenotypic traits, comparisons are often made with reference to type species, which relies on experimental accuracy and therefore standard protocols should be implemented to reduce analytical variability.

Genotypic characteristics: 16S rRNA gene sequencing, GC content analyses and DNA-DNA hybridisation. Since the first early classification studies based on DNA sequencing in the 1960's, the use of gene markers for bacterial identification has increased rapidly. The 16S rRNA gene is most commonly used to infer phylogenies as its essential nature and slow rate of mutation make it an ideal taxonomic marker gene. Following PCR amplification of the gene using universal primers, sequences

can be aligned and compared with those in public databases using software such as BLAST and CLUSTAL W. Phylogenies can then be constructed using, among others, Neighbour-Joining, Maximum Parsimony and Maximum Likelihood methods, to determine the new isolate's taxonomic positioning and relatedness with its neighbours. Traditionally, 16S rRNA gene sequence similarities of >97% and >95% have been used to justify that two organisms belong to the same species and genus, respectively (Stackebrandt and Goebel, 1994). However, the species threshold has been redefined more recently as 98.65% and this cut-off value likely varies depending on the genera being studied (Kim *et al.*, 2014; Rossi-Tamisier *et al.*, 2015). Particularly when 16S rRNA gene identities are above 97%, caution should be taken when inferring taxonomic relationships without performing additional complementary genotypic analyses (Fox *et al.*, 1992; Tindall *et al.*, 2010). 16S rRNA gene sequencing remains a fundamental technique in bacterial identification and should be used, where possible, as one component of a polyphasic approach to bacterial classification. This single tool is particularly useful, however, for inferring the taxonomic affiliations of non-culturable bacteria as pioneered first by Giovannoni *et al.* (1990), Torsvik *et al.* (1990), Ward *et al.* (1990) and Schmidt *et al.* (1991).

Comparisons of the DNA base composition of organisms can be used as indicators of their relatedness. The GC content, often shown as a percentage, represents the combined proportion of guanine and cytosine nucleotides in a given gene, region or entire genome. The GC content acts as a genomic 'signature' although intra-species variations in the composition of nitrogenous bases may be due to biases and selective pressures (Sueoka, 1988; Foerstner *et al.*, 2005; Lassalle *et al.*, 2015). Experimental techniques such as thermal denaturation, melting profiles and HPLC are indirect methods of GC content determination and thus are reliant on procedural accuracy (Marmur and Doty, 1962; Owen *et al.*, 1969; Mesbah *et al.*, 1989). The determination of GC content from genome sequencing data has been shown to be more reliable. Though a 3-5 mol% difference in the genomic GC content is traditionally used as a threshold for inter-species delineation, this difference was shown to be just 1% when using genome sequencing data (Mesbah *et al.*, 1989; Rosselló-Mora and Amann, 2001; Meier-Kolthoff *et al.*, 2014). It's important to remember that GC content differences are most useful as an indicator that two organisms are not in fact closely

related. Identical GC contents must be carefully interpreted alongside other data as they do not take into account the arrangement of these nucleotides within the genome.

Through DNA-DNA hybridisation (DDH) techniques, the degree of similarity between the DNA sequences of two organisms can be determined. The extent to which two genomes will hybridise with one another is a direct indication of their sequence similarity. *In vitro* DDH relies on labour intensive methods involving temperature-dependant DNA denaturation and subsequent reassociation. In recent years, advances in genome sequencing have led to the development of digital DDH (dDDH) techniques. Sequenced-based approaches are quickly replacing the wet-lab DDH methods as more advanced algorithms successfully and reproducibly emulate *in vitro* results. Whether determined experimentally or computationally, a DDH similarity of over 70% indicates that the bacteria belong to the same species (Moore *et al.*, 1987; Meier-Kolthoff *et al.*, 2013). Two commonly used methods of *in silico* DDH include Genome-to-Genome distance (GGD) (Henz *et al.*, 2004; Auch *et al.*, 2010a) and Average Nucleotide Identity (ANI) (Goris *et al.*, 2007). Average Nucleotide Identity (ANI) is calculated between conserved genes of two sequenced genomes. Genomes are partitioned into ~1000bp fragments which are queried against each other using BLASTN. High-scoring segment pairs sharing high levels of nucleotide similarity, are then used for ANI calculations. Results show a good correlation with *in vitro* DDH estimation and 16S rRNA gene similarities (Goris *et al.*, 2007; Rodriguez-R and Konstantinidis, 2014). The GGDC server calculates genome-to-genome distances (GGD) (Henz *et al.*, 2004; Auch *et al.*, 2010a). Auch *et al.* compared a number of approaches and algorithms for the calculation of GGD which led to the development of the GGDC server (Auch *et al.*, 2010a; Meier-Kolthoff *et al.*, 2013). dDDH calculations using the GGDC server are often used preferentially as they are considered one of the most accurate phylogenomic methods and have been shown to outperform ANI (Meier-Kolthoff *et al.*, 2013).

2.3 Materials and methods

2.3.1 Metabolic, physiological and biochemical characterization of Alphaproteobacterium strain 5N

General culture conditions. Alphaproteobacterium strain 5N was isolated from suboxic intermediate waters (120m) in the northwest Arabian Sea (Figure 2.1) (Wyman *et al.*, 2013). Strain 5N was grown routinely in liquid culture at 33°C in an orbital incubator (200 rpm) in ASW:LB (3:1); a modification of ASW medium (Wyman *et al.*, 1985) amended with 25 % (vol/vol) Luria-Bertani medium (10 g.L⁻¹ tryptone, 5 g.L⁻¹ yeast extract, 10 g.L⁻¹ NaCl), 1 ml.L⁻¹ A5 trace metal solution (Stanier *et al.*, 1971) and 0.5 ml L⁻¹ f/2 vitamin solution (Guillard and Ryther, 1962) (Appendix Tables 8.11 and 8.12).

Anaerobic culture conditions. For all fermentation tests the growth medium consisted of standard ASW medium amended with 1 g.L⁻¹ ammonium chloride, 1 g.L⁻¹ yeast extract, 2 g.L⁻¹ carbon source, 1 ml.L⁻¹ A5 trace metal solution (Stanier *et al.*, 1971) and 0.5 ml L⁻¹ f/2 vitamin solution (Guillard and Ryther, 1962). Denitrification medium consisted of standard ASW medium amended with 1 g.L⁻¹ ammonium chloride, 1 g.L⁻¹ yeast extract, 2 g.L⁻¹ sodium acetate, 2 g.L⁻¹ sodium nitrate, 1 ml.L⁻¹ A5 trace metal solution (Stanier *et al.*, 1971) and 0.5 ml L⁻¹ f/2 vitamin solution (Guillard and Ryther, 1962). 0.5 mg.L⁻¹ Resazurin (sodium salt, 0.5 mg.L⁻¹) was added as a colorimetric redox indicator in addition to 0.1 g.L⁻¹ sodium thioglycolate as a reducing agent to remove residual oxygen. Bacto Agar (Difco) at a final concentration of 10 g.L⁻¹ was added to media when required. For both fermentation and denitrification tests, the sterile growth medium was degassed by boiling and, once cooled, dispensed aseptically into Universal or Bijou glass bottles with lined screw caps. Bottles were filled close to the brim, inoculated with 200µL overnight culture, sealed tightly without delay, and incubated at 33 °C for 48 hours. For all anaerobic tests, a Durham tube was submerged in the medium to capture any gases generated through metabolic processes.

Aerobic controls were performed alongside all tests. A control anaerobic culture, with acetate as the sole carbon source but no added nitrate, was used to establish the extent of any aerobic respiration using residual dissolved oxygen. As acetate cannot be further fermented, any growth could not result from fermentation but would indicate

aerobic respiration of the carbon source using oxygen remaining in the medium. Growth on any residual oxygen following degassing and inoculation was negligible in all cases.

PCR amplification of *nosZ* from alphaproteobacterium strain 5N. Genomic DNA from a clonal culture of strain 5N was isolated using the ZR fungal/bacterial DNA miniprep™ kit according to the suppliers' recommendations (Zymo Research Corp., Irvine, California, USA). A PCR was carried out on genomic DNA to confirm the presence of *nosZ*, a gene indicative of the strains capacity to fully denitrify. 1µl purified DNA (diluted 1:100) was added to 12.5 µl of 2 × MyTaq™ Red Mix (Bioline reagents, London, UK) amended with 50pmol forward and reverse primer mix (StanosF and StanosR, Appendix Table 8.1) (Wyman *et al.*, 2013) and diluted to a final volume of 25µl with sterile dH₂O. PCR thermocycling conditions were as follows: initial denaturation at 95 °C for 2 min followed by 30 amplification cycles of a 94 °C denaturation step for 30 s, a 30 s annealing step at 52 °C and 72°C extension for 45 s and concluding with a final extension step for 10 min at 72°C. Following amplification, the presence of the 252bp PCR product expected was investigated by resolution on a 1% (w/vol) agarose gel stained with 0.5 µg.mL⁻¹ ethidium bromide.

Structural, physiological and biochemical characterization of alphaproteobacterium strain 5N. Gram staining was carried out using standard microbiological techniques. Motility was investigated by the hanging drop microscopic technique using a magnification of x 400. API 20 NE (NE = Non-Enteric) test strips (BioMérieux, Durham, USA) were used to determine biochemical characteristics. A single colony from a fresh agar plate was resuspended in 2 mL 2.5 % (wt:vol) sterile, saline solution to an OD₆₀₀ of 0.08. An aliquot (200 µL) of the cell suspension was added to an ampule of AUX medium (BioMérieux, Durham, USA) and the diluted suspension was used to inoculate the API strip following the suppliers' recommendations. Tests were incubated at 31 °C and results recorded after both 24 and 48 hours.

General metabolic requirements were determined through growth experiments. To test the capacity of strain 5N to use different carbon substrates, replicate aerobic cultures (n = 3) were grown at 33°C in an orbital incubator (200 r.p.m) in 30 mL nitrate-free ASW medium amended with 2.26 g.L⁻¹ NH₄Cl, 0.5 g.L⁻¹ yeast extract, 2 g.L⁻¹ of the

carbon (and energy) source under test. Prior to inoculation, the cell pellet was washed once in nitrate-free ASW to remove residual medium. To test the capacity to use various nitrogen sources, replicate cultures were grown in 30 ml nitrate-free ASW amended with 2 g.L⁻¹ of the N-source under test, 0.5 g.L⁻¹ yeast extract, 2 g.L⁻¹ sodium acetate and examined for growth after 24 hours. Growth was measured turbidometrically at 600 nm. Following these growth experiments, the carbohydrate metabolism growth tests were complemented with API 50CH test strips (BioMérieux, Durham, USA) to test for the utilization of a wider range of substrates.

Fatty acid and polar lipids analysis. The fatty acid and polar lipid profiles of strain 5N were analysed using cells harvested from cultures grown in ASW:LB (3:1) at 33°C. Cells were collected at an OD₆₀₀ of 1 by centrifugation of 50 mls of culture suspension and the pellet obtained was freeze dried. Cellular fatty acid extraction and analysis was performed using the Sherlock MIS (MIDI Inc, Newark, USA) system by the Identification Service at DSMZ, Braunschweig, Germany. Polar lipid extraction and analysis was also performed by the same laboratory using protocols modified from Bligh and Dyer (1959), and separated by two dimensional silica gel thin layer chromatography (Macherey-Nagel Art. No. 818 135).

Southern blot detection of *cbbL*, the gene encoding ribulose-1,5-bisphosphate carboxylase large subunit. Restriction digests were performed with 0.5 µg genomic DNA from alphaproteobacterium strain 5N and its close relative, *Labrenzia aggregata* strain IAM12614, using 0.5 units of each of the following enzymes: EcoRI, XbaI, XhoI, HindIII, PstI, KpnI, and BamHI. After digestion at 37 °C for one hour, digests were resolved on a 1% (w/vol) agarose gel alongside uncut genomic DNA and lambda HindIII markers. The gel was washed in 0.25M HCl on a rotating platform for 30 min, rinsed with dH₂O and then equilibrated with denaturation solution (1.5M NaCl, 0.5M NaOH) for 2 x 15 mins before two further washes of 20mins each in neutralisation solution (1.5M NaCl, 0.5M TrisHCl pH7). The denatured DNA was transferred to a positively-charged nylon membrane by Southern blotting using a 10x SSC transfer buffer. The membrane was air-dried and fixed with UV (302 nm) for 15 sec using a transilluminator. The *cbbL* probe was generated by PCR using purified DNA from the alphaproteobacterium, *Rhodobacter sphaeroides*, in combination with the Form1CF and Form1CR primers (Appendix Table 8.1) and labelled during amplification with digoxigenin-dUTP using the DIG High Prime probe synthesis kit (Roche Diagnostics

Ltd). The blot was hybridised overnight at 65°C in pre-warmed hybridization buffer (5xSSC, 0.1%N-lauroylsarcosine, 0.02% SDS, 1% blocking reagent) containing 25 ng.mL⁻¹ probe (denatured for 10 min at 98°C). The blot was washed twice in a low stringency solution (2x SSC, 0.1% SDS) at room temperature and twice in a high stringency wash (0.5x SSC, 0.1% SDS) at 68°C for 30 min each. Probe hybridization was detected with 150 mU.mL⁻¹ anti-digoxigenin-AP FAB fragments (Roche Diagnostics Ltd) and the chromogenic alkaline phosphatase reporter substrates NBT/BCIP following the suppliers' recommendations.

2.3.2 Phylogenetic and taxonomic analyses

The closely related 16S rRNA gene from the alphaproteobacterium strain 4N (>99.7 % identity to strain 5N) was amplified and sequenced previously by Wyman *et al.* (2013). The 1499bp sequence was aligned with that of strain 5N and compared with 16S rRNA sequences available from the GenBank database (NCBI Coordinators 2017). Multiple sequence alignment with closely related species was carried out using ClustalΩ (Sievers *et al.*, 2011) and phylogenetic trees were generated using MEGA10 using maximum likelihood (Kumar *et al.*, 2017).

Whole genome sequencing of strain 5N was performed and is described in greater detail in Chapter 3. The draft genome is 5.9Mbp in length and the entire sequence dataset was used in the present investigation for whole genome DNA-DNA hybridisation, calculation of the G+C content, and in searches for the presence of genes characteristic of members of the genus, *Labrenzia*, including the *cox* and *puf* gene clusters. *In silico* DNA-DNA hybridization (DDH) was performed using the GGDC web server algorithms (Auch *et al.*, 2010b; Meier-Kolthoff *et al.*, 2013). The commonly used DDH estimate of >70% identity was selected as the threshold for species delineation. Intergenomic distances were calculated using formula 2 from the GGDC software, that estimates the DDH value by dividing the sum of all identities found in high-scoring segment pairs (HSPs) by the HSP length. This formula was selected as it does not take genome length into account when calculating intergenomic distances. Therefore, it is the most suitable analytical protocol for use with draft genomes. Average nucleotide identity (ANI) was also assessed, via the online ANI calculator, using reciprocal best hits (2-way ANI) between the genomes (Goris *et al.*, 2007;

Rodriguez-R and Konstantinidis, 2016). ANI values exceeding 95% indicate that the two genomes compared share sufficient overall similarity to come from members of the same species.

2.4 Results and Discussion

2.4.1 Morphological, physiological and biochemical characteristics of alphaproteobacterium strain 5N.

Selected characteristics of strain 5N were compared with the six taxonomically-described type strains of the genus *Labrenzia*; *Labrenzia aggregata* strain IAM12614, *L. suaedae* YC6927^T, *L. alexandrii* strain DFL-11^T, *L. marina* KCTC 12288^T, *L. alba* CECT5095 and *L. salina* Cs25^T (Table 2.1). Strain 5N produces Gram negative, rod shaped, motile cells. Young colonies are cream in colour, while more mature colonies develop a pinkish brown centre after a few days' incubation. Strain 5N is halophilic with an obligate requirement for NaCl and an optimal salt concentration for growth in the range 2.5 – 3.5% (w/v). The optimal growth temperature for strain 5N is 33 °C (range 15 – 40 °C). The analysis of genome sequencing data for strain 5N (Chapter 3) revealed this strain does not harbour copies of the photosynthetic reaction centre genes *pufLM* or bacteriochlorophyll BChla genes variably present among members of the genus (Biebl *et al.*, 2007). However, the genome contains copies of the *cox* genes required for CO oxidation (discussed in more detail in Chapter 3) as do all the type strains of *Labrenzia* (Weber and King, 2007).

The strain was able to grow aerobically using a broad range of carbon and nitrogen sources. Strain 5N grew aerobically using acetate, D-adanitol, L-arabinose, D-arabitol, arbutine, butyrate, D-cellobiose, citrate, erytol, esculin, D-fructose, L-fructose, galactose, gentiobiose, 2-keto-gluconate, 5-keto-gluconate, methyl- α -D-glucopyranoside, glucose, glutamate, glutamine, glycerol, inositol, D-maltose, mannitol, methyl- α -D-manopyranoside, manose, D-melibiose, potassium gluconate, proline, pyruvate, rhamnose, ribose, salicin, sorbitol, succinate, D-trehalose, D-turanose, D-xylose and D-zaharose as sole carbon sources. The strain did not show growth on either ascorbate or malate or when an organic C-source was not provided. Ribulose-1,5-bisphosphate carboxylase large subunit, *cbbL*, was not detected in strain 5N. Strain 5N could utilise all of the N sources investigated, including ammonium, urea, nitrate, nitrite, proline, glutamate and glutamine as sole nitrogen sources.

Anaerobic growth was tested under both fermentative and denitrifying conditions. None of the carbon sources tested supported fermentation, confirming that this organism is incapable of fermentative growth. After 48 hours, the denitrification tests were positive for all the carbon sources that supported growth under aerobic

conditions. Gas produced via the denitrification pathway accumulated in Durham tubes placed in the vials. These tests confirm that this organism is capable of growth under anaerobic conditions only when nitrate (and, most likely, other Nox species) is provided in addition to a carbon source. Thus, this organism is a non-fermentative, heterotrophic, denitrifier.

API 20NE strip results showed the strain was incapable of indole production from tryptophan, arginine dihydrolase activity, and confirmed the absence of glucose fermentation (Table 2.1). Results were positive for protease, urease, β -galactosidase and β -glucosidase activity. The strain was also oxidase positive and could reduce nitrate beyond nitrite to gaseous end products. The presence of *nosZ*, encoding nitrous oxide reductase (NosZ), the last step in the complete denitrification pathway, was confirmed by PCR (Figure 2.2). Earlier work showed that N₂O accumulates in the growth medium (and the overlying atmosphere) when the co-isolate, strain 4N, is incubated under denitrifying conditions in the presence of ethene (acetylene, C₂H₂); an inhibitor of NosZ (Wyman *et al.*, 2013). Both N₂O and N₂ were detected during stable isotope experiments with strain 5N using ¹⁵NO₃⁻ under denitrifying growth conditions (see Chapter 5) confirming that, like strain 4N, it is a complete denitrifier.

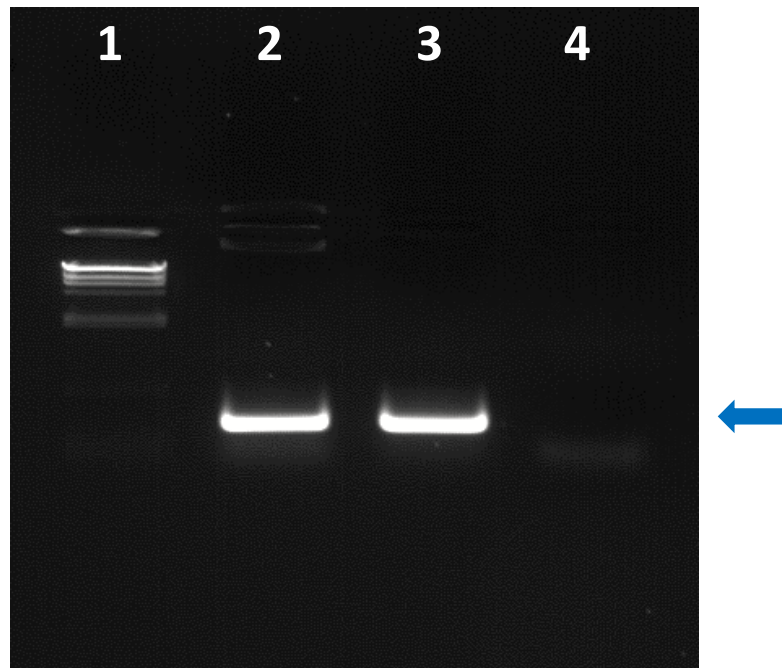


Figure 2.2. Analytical agarose gel of a PCR targeting *nosZ* in DNA extracted from strain 5N. Lane 1, Lambda *Hind*III ladder, Lanes 2 and 3, strain 5N; Lane 4, negative control. The arrow indicates the expected position (Rf) of the 252 bp target PCR product.

Table 2.1. Characteristics of alphaproteobacterium strain 5N compared with the type strains of the *Labrenzia* genus.

Strains: **1**, Strain 5N; **2**, *L. aggregata* strain IAM12614; **3**, *L. suaedae* YC6927^T; **4**, *L. alexandrii* strain DFL-11^T; **5**, *L. marina* KCTC 12288^T; **6**, *L. alba* CECT5095; **7**, *L. salina* Cs25^T. Data from: strain 5N, this study; a, Bibi *et al.*, 2014; b, Uchino *et al.*, 1998; c, Biebl *et al.*, 2007; d, Kim *et al.*, 2006; e, Pujalte *et al.*, 2005; f, Camacho *et al.*, 2016. +, positive; -, negative; BChl a, bacteriochlorophyll a; *coxL*, carbon monoxide dehydrogenase large subunit gene; ND, no data.

Characteristic	1 Strain 5N	2 <i>L. aggregata</i> ^{abc}	3 <i>L. suaedae</i> ^a	4 <i>L. alexandrii</i> ^{ac}	5 <i>L. marina</i> ^{acd}	6 <i>L. alba</i> ^{ace}	7 <i>L. salina</i> ^f
Isolation	Seawater	Marine sediment	Halophyte	Dinoflagellate	Seawater	Oyster	Halophyte
Colony appearance	Cream	White/cream	Cream	Pale pink	Cream	White	Cream
Temperature range (°C)	15-40	15-37	15-37	15-37	15-40	13-28	15-40
Salinity range (% NaCl, w/v)	0.5-7 %	0.3-6 %	0-9 %	1-10 %	1-7 %	1-8 %	1-11 %
Reduction of nitrate to nitrite	+	+	+	-	+	+	-
Reduction of nitrate to N ₂	+	+	-	-	-	+	-
Indole production	-	-	+	-	-	-	ND
Urease	+	+	-	-	-	-	ND
<i>pufLM</i> genes	-	-	+	+	+	ND	-
BChl a	-	-	-	801 and 865nm	ND	-	+
<i>coxL</i> genes	+	+	+	+	+	+	+
DNA G+C content (%)	59%	59%	58.50%	56%	60%	59%	62.40%
16S rRNA gene similarity to Strain 5N		99.7%	97.3%	97.7%	98.5%	98.6%	98.5%

2.4.2 Cellular fatty acids and polar lipids analysis

The cellular fatty acid profile of strain 5N was compared with that of the *Labrenzia* type strains *L. alba* CECT5095, *L. aggregata* IAM12614, *L. alexandrii* strain DFL-11^T, *L. marina* KCTC 12288^T, *L. salina* Cs25^T and *L. suaedae* YC6927^T (Table 2.2). The fatty acids of strain 5N comprise C_{16:1} ω7c, C_{16:0}, 18:1 ω7c, C_{18:0}, 11-methyl C_{18:1} ω7c, C_{20:1} ω7c, C_{20:0} and the hydroxy fatty acids C_{14:0} 3-OH (ester linked) and C_{18:0} 3-OH which is consistent with the typical profile reported for the *Labrenzia* genus (Biebl *et al.*, 2007). The unsaturated fatty acid C_{18:1} ω7c, annotated as part of summed feature 8 (C_{18:1} ω7c and/or C_{18:1} ω6c), was the most abundant (79.6%), as it is in other members of the Alphaproteobacteria. Summed feature 2 (iso-C_{16:1} I and/or C_{14:0} 3-OH; 2.98%), C_{18:0} (8.21%), C_{20:1} ω7c (3.11%) and 11-Methyl C_{18:1} ω7c (2.46%) were also present in readily detected amounts. Previous fatty acid analyses of members of the *Labrenzia* genus have shown some inconsistency in the annotation of the latter unsaturated fatty acid, which is recorded as either 11-methyl C_{18:1} ω7c (Camacho *et al.*, 2016; Bibi *et al.*, 2014; this study) or 11-methyl C_{18:1} ω6t (Biebl *et al.*, 2007).

Table 2.2. Cellular fatty acid composition of alphaproteobacterium strain 5N and type strains of the genus *Labrenzia*.

Strains: **1**, strain 5N; **2**, *L. aggregata* IAM12614; **3**, *L. alba* CECT5095; **4**, *L. alexandrii* strain DFL-11^T; **5**, *L. marina* KCTC 12288^T; **6**, *L. salina* Cs25^T; **7**, *L. suaedae* YC6927^T. Data shown as the percentage of total cellular fatty acids. TR, Trace (<1%); -, not detected. Summed features are groups of fatty acids that cannot be easily separated and are treated as being combined. Summed feature 2, iso-C_{16:1} I and/or C_{14:0} 3-OH; summed feature 3, C_{16:1} v7c and/or C_{16:1} ω6c; summed feature 8, C_{18:1} ω7c and/or C_{18:1} ω6c. Data is shown as mean values ±SD of two determinations for this study and that of Camacho *et al.* (2016).

Fatty acid	1 Strain 5N	2 <i>L. aggregata</i>	3 <i>L. alba</i>	4 <i>L. alexandrii</i>	5 <i>L. marina</i>	6 <i>L. salina</i>	7 <i>L. suaedae</i>
C _{10:0}		-	-	-	TR		-
C _{12:0}	0.34±0.0	-	TR	-	2.1		-
C _{16:0}	1.185±0.01	0.7	1.6	-	2	TR	2.3
C _{18:0}	8.165±0.06	5.6	7.4	4.1	7.6	7.6±0.1	2.7
C _{16:0} N-alcohol	-	-	-	3	2		-
C _{20:1} ω7c	3.095±0.02	4.7	6.8	12.9	5.2	11.2±0.3	-
C _{18:1} ω9c	-	-	-	1.2	-		-
11-Methyl C _{18:1} ω6t	-	15.2	-	-	-		-
11-Methyl C _{18:1} ω7c	2.445±0.02	-	3.5	7.8	11.2	19.0±0.0	10.4
C _{18:0} 3-OH	1.055±0.0	2.3	-	-	2.3	TR	8.1
C _{19:0} cyclo	-	-	-	-	-	-	-
C _{19:0} cyclo ω8c	-	-	-	-	6.1	2.8±0.1	-
Summed features:							
2	2.965±0.02	2.4	7.2	11.7	4.9	2.3±0.2	11.6
3	0.36±0.0	0.6	-	-	10.1	-	-
8	79.175±0.60	76.6	72.6	61.5	46.1		65
Data source	This study	Biebl <i>et al.</i> , 2007	Bibi <i>et al.</i> , 2014	Bibi <i>et al.</i> , 2014	Bibi <i>et al.</i> , 2014	Camacho <i>et al.</i> , 2016	Bibi <i>et al.</i> , 2014

The polar lipid profile of strain 5N revealed the presence of phosphatidylglycerol (PG), diphosphatidylglycerol (DPG), two glycolipids (GL), phospholipid (PL) and two aminolipids (AL) (Figure 2.3). The results obtained highlight some differences between strain 5N and the profile of members of the *Labrenzia* genus that have been analysed to date. Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) (or phosphatidylmonomethylethanolamine, PMME) were not detected although the presence of the latter may be masked and indistinguishable from other PLs by thin layer chromatography (Biebl *et al.*, 2007; Bibi *et al.*, 2014). Sulphoquinovosyldiacylglyceride, which occurs in some members of the *Labrenzia* genus (Bibi *et al.*, 2014), was not detected in the profile of strain 5N.

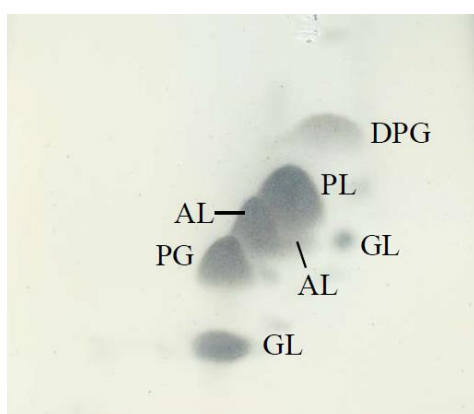


Figure 2.3. Polar lipids of alphaproteobacterium strain 5N. AL, aminolipid; DPG, diphosphatidylglycerol; PG, phosphatidylglycerol; GL, glycolipid; PL, phospholipid.

2.4.3 Taxonomic positioning of alphaproteobacterium strain 5N.

16S rRNA analysis. The entire 1499bp 16S rRNA small subunit gene sequence of strain 5N was used to interrogate the NCBI database using BLASTN default settings. Among the top 100 hits, 61% of the matches belonged to the *Labrenzia* genus, 10% to *Stappia* and the remainder (19 %) were from uncharacterised isolates or uncultured bacteria. The 16S rRNA gene was aligned and compared with that from the type strains of each of the six recognised *Labrenzia* species. The 16S sequence from strain 5N showed an identity of 99.7% with *L. aggregata* IAM12614, 97.3% with *L. suaedae* YC6927^T, 97.7% with *L. alexandrii* strain DFL-11^T, 98.6% with *L. marina* KCTC 12288^T, 98.5% with *L. alba* CECT5095 and 98.5% *L. salina* Cs25^T.

Alphaproteobacterium strain 5N, therefore, appears to be most closely related to the type strain, *L. aggregata* IAM12614. Across all other *Labrenzia* species sequenced to date and entered in the databases (June 2020), the highest 16S rRNA gene nucleotide identity was found with the strains designated *L. aggregata* RMAR6-6 (100%) and *Labrenzia* sp. CP4 (100%). The neighbour-joining tree shows the positioning of strain 5N within the genus *Labrenzia* and related to other Alphaproteobacteria (Figure 2.4). Strain 5N clearly clusters with other members of the *Labrenzia* genus, however the resolution is very low among members of the genus and this highlights the necessity for supplementary analyses to correctly classify this strain.

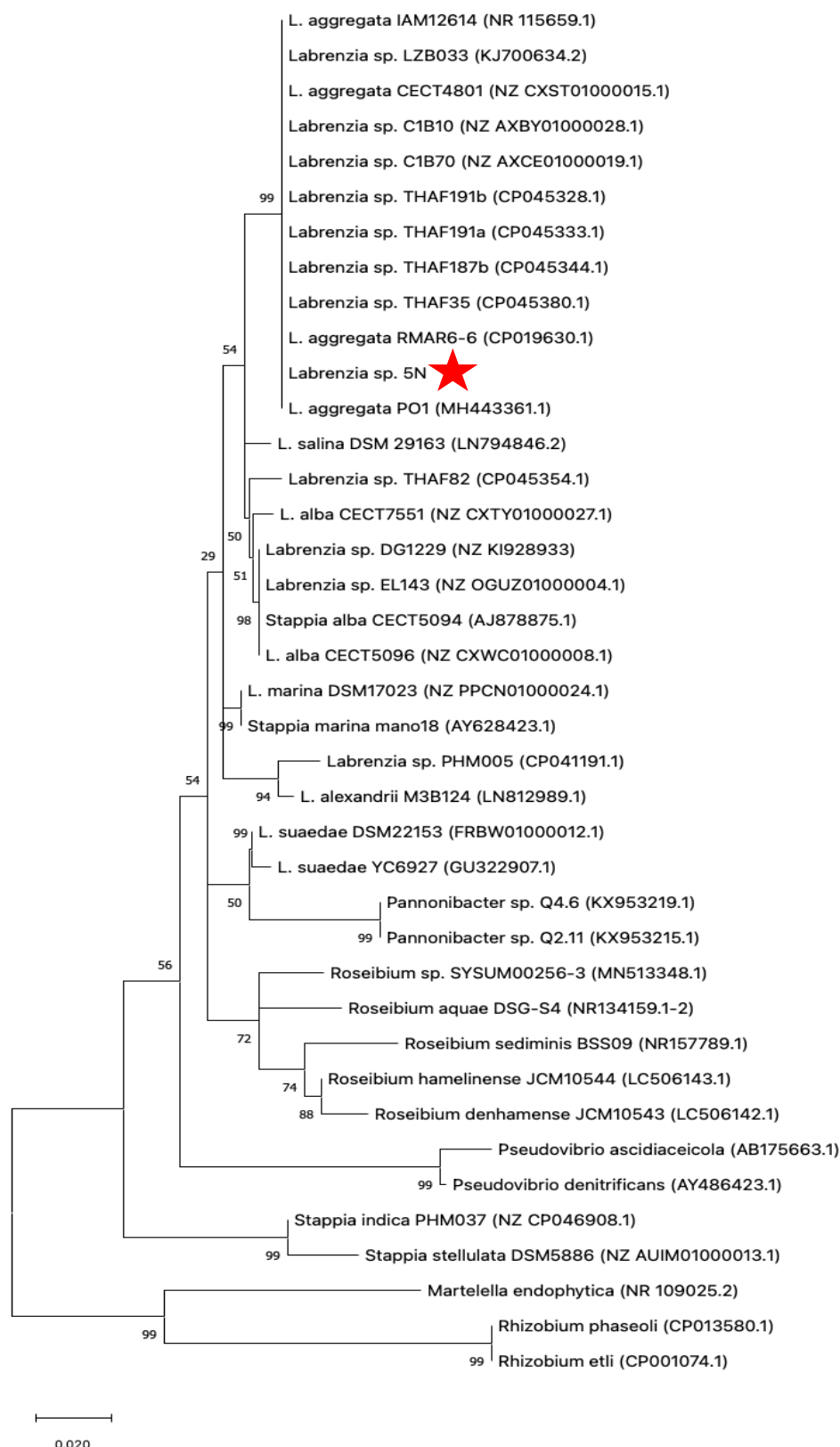


Figure 2.4. Evolutionary analysis of marine alphaproteobacterium strain 5N 16S rRNA and *Labrenzia* type strains and related species. The positioning of strain 5N is indicated by a star. Bootstrap support values from 1000 Maximum likelihood bootstrap replicates are shown as percentages. NCBI accession numbers are provided in brackets following the species name.

Whole genome phylogenetic analysis. 16S rRNA sequences were highly similar to strain 5N for many of *Labrenzia* species analysed and therefore additional molecular analyses were required to confidently assign this strain taxonomically at the species level. Whole genome sequence similarity via *in silico* digital DNA-DNA hybridization (dDDH) and average nucleotide identity (ANI) were calculated using the genome sequence data for this strain (Chapter 3). Whole genome comparisons with the six *Labrenzia* species type strains by dDDH estimates and ANI analyses both indicate that strain 5N does not belong to any of the currently recognised species (Supplementary Tables 8.2 and 8.3). dDDH similarities between strain 5N and the type strains *L. aggregata* IAM12614, *L. alexandrii* strain DFL-11^T, *L. alba* CECT5095, *L. marina* KCTC 12288^T and *L. suaedae* YC6927^T were 38.5% (± 2.5), 25.9% (± 2.5), 25.2% (± 2.5), 26.5% (± 2.5) and 23.4% (± 2.5) respectively. These dDDH values are significantly lower than the 70% threshold conventionally used to delineate species. Similarly, ANI comparisons with strain 5N were also below the 95% species delineation threshold for *L. aggregata* IAM12614 (Two-way ANI: 87.30%, SD: 4.14%), *L. alexandrii* strain DFL-11^T (Two-way ANI: 79.83%, SD: 4.79%), *L. alba* CECT5095 (Two-way ANI: 79.47%, SD: 4.24%), *L. marina* KCTC 12288^T (Two-way ANI: 80.92%, SD: 4.31%) and *L. suaedae* YC6927^T (Two-way ANI: 79.18%, SD: 4.56%). Whole genome sequence data was not available for the type strain, *L. salina* Cs25^T. However, the low 16S rRNA identity between strain 5N and *L. salina* Cs25^T (98.5%), 3.4% difference in GC content (Table 2.1), distinct fatty acid profiles (Table 2.2) and the inability *L. salina* Cs25^T to denitrify all suggest strain 5N does not belong to this species either.

However, the genome of strain 5N showed high similarity with the two strains with which it shares highest 16S rRNA identity; *L. aggregata* RMAR6-6 (DDH: 86.60% [84 - 88.8%]; Two-way ANI: 98.12%, SD: 1.40%) and *L. sp.* CP4 (DDH: 83.40% [80.6 - 85.9%]; Two-way ANI: 97.64%, SD: 1.36%) (Appendix Tables 8.2 and 8.3). Results suggest that these three organisms all belong to the same novel species, since, in contrast to the *Labrenzia* type species, their DDH values are all greater than 70%. *L. aggregata* RMAR6-6 and *L. sp.* CP4 have previously been suggested to belong to the same species (Kačar *et al.*, 2019). DDH estimates also suggest *Labrenzia aggregata* RMAR6-6 has been misassigned to the species *L. aggregata* despite being named in the NCBI database as such.

2.5 Conclusions

This study aimed to provide a comprehensive characterisation of alphaproteobacterium strain 5N and to classify this Arabian sea isolate. The Arabian Sea is an area of intense denitrification, responsible for approximately 10% of global marine N losses (Fauzi *et al.*, 1993). *Labrenzia*-type denitrifying bacteria, closely related to the model strain used in this study, have a widespread distribution throughout the Arabian Sea and are not restricted to the OMZ (Wyman *et al.*, 2013). *Labrenzia*-type organisms are estimated to make up to two thirds of the overall community of denitrifiers at some depths (Ward *et al.*, 2009; Lam *et al.*, 2011; Wyman *et al.*, 2013). Knowledge of these organisms and their metabolic capacity will enhance understanding of their contribution to biogeochemical and nutrient cycling in these waters.

The phylogenetic, biochemical and morphological characteristics of alphaproteobacterium strain 5N support the classification of this isolate within the *Labrenzia* genus. *Labrenzia* spp. are traditionally members of the family *Rhodobacteraceae*, order Rhodobacterales and class Alphaproteobacteria. Recent genome-wide phylogenies have however proposed *Labrenzia* species should instead be re-named as members of the genus *Roseibium* cob. Nov., which would be placed within the newly described Stappiaceae family as part of the order Rhizobiales (Hördt *et al.*, 2020). Strain 5N exhibits key traits characteristic of *Labrenzia* spp. including an optimal growth pH of 8-8.5, optimal salinity of around 2.5% and an obligate requirement for NaCl. Strain 5N is a halophytic, motile, facultative anaerobe, incapable of fermentation. API 20NE strip results showed that the strain is incapable of indole production from tryptophan, glucose fermentation and arginine dihydrolase activity. Results were positive for protease, urease, β -galactosidase, oxidase and β -glucosidase activity. The G+C content of strain 5N is within the 56-60 mol% expected for *Labrenzia* spp. (Biebl *et al.*, 2007). The strain can reduce nitrate fully to N₂ while the presence of *coxLMS* genes further suggest that this strain has the ability to oxidise carbon monoxide, as can all the type strains of *Labrenzia* (Weber and King, 2007). The genome of strain 5N does not contain the photosynthetic reaction centre genes *pufLM* genes, bacteriochlorophylls or *cbbL* that are of more variable distribution within the *Labrenzia* genus (Biebl *et al.*, 2007).

Proposed classification of strain 5N as a novel species of *Labrenzia*. The combination of phylogenetic, physiological, biochemical, fatty acid and lipid data suggest that strain 5N represents a novel species within the *Labrenzia* genus and for which we propose the name *Labrenzia* sp. strain 5N. Considering their low DNA-DNA relatedness with any of the described *Labrenzia* type strains we also suggest that *L. aggregata* RMAR6-6 and *Labrenzia* sp. CP4, the closest relatives of strain 5N, should be included in this species group. Details of this preliminary classification, the isolation data and general features of *Labrenzia* sp. strain 5N, are presented in Table 2.3 following MIGS recommendations (Field *et al.*, 2008).

Table 2.3. Classification and general features of *Labrenzia* sp. strain 5N according to the MIGS recommendations (Field *et al.*, 2008). Evidence codes used are taken from the Gene Ontology codes (Ashburner *et al.*, 2000). TAS: traceable author statement (literature source stated); NAS: non-traceable author statement (widely accepted trait); IDA: Inferred from direct assay.

MIGS-ID	Property	Description	Evidence code
MIGS-2	Classification	Domain <i>Bacteria</i>	
		Phylum <i>Proteobacteria</i>	TAS (Bibi <i>et al.</i> , 2014)
		Class <i>Alphaproteobacteria</i>	TAS (Bibi <i>et al.</i> , 2014)
		Family <i>Rhodobacteraceae</i>	TAS (Bibi <i>et al.</i> , 2014)
		Genus <i>Labrenzia</i>	TAS (Biebl <i>et al.</i> 2007; Bibi <i>et al.</i> 2014)
	Gram stain	Negative	IDA
	Cell shape	Rod	IDA
	Motility	Motile	
MIGS-4	Geographic Location	Arabian Sea	TAS (Wyman <i>et al.</i> , 2013)
4.1	Latitude	24.19 N	
4.2	Longitude	58.17 E	
4.3	Depth		
MIGS-5	Sample collection	2001	
MIGS-6	Habitat	Marine	
6.1	temperature	33°C	IDA
6.2	pH optimum	8-8.5	IDA
6.3	Salinity optimum	2.5% NaCl (w/v)	IDA
MIGS-15	Biotic Relationship	Free-living and with host	IDA/NAS
MIGS-19	Trophic Level	Chemoheterotroph	NAS
MIGS-22	Oxygen requirement	Facultative anaerobe	IDA

Chapter 3:

Whole genome analysis of *Labrenzia* sp. strain 5N reveals insights into the strain's metabolic potential and unveils the wider distribution of “denitrification plasmids” across the genus

3.1 Abstract

Labrenzia sp. strain 5N is a non-fermentative, biofilm-forming, alphaproteobacterium isolated by enrichment culture of seawater sampled from sub-oxic depths in the northwest Arabian Sea. It was selected for genome analysis as a representative of a major clade of denitrifiers associated with the diazotrophic cyanobacterium, *Trichodesmium*: the major source of biologically-fixed nitrogen in the tropical/subtropical oceans. Whole Genome Sequencing was performed to provide insights into the metabolic potential of its epibiotic denitrifying community, with a particular focus on N cycling potential. Genome sequencing was performed on an Illumina MiSeq system using a 250bp paired end protocol. The *Labrenzia* sp. strain 5N genome consists of a single circular chromosome (5.9 Mb) and two putative mega-plasmids (approximately 410 kb and 135 kb). It has an overall GC-content of 59%, encoding 6133 predicted proteins, 3 rRNAs and 48 tRNAs. The strain has the genetic potential to degrade a range of aromatic compounds, oxidise carbon monoxide, assimilate and synthesise the osmolyte DMSP, and is capable of the full denitrification of nitrate to dinitrogen gas. The denitrification genes of strain 5N are distributed between the chromosome and the ~410 kb mega-plasmid. Notably, this large plasmid exhibits high levels of synteny with similarly-sized plasmids identified to date in nine other *Labrenzia* strains. The second, smaller plasmid is a conjugative plasmid. It shows large scale synteny with the conjugal plasmids carried by six of the same nine *Labrenzia* strains that also harbour the “denitrification plasmid”. These observations suggest that the conjugative plasmid might mediate the horizontal transfer of the much larger “denitrification plasmid”. This study highlights the versatility of strain 5N through its genetic capacity to perform a wide range of environmentally and climatically important processes. Furthermore, results support evidence for the plasmid-based

transfer of denitrification genes among members of the *Labrenzia* genus, and, perhaps, between other members of the marine *Rhodobacterales/Rhizobiales*.

3.2 Introduction

Genome studies of marine microorganisms. Marine bacteria encompass a vast genetic diversity that at the present time remains largely undescribed and underexploited. Whole genome studies enable the reconstruction of metabolic pathways, unveiling a comprehensive picture of the potential functional capacity of the species of interest. *Labrenzia* spp. are important members of the *Rhodobacterales* that are found in a wide range of marine environments globally (Weber and King, 2007). Many laboratory strains of *Labrenzia* spp. were isolated originally from coastal and open waters, from marine sediments, or from water treatment facilities. A significant number have been found living in association with other organisms including algae, dinoflagellates, halophytes like seagrasses, and various invertebrates including molluscs, sponges, and corals (Pujalte *et al.*, 2005; Kim *et al.*, 2006; Weber and King, 2007; Bibi *et al.*, 2014). Investigating the genetic and metabolic capabilities of members of the *Labrenzia* genus is of considerable interest, therefore, when one considers the diversity of habitats they occupy, the broad geographic range of these organisms, and in the light of the emerging discoveries of new *Labrenzia* strains worldwide, in particular.

The genomes of a number of *Labrenzia* strains have been sequenced, including the type strains of each of the five recognised species. Genome analyses have already unveiled the large biotechnological potential among members of the genus for applications in industrial, environmental, and pharmaceutical sectors (Wang *et al.*, 2016; Curson *et al.*, 2017; Rodrigues *et al.*, 2018; Kačar *et al.*, 2019). In addition, *Labrenzia* spp. have been shown to contribute to the climatically important production of dimethylsulfoniopropionate (DMSP) and its derivative, dimethyl sulfide (DMS), as well as nitrous oxide (Coates and Wyman, 2017; Curson *et al.*, 2017). Research into the genus has gained increasing momentum in recent years, with one third of all publicly available (June 2020) *Labrenzia* genomes having been DNA sequenced since 2019.

Genome sequencing of *Labrenzia* sp. strain 5N; a representative of denitrifying community associated with the diazotrophic cyanobacterium, *Trichodesmium*.

Here, the whole genome sequence of *Labrenzia* sp. strain 5N is presented. This strain was selected for sequencing because of its potential role in the cycling of nitrogen in oceanic surface waters. *Labrenzia* sp. strain 5N was originally isolated from a suboxic seawater enrichment but its close relatives have since been found within colonies of the N₂-fixing cyanobacterium, *Trichodesmium*, also (Wyman *et al.*, 2013). The microbial communities harboured by *Trichodesmium* are diverse, both in terms of the range of individual genera present and, collectively, their extensive metabolic potential. Between 8×10^8 and 2.6×10^{11} bacterial cells ml⁻¹ are estimated to live in close association with *Trichodesmium* colonies (Paerl, 1982; Sheridan *et al.*, 2002), amongst which, assorted representatives of the Bacteroidetes, Alpha- and Gamma-proteobacteria as well as other Cyanobacteria predominate (Hewson *et al.*, 2009). Following the discovery of these extensive communities of epibionts, it has become increasingly recognised that the *Trichodesmium* community functions as a cooperative metabolic network (Lee *et al.*, 2017). Metabolic pathways taking place within the *Trichodesmium* consortium are likely to be complex interactions between the microbial epibionts present and the cyanobacterial host. Hence, metabolic processes carried out by one species will most likely impact the growth of another, building a network of intra-species interactions. Associated bacteria have the potential to influence *Trichodesmium* growth directly in terms of its CO₂ and N₂ fixation capacity and, more widely, in regulating the flux of essential nutrients, impacting marine biogeochemical cycling at a larger scale. The present genome study provides insights into the metabolic potential of a representative denitrifying epibiont, strain 5N, and the functional diversity it contributes to these potentially important N-cycling hotspots. Studying the genome of this *Labrenzia* strain will provide a better understanding of the metabolic capacity of the wider denitrifying communities found within the colonies (Coates and Wyman, 2017) and how they may interact with other epibionts, as well as with the *Trichodesmium* host itself.

Horizontal gene transfer in marine systems. Horizontal gene transfer (HGT) represents an important mechanism for genetic material exchange between bacteria. The HGT mechanisms of transformation, transduction and conjugation all contribute

to the passage of DNA from one cell to another. HGT is a key evolutionary process in bacteria, enabling organisms to share genes encoding desirable traits that might increase survival or enable adaptation to specific niches (Treangen and Rocha, 2011; Burmeister, 2015). The acquisition of new genes, groups of genes, or even entire replicons, can greatly shape bacterial evolution by crossing genus/family boundaries (Patzelt *et al.*, 2016; Petersen and Wagner-Döbler, 2017; Petersen *et al.*, 2019). As self-replicating, mobile replicons, plasmids represent ideal vectors for the transport of genetic material between bacteria within natural environments (Funnell and Phillips, 2004). Although it may be coordinated, plasmids replicate independently from the host genome. They encode essential genes involved in the replication of the plasmid itself, as well as non-essential genes encoding potentially desirable new phenotypes. Such traits often include antibiotic or heavy metal resistance and, sometimes, more complex metabolic functions like nitrogen fixation and the ability to photosynthesize (Silver and Misra, 1988; Petersen *et al.*, 2012).

The costs to host cells of maintaining plasmids are high. It places additional demands on the hosts DNA replication and repair machinery, while plasmid protein synthesis competes with the hosts transcriptome for access to its ribosomes. As a result, it is thought that for a plasmid to be maintained in the long term, the benefits and additional traits contributed by the plasmid to the host must outweigh the costs associated with its maintenance and upkeep (Harrison and Brockhurst, 2012). Should the genes encoded by a plasmid not be of benefit to the organism, at least not in its current environment, the plasmid may be lost from the host cell. Equally, any highly desirable traits encoded by the plasmid might be acquired by the host and incorporated into its own chromosome, eliminating the need for continuous plasmid maintenance (Bergstrom *et al.*, 2000). Despite this, plasmids are frequently present in bacteria, and studies have shown that they can remain in the host cell for hundreds of generations even in the absence of selection (Subbiah *et al.*, 2011). The long-term maintenance of plasmids with non-essential traits is widely studied and suggests some co-adaptation between the plasmid and host bacterium to enable their maintenance. Plasmids may reduce in size (and metabolic cost) by losing genes that are not required by the host directly and/or plasmid gene expression may be down-regulated to lessen the physiological impact that harbouring extra-chromosomal DNA has on the host

(Harrison and Brockhurst, 2012). Such adaptations may enable plasmids to persist even when not under positive selection by environmental conditions.

Genetic exchange in marine environments. The occurrence of plasmids in aquatic systems is far less studied than is the case for terrestrial systems, though investigators have shown they are present in up to 52% of aquatic bacteria (Hermansson *et al.*, 1987; Sobecky, 2002). Despite their widespread distribution among marine bacteria, the transfer of plasmids via conjugation remains little studied in the oceans to date. Recent work, however, has demonstrated that this phenomenon is indeed active (Petersen and Wagner-Döbler, 2017). It revealed that syntenic plasmids found in three distinct species of the *Roseobacter* clade; *Dinoroseobacter shibae* DFL12^T, *Confluentimicrobium naphthalenivorans* and *Roseovarius indicus*, were acquired from another host via independent transfer events. Plasmid transfer by conjugation is evidently a potentially important process in shaping bacterial evolution in marine systems. Plasmid transfer is fundamental to bacterial adaptability, enabling cells to rapidly acquire exogenous genetic material along with the new phenotypes they encode.

Many members of the *Rhodobacteraceae* (which includes the abundant *Roseobacter* group) are known to harbour a number of natural plasmids; up to 12 per cell in *Marinovum algicola* (Pradella *et al.*, 2010). Among *Roseobacter* strains, plasmids confer important traits such as toxin production which enables the killing of phytoplankton cells to release vital nutrients, promoting the formation of biofilms, and the capacity to carry out aerobic anoxygenic photosynthesis (Petersen *et al.*, 2013; Wang *et al.*, 2015). It follows that the additional traits encoded by plasmid genes more widely among the Rhodobacterales may also make an important contribution to their global success in marine environments. As members of the Rhodobacterales readily colonise surfaces, forming biofilms, they present prime candidates for plasmid transfer via conjugation in the marine environment. They are frequently found living in close association with primary producers including diatoms, algae and dinoflagellates (Fiebig, Pradella, Petersen, Michael, *et al.*, 2013; Schwenk *et al.*, 2014). Living in such tight micro-communities exposes these microbes to a wide diversity of other bacteria in with which they are in intimate contact. This close physical contact greatly facilitates

the exchange of genetic material, such as plasmids. These dense micro-communities must generate high competition for resources such as nutrients and access to oxygen to fuel aerobic respiration. Thus metabolic versatility in such an environment is essential to enable microbes to adapt and refine their metabolic strategy when the immediate conditions require so.

The wide diversity and range of environmental conditions within which *Labrenzia* species thrive is testament to their versatility. It might also be an indicator of an underlying genetic plasticity. *Labrenzia* spp. are often found suspended on particles, within sediments, or in pseudobenthic association with other host organisms; habitats that increase the probability of direct contact with a myriad of other microbial species. This degree of intimacy presents an enhanced chance of genetic exchange by either transformation or plasmid-mediated conjugation. Mindful of the epibiotic lifestyle of the *Labrenzia*-like *Trichodesmium*-associates (and their potential biogeochemical importance in N-cycling), this study focussed not just on the core genome of the representative strain 5N but also on the other genetic elements it harbours.

3.3 Methods

3.3.1 Genome sequencing

Growth conditions and genomic DNA isolation. A 30 ml late logarithmic phase culture grown in ASW medium (Wyman *et al.*, 1985) amended with 25% (vol/vol) Luria-Bertani broth in an orbital incubator (200 rpm) at 33 °C, was harvested by centrifugation. Total DNA was extracted from the cell pellet using the ZR fungal/bacterial DNA miniprep™ kit as recommended by the suppliers (Zymo Research Corp., Irvine, California, USA).

Genome sequencing and assembly. Genomic DNA concentration was estimated using a PicoDrop UV-Vis spectrophotometer (Picodrop Ltd., Saffron Walden, UK) and its integrity and purity were confirmed by visualisation on a 1% (w/v) agarose gel. The sequencing of the *Labrenzia* sp. strain 5N genome was performed by MicrobesNG (Birmingham) on an Illumina MiSeq using a 250bp paired end protocol (Illumina, Inc., San Diego, CA, USA). Data quality were determined by mapping the reads using the Burrows-Wheeler Aligner mem (BWA mem) software package (Li and Durbin, 2010; Wood and Salzberg, 2014). Read adaptor trimming was carried out

using Trimmomatic 0.30 and *de novo* assembly of the edited reads was performed using SPAdes version 3.8.0 (Bankevich *et al.*, 2012; Bolger *et al.*, 2014). Contigs were ordered against a reference genome (*Labrenzia aggregata* RMAR6-6) using MAUVE (Darling *et al.*, 2010).

Genome annotation. RAST was used for the automated annotation of the genome (Aziz *et al.*, 2008) and supplemented where required by manual annotation. Functional annotation was performed using WebMGA to assign the protein coding sequences (CDS) to clusters of orthologous groups (COGs) (Wu *et al.*, 2011). The CGView Server was used to generate a genome map for visualisation of genome features (Grant and Stothard, 2008). Genomes were visualised and compared with those of closely related organisms using the Artemis Comparison Viewer (Carver *et al.*, 2005). Genome annotation and pathway reconstruction were also performed for a selection of these comparator species using whole genome sequencing data publicly available from the GenBank genome database.

Whole genome phylogenetic analyses. *In silico* DNA-DNA hybridization (DDH) was performed using algorithms available on the GGDC web server (Auch *et al.*, 2010b; Meier-Kolthoff *et al.*, 2013). The most commonly used DDH value of >70% was adopted as the threshold for species delimitation. Average nucleotide identity (ANI) was also assessed, via the online ANI calculator, using reciprocal best hits (2-way ANI) between the genomes (Rodriguez and Konstantinidis, 2016). ANI values exceeding 95% are considered to indicate two genomes share sufficient similarity to belong to the same species.

3.3.2 Detection of Natural Plasmids

Computational methods for the detection of putative plasmids in the *Labrenzia* sp. 5N genome. Putative non-chromosomal contigs from *Labrenzia* sp. strain 5N were initially identified via whole genome sequence comparisons with closely related *Labrenzia* strains. The *de novo* assembly was first mapped against the genomes of both *Labrenzia aggregata* RMAR6-6 and *Labrenzia* sp. CP4 using MAUVE (Darling *et al.*, 2010). A BLASTN search was also performed for all twenty-nine *Labrenzia* sp. strain 5N contigs against the NCBI database, to determine which contigs matched most closely with previously mapped chromosomal or plasmid sequences. The closest

matching plasmids selected from the GenBank database were then used for further comparative analyses. To complement this, the guanine-cytosine (GC) content and sequencing coverage was calculated manually for each contig and compared between putative chromosomal and plasmid contigs to further authenticate their identification.

Experimental *Labrenzia* sp. strain 5N plasmid isolation. *Labrenzia* sp. strain 5N was grown aerobically in an orbital incubator (200 r.p.m.) for 24 hours at 33°C in ASW medium (Wyman *et al.*, 1985) amended with 25% (vol/vol) Luria-Bertani broth (10 g.L⁻¹ NaCl, 10 g.L⁻¹ tryptone, 5 g.L⁻¹ yeast extract). The cells from 5 ml of culture were harvested by centrifugation at 16,000g at room temperature for 2 min. The cell pellet was washed twice in 100% ASW medium before carrying out plasmid isolation using the BAC DNA Miniprep kit following the suppliers recommended protocol (Zymo Research, Irvine, USA). The purified plasmids were resolved on a 0.5% (w/v) agarose gel stained with 0.5 µg/mL ethidium bromide.

Detection of syntenic plasmids. Syntenic replicons shared between *Labrenzia* sp. 5N and other *Labrenzia* spp. sequences available in the GenBank database were detected initially from the results of BLASTN searches of entire contigs as described above. Whole genome sequence data were downloaded from the NCBI server for these species. Syntenic comparisons were then performed using a combination of BLAST searches, sequence alignments using MAUVE, and manual comparisons of the replicons.

***Labrenzia* sp. strain 5N DNA extraction.** DNA was extracted from *Labrenzia* sp. strain 5N by two separate methods to confirm equal extraction efficiency of all replicons. DNA was extracted from whole cells, as described above, using the ZR fungal/bacterial DNA miniprep™ kit. Additionally, DNA was also extracted from an overnight culture of strain 5N by lysing the cells at 98 °C for 10 minutes to release DNA. DNA from both extractions was diluted 1:1000 for use in qPCR.

The regulation of *Labrenzia* sp. strain 5N plasmid 1 copy number in cultures grown under denitrifying conditions was also examined since, with the exception of *napA*, the remaining denitrification genes are harboured by this plasmid. *Labrenzia* sp. strain 5N was grown under anaerobic conditions in denitrification medium (see Chapter 2) containing nitrate as the terminal electron acceptor. After 48 hours growth, cells were harvested by centrifugation and DNA was extracted as described above for the aerobic

cultures. DNA for the aerobic control and denitrifying experimental cultures was diluted 1:1000 for use in qPCR as before.

Generation of qPCR standards for plasmid1 copy number determination. *nosZ* and *narB* were amplified from Labrenzia strain 5N genomic DNA by the PCR. 25µL reactions were prepared using 1x MyTaq™ Red Mix (Bioline Reagents Ltd, London, UK) and contained 4 ng DNA, 2 mM MgCl₂ and 0.5 µM of each primer (Appendix Table 8.1). PCR conditions were as follows: initial denaturation at 95°C for 2 min, 30 amplification cycles (94°C for 30s, 55°C for 30s, 72°C for 20s) and a final extension step for 10 min at 72°C. The products were resolved on a 2% (w/vol) agarose gel stained with 0.5 µg/mL ethidium bromide and the desired fragments were excised from the gel and DNA purified using the Wizard® SV Gel and PCR Clean-Up System (Promega).

Fragments were cloned into the pCR2.1 vector using the Rapid DNA Dephos & Ligation Kit (Roche, Germany). The resulting plasmids pCR2.1-*nosZ* and pCR2.1-*narB* were transformed into One Shot® TOP10 chemically competent *E. coli* cells as recommended by the suppliers (Invitrogen, ThermoFisher, Loughborough, UK). The transformed cells were expressed at 37°C for 2 hours in an orbital incubator (200 rpm), inoculated on agar plates containing 100 µg.ml⁻¹ Ampicillin (Ap) and incubated overnight at 37 °C. Recombinant transformants were selected by blue-white screening and the presence of an insert was verified by colony PCR targeting either *narB* or *nosZ* using the PCR conditions described above. Selected recombinant colonies were subcultured and grown overnight in selective LB medium (100 µg.ml⁻¹ Ap) at 37°C and plasmids were purified using the NucleoSpin® Plasmid kit (Macherey-Nagel, Düren, Germany) as per the suppliers' recommendations.

The plasmids were linearized by digesting with 1 unit of the restriction enzyme HindIII per 1µl DNA overnight at 37 °C (New England Biolabs, MA, USA). Digestion was verified by resolution on a 1% (w/v) agarose gel stained with 0.5 µg/mL ethidium bromide. The linearized plasmid fragments were recovered from the gel and purified using the Wizard® SV Gel and PCR Clean-Up System (Promega). The linearized plasmids were quantified using a Picodrop UV-Vis spectrophotometer and copy numbers in the preparations were calculated based on the following equation (Whelan *et al.*, 2003).

Number of copies = (quantity * 6.022x10²³) / (length * 1x10⁹ * 650)

(Quantity of template DNA in ng; length of template in bp)

A 10-fold dilution series for each linearized plasmid was used to generate qPCR standard curves for both *nosZ* and *narB*.

qPCR reactions. The QuantiNova SYBR Green PCR kit (Qiagen) was used for qPCR reactions in combination with a Stratagene Mx3000p thermocycler (Agilent Technologies, Santa Clara, USA). Reactions were prepared as follows: 10µl 2x QuantiNova SYBR Green PCR master mix, 0.7µM primer 1, 0.7µM primer 2, 10µl template DNA. A two-step cycling protocol was used for all qPCR reactions, with the following cycling conditions: initial activation step at 95°C for 2 minutes, 40 amplification cycles (95°C for 5s and 60°C for 10s) followed by melting curve analysis (95°C for 1 minute, 55°C for 30 seconds followed by a ramp to 95°C at 2.5°C per minute).

3.3.3 Codon usage bias analysis of the *Labrenzia* sp. strain 5N replicons

Relative Synonymous Codon Usage analysis. Relative synonymous codon usage (RSCU) values were calculated for all protein coding genes located on the chromosome and plasmids of *Labrenzia* sp. strain 5N, and the comparator strains, *Labrenzia aggregata* RMAR6-6 and *Labrenzia* sp. CP4 (Sharp and Li, 1987). The RSCU for a codon is equal to the number of times that codon is used within a gene divided by how often that codon would be expected to be used in the absence of any codon bias. For example, the four possible codons for glycine would have an equal frequency (0.25) in the absence of bias. Mean RSCU values were then calculated for each of the replicons. RSCU values were calculated by the CAIcal server using the following equation (1) (Puigbò *et al.*, 2008):

$$(1) \quad RSCU_i = \frac{X_i}{\frac{1}{n} \sum_{i=1}^n X_i}$$

Where n is the number of possible synonymous codons for the particular amino acid and X_i is the number of times each codon (i) occurs within a gene.

RSCU was selected as the primary analysis of codon usage to minimize coding sequence length biases. Analyses were repeated, however, using both relative adaptiveness (w_i) values and the Codon Adaptation Index to confirm the consistency of the results. An RSCU value of 1 shows no bias in the usage of that codon, whereas values greater or less than 1 show a positive or negative codon usage bias, respectively. Methionine, Tryptophan and the three stop codons (TAA, TAG, and TGA) were excluded from the analysis, as their RSCU values will always be equal to 1. Additionally, any genes shorter in length than 350bp were omitted from analyses to avoid any bias introduced by short open reading frames (Duret and Mouchiroud, 1999; Chen, 2013).

Multivariate analysis of codon usage bias. A principal component analysis (PCA) was chosen as the multivariate statistical method to examine relationships between variables (RSCU values) and samples, enabling exploration of codon usage patterns among replicons (Médigue *et al.*, 1991; Romero *et al.*, 2000; Gupta and Ghosh, 2001). RSCU values for each replicon were averaged and an 8x59 RSCU matrix (Appendix Table 8.4) was used as the principal component analysis data, with columns representing codons and rows representing replicons. A PCA transforms the RSCU variables into principal components, which are uncorrelated variables, in order to identify trends in data sets with a high number of variables. Prcomp in R was used for the PCA analysis.

A heatmap was generated for *Labrenzia* sp. 5N, *Labrenzia aggregata* RMAR6-6 and *Labrenzia* sp. CP4 replicons, from the RSCU values of the 59 codons, using Euclidean distance and average linkage clustering module. The heatmap was produced using the ClustalVis webtool (Metsalu *et al.*, 2015). Each rectangle represents a single codon, for each of the 8 replicons. For each replicon, synonymous codon usage bias was visualised by a colour gradient, where red shades represent a high RSCU value (i.e. a preferred synonymous codon) and blue shades represent lower RSCU values (i.e. a synonymous codon used less frequently in a particular replicon).

3.4 Results and Discussion

3.4.1 Genome summary and general features

Genome properties. DNA sequencing generated 839,082 reads with a median insert size of 414 bp and 56.8-fold coverage. The draft genome comprised 6,464,235 bp and was composed of 29 contigs, with an average GC content of 59%, 3 rRNAs, 48 tRNAs and 5929 coding sequences, accounting for a coding density of 89%. These results are within the expected ranges for a member of the *Labrenzia* genus. The mean total genome size of the 34 sequenced *Labrenzia* strains (June 2020) is 6.3Mb (± 0.7 Mb) and encodes an average of 5873 (± 1002) proteins. The mean GC content of *Labrenzia* strains, calculated from genome sequencing data, is 58.03% (± 1.78) which is consistent with the genus description of 56-60 % (Biebl *et al.*, 2007). Strain 5N harbours a circular chromosome (5,918,759-bp) as well as 2 putative megaplasms, designated plasmid 1 (410,093-bp) and plasmid 2 (135,383-bp), that encode 370 and 133 proteins respectively. Plasmids have previously been identified in other members of the genus (Biebl *et al.*, 2007; Fiebig, Pradella, Petersen *et al.*, 2013; Wang *et al.*, 2016; Rodrigues *et al.*, 2018; Kačar *et al.*, 2019). As detailed in section 3.4.4, a total of twenty-three putative plasmids, ranging from ~60 to ~540-kb in size, were detected across 17 distinct *Labrenzia* strains (Appendix Table 8.5). Sequencing project information is shown in Table 3.1 and genome statistics are shown in Table 3.2.

The draft genome sequence of *Labrenzia* sp. strain 5N has been deposited in GenBank (Accession number: NZ_JAAZTM000000000). The GC skew of the chromosome (and other features) was displayed on a genome map (Figure 3.1) and reflects compositional differences in nucleotide abundance. The GC skew represents the excess of cytosine over guanine in each region, and is calculated as $(C - G)/(C + G)$ (Lobry, 1996). Selective pressures from bidirectional DNA replication are exerted on the base composition of different parts of a genome (Lobry and Louarn, 2003). As replication starts from the origin of replication (*ori*) and ends at the terminus (*ter*) opposite, this selection can result in biases in the composition of the leading and lagging strands (Lobry, 1996; Rocha, 2004). The leading and lagging strands in DNA replication have a positive and negative GC skew, respectively (Grigoriev, 1998). As such, this asymmetrical nucleotide content can be used to locate the positions of *ori* and *ter* which are located at the junctions of the GC positive and negative regions (Grigoriev, 1998). In the chromosome of strain 5N, the boundaries between these two

regions are clearly visible on the genome map (Figure 3.1) and were determined by computational methods to be centred around 1,745,811 bp (*ori*) and 4,835,007 bp (*ter*) respectively (Appendix Figure 8.1), using the GenSkew server (<http://genskew.csb.univie.ac.at/>).

On the other hand, plasmid replication can be either unidirectional, bidirectional or by a rolling circle replication (RCR) mechanism (Arakawa *et al.*, 2009). The two circular plasmids harboured by strain 5N are RepABC-type plasmids and, therefore, replicate by RCR (Khan, 2005). Due to the nature of RCR, during which the single strand being replicated acts as the leading strand only, this class of plasmids does not display the GC skew that was observed for the chromosome (Appendix Figures 8.2 and 8.3).

Table 3.1. Sequencing project information based on MIGS classifications.

MIGS ID	Property	Term
MIGS-28	Libraries used	Illumina
MIGS-29	Sequencing platforms	Illumina MiSeq
MIGS-30	Assemblers	SPAdes version 3.8.0
MIGS-31	Finishing quality	High quality draft
MIGS-31.2	Sequencing coverage	57x
MIGS-31.3	Contig count	29
MIGS-32	Gene calling method	RAST
	Genbank ID	
	Genbank date of release	NZ_JAAZTM000000000 8 th April 2020
	BIOPROJECT	PRJNA622454
MIGS-13	Project relevance	Environmental, biogeochemical cycling of N

Table 3.2. Genome Statistics

Attribute	Value	% of total genome
Total genome size (bp)	6464235	100.0
DNA coding region (bp)	5753655	89.0
DNA G+C content (bp)	3811959	59.0
Total genes	5929	100.0
Protein-coding genes	5839	98.4 ^a
RNA genes	51	
rRNA operons	3	
tRNA genes	48	
Genes with function prediction	4710	79.4 ^a
Protein coding genes assigned to COGs	5611	96 ^b

^aProportion calculated based on the total number of genes. ^bProportion calculated based on the total number of protein coding genes

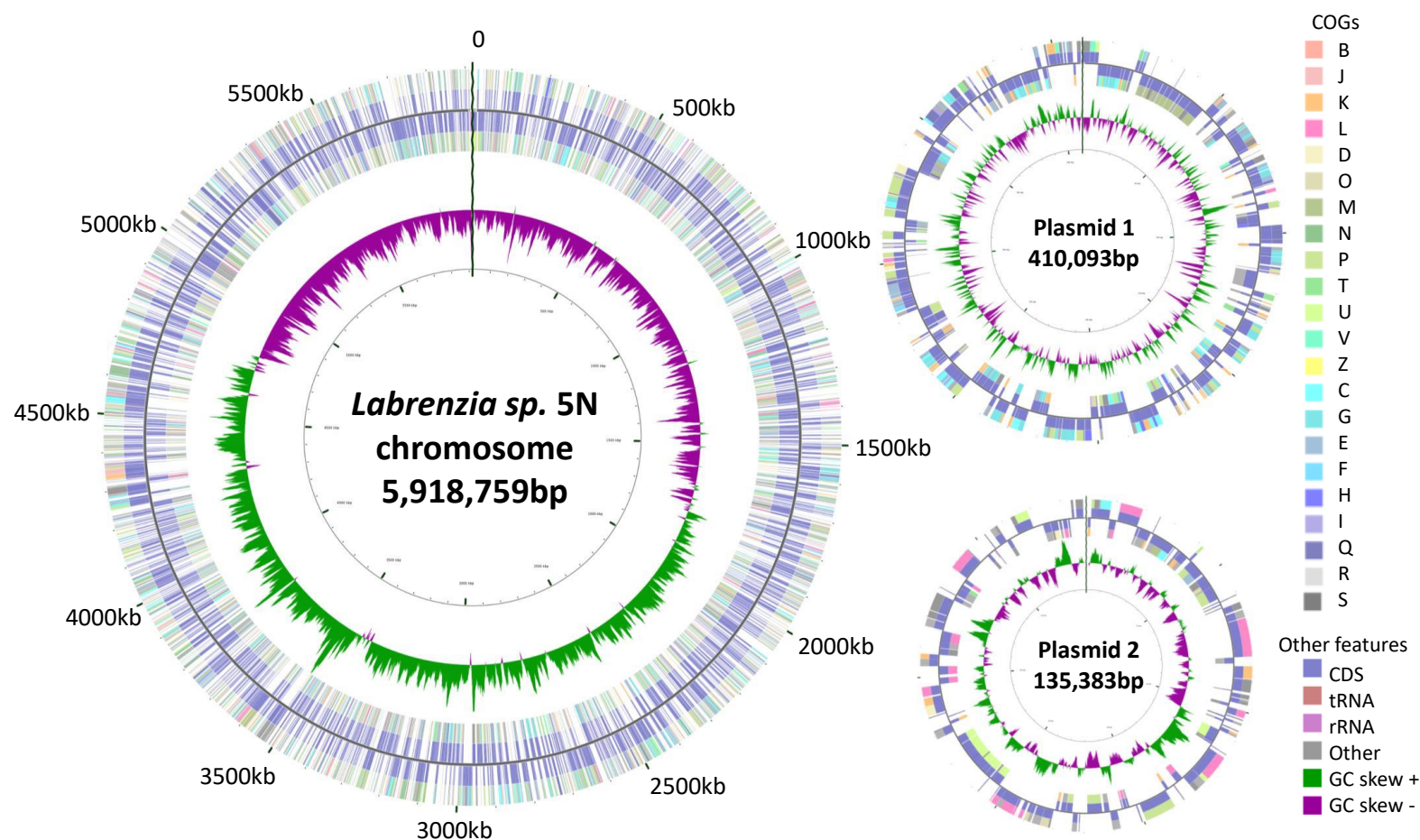


Figure 3.1. Genome map of *Labrenzia* sp. 5N, including the chromosome (left), plasmid 1 (top right) and plasmid 2 (bottom right). From outside to centre: CDS on positive strand coloured according to their COG functional categories; CDS and RNA genes on positive strand; CDS and RNA genes on negative strand; CDS on negative strand coloured according to their COG functional categories; GC skew. Genome maps were generated by CGView. Descriptions of COG functional categories are given in Appendix Table 8.6.

Identification of natural plasmids in *Labrenzia* sp. strain 5N. It was demonstrated by experiment and also by *in silico* methods that *Labrenzia* sp. strain 5N harbours two natural plasmids. The draft genome of *Labrenzia* sp. strain 5N is composed of a circular chromosome of 5.9-Mb and two presumed megaplasms (Plasmid 1: ~ 410-Kb and plasmid 2: ~ 135-Kb). Initially, all strain 5N contigs were searched against known plasmid sequences in the NCBI database. Certain contigs of *Labrenzia* sp. strain 5N showed very high identity with known plasmids from other *Labrenzia* strains. More detailed comparisons were performed using MAUVE (See 3.4.4 for more detail) and led to the identification of the two presumed megaplasms. Sequencing coverage of each non-chromosomal replicon identified also confirmed their correct classification as plasmid sequences. Contigs originating from plasmid DNA as opposed to chromosomal DNA may have a different, often higher, sequencing coverage. This may occur because differences in the initial ratio of chromosomal to plasmid DNA can result in unequally represented replicon sequences (Becker *et al.*, 2016). Mean sequencing coverage for the *Labrenzia* sp. strain 5N chromosome was 27.5x (± 4.94), whereas coverage was significantly higher for plasmid 1 (41.6 ± 6.06) and plasmid 2 (51.5 ± 7.21).

Experimentally, these plasmids were also successfully purified and visualised by gel electrophoresis (Figure 3.2). As expected, both plasmids are larger in size than the largest Lambda HindIII fragment (~23-kb) indicated in the marker lane. The supercoiled nature of these non-linearised plasmids is reflected by their faster migration than expected given their known sizes (~410-kb and ~135-kb). Plasmid yield was low considering the 5ml culture sample required for the plasmid purification kit used (BAC DNA Miniprep kit, Zymo Research), suggesting both plasmids are present at low copy number.

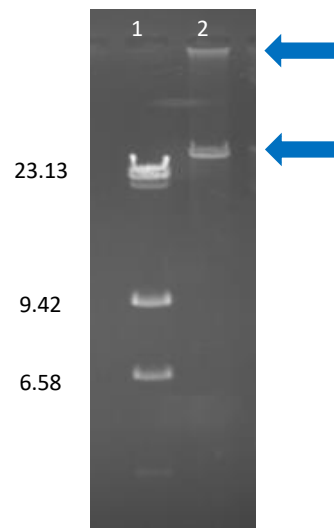
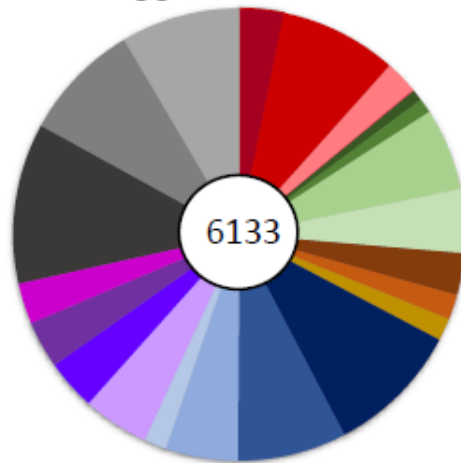


Figure 3.2. Analytical agarose gel analysis of the purified natural plasmids of *Labrenzia* sp. strain 5N. Lane 1, Lambda HindIII marker; Lane 2, un-digested plasmid purification. Arrows indicate the locations of the two plasmids.

COG analysis. The whole genome of *Labrenzia* sp. strain 5N contains 6133 predicted coding sequences, of which 5611 (91.5%) were assigned to clusters of orthologous groups (COGs) with known functions (Figure 3.1; Appendix Table 8.6). Overall, the largest functional groups in the *Labrenzia* sp. strain 5N genome were from those COGs for amino acid transport and metabolism (category E, 9.59%), transcription (category K, 8.4%), carbohydrate transport and metabolism (category G, 7.79%), signal transduction mechanisms (category T, 5.9%) and energy production and conversion (category C, 5.15%) (Figure 3.3). Combined, these five COGs represent 54% of all of the coding sequences (CDS) assigned to functional categories, demonstrating the wide metabolic capacity of this strain. COG properties were compared with other related organisms of interest using data available (July 2020) from the IGM database (Markowitz *et al.*, 2012). Comparisons show that the twenty-two other genome-sequenced members of the *Labrenzia* genus are of comparable versatility to strain 5N. The five most important COG categories in strain 5N were also the most abundant in the other *Labrenzia* species (Means given for 22 *Labrenzia* strains. Categories E: 11%, G: 9%, K:9%, C: 6%, T: 5%). Similarly, among the wider Rhodobacteraceae family (1146 representative genomes in the IGM database), the largest COG group includes proteins involved in amino acid transport and metabolism (E: 13.3%). Strain 5N, and other *Labrenzia* species, show a slightly lower capacity for energy production and conversion when compared with other Rhodobacteraceae (C: 7.8%), whereas carbohydrate transport and metabolism plays a more important role (G: 7.5%). COG group K (transcription), which includes RNA polymerase and assorted transcription factors, represents 8.4% of the total CDS in strain 5N compared with 7.4% in other Rhodobacteraceae, which suggests a higher level of genetic regulation than that found on average within this alphaproteobacterial family.

A) All protein-coding genes



B) Poorly categorised genes removed

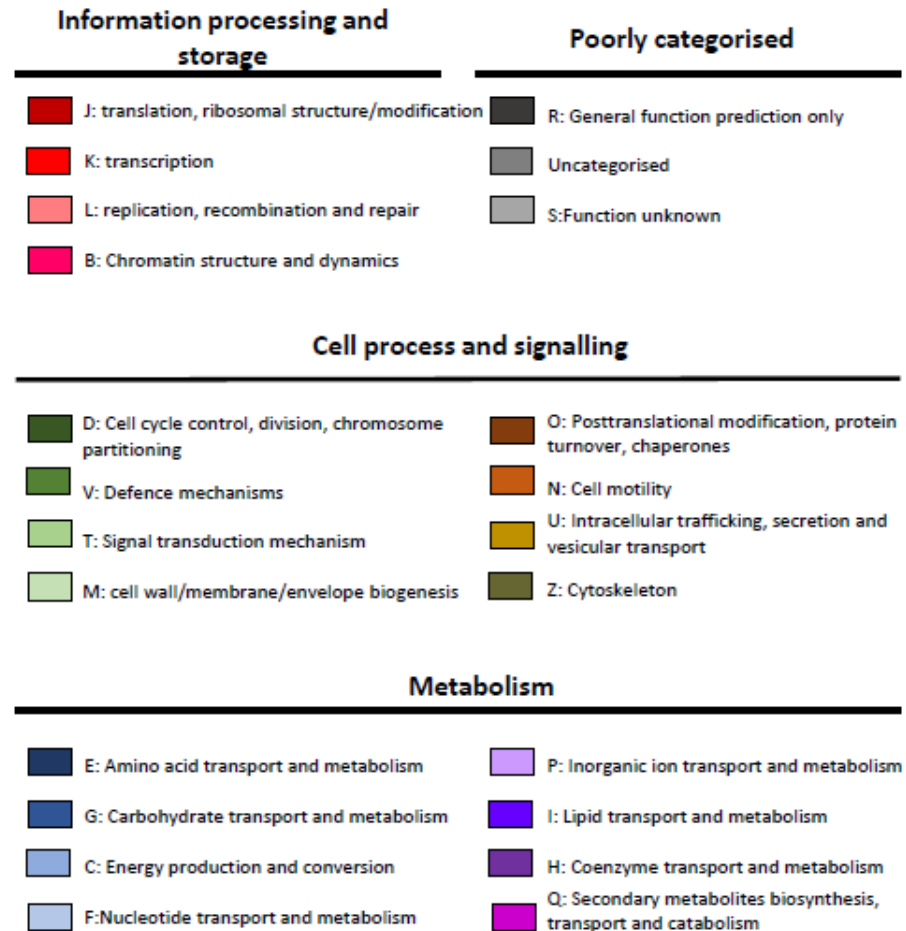
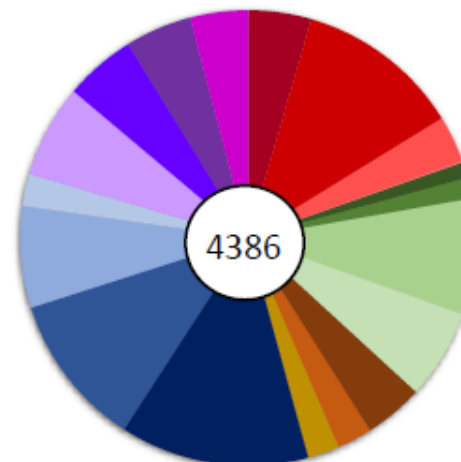


Figure 3.3. Functional classification of the protein-coding genes of *Labrenzia* sp. strain 5N. Each CDS was assigned to a COG functional category and coloured based on this classification. In the centre of the pie chart is the number of genes. A) All 6133 protein-coding genes identified in the genome. B) The 4386 genes which have been assigned a functional COG category.

3.4.2 Insights into the metabolism of *Labrenzia* sp. strain 5N

Central Metabolism. *Labrenzia* sp. strain 5N was shown by experiment to be metabolically versatile and has the capacity to use a wide range of carbon and nitrogen substrates (Chapter 2). The central metabolism of strain 5N was reconstructed from the genome (Figure 3.4) and compared with that of closely related strains of interest. Strain 5N lacks fructose-6-phosphate kinase (EC 2.7.1.11), the glycolytic enzyme necessary for the conversion of D-fructose-6-phosphate to D-Fructose-1-6-bisphosphate, as do the related strains *Labrenzia aggregata* RMAR6-6, *Labrenzia aggregata* IAM12614, *Labrenzia* sp. CP4, *Polymorphum gilvum* SL003B-26A1T and *Roseibium* sp. TrichSKD4. By contrast, *Labrenzia alexandrii* and *Labrenzia* sp. DG1229 have the potential enzymology to express fully functional glycolytic and gluconeogenesis pathways. Strain 5N has a complete pentose phosphate pathway, however, suggesting that this strain uses this alternative route to convert Glucose-6-P to Glyceraldehyde-3-P where, at this point, it can re-enter the glycolytic pathway and be further metabolised to Acetyl-CoA.

Labrenzia sp. strain 5N contains the genes for both the oxidative and non-oxidative phases of the pentose phosphate pathway. The latter enables the generation of ribose 5-P required for the synthesis of DNA/RNA while the oxidative phase provides reducing power (NADPH) to meet the demand of anabolic reactions. Strain 5N does not have an operational glyoxylate cycle, since it lacks isocitrate lyase. However, strain 5N does have genes encoding the necessary enzymology for a complete ethylmalonyl-CoA pathway, first discovered in *Rhodobacter sphaeroides*, and used by a number of isocitrate lyase-deficient bacteria for growth on acetate (and other substrates like succinate) and for acetyl-CoA assimilation (Erb *et al.*, 2007).

Labrenzia sp. strain 5N has the genetic capacity to catabolise Dimethylsulfoniopropionate (DMSP), an important precursor of the climatically important gas dimethylsulfide (DMS), as well as to utilise DMS itself. Relevant genes include *dmdB* (3-methylmercaptopropionyl-CoA ligase); *dmdD2* (3-methylthioacryloyl-CoA hydratase 2), and *dddL* (DMSP lyase). Like other *Labrenzia* spp., strain 5N can also synthesise DMSP as do many marine phytoplankton (mainly dinoflagellates and haptophytes) and the cyanobacterium, *Trichodesmium* (Curson *et al.*, 2017; Mcparland and Levine, 2018). As well as its role as an osmolyte, DSMP acts as a chemotaxant (Seymour *et al.*, 2010) with the potential to recruit not only zooplankton

but also nearby heterotrophic bacteria, such as *Labrenzia* spp., that could potentially utilise it as a carbon and sulfur source (Kiene and Linn, 2000).

Additionally, strain 5N encodes the suite of *cox* genes required for the oxidation of CO, which is a characteristic feature of the genus *Labrenzia* (Weber and King, 2007). CO is a reactive trace gas which indirectly influences the lifetime of climatically important gases like methane by impacting tropospheric OH levels (Thompson and Cicerone, 1986; Daniel and Solomon, 1998). Current atmospheric CO concentrations, produced both by natural and anthropogenic sources, range between 0.05 and 0.35ppm (Crutzen and Gidel, 1983). The oceans contribute a relatively small proportion of the atmospheric reservoir (Swinnerton *et al.*, 1970; Conte *et al.*, 2019), since the majority is oxidised *in situ* by marine bacteria thereby limiting overall outgassing (Zafiriou *et al.*, 2003).

Under aerobic conditions, CO can be oxidised to CO₂ by CO oxidoreductase (*cox*). In carboxydotrophs, the CO₂ produced is then assimilated via ribulose-1,5-bisphosphate carboxylase oxygenase (RubisCO) in support of growth (King and Weber, 2007). By contrast, carboxydovores do not possess a functional RubisCO enzyme and cannot utilise CO as a carbon source but may still benefit from the additional energy generated from CO oxidation (King, 2003; King and Weber, 2007). Studies with the *Roseobacter* species, *Ruegeria pomeroyi* DSS-3, demonstrated that this strain, which harbours *cox* genes but not RubisCO, is capable of CO oxidation but requires an additional C source for growth (Cunliffe, 2013). Despite being present in some *Labrenzia* isolates (King, 2003), a functional RubisCO was not detected in strain 5N (Chapter 2) and therefore it appears unlikely that it would be capable of utilising CO as a combined C and energy source. Perhaps more importantly, CO oxidation has been shown to be an important means of survival in carboxydovores starved of organic C sources (Cordero *et al.*, 2019). This role in C starvation might be a more likely benefit to strain 5N since, at oceanic concentrations, the volumes of CO₂ produced via CO oxidation are likely to be minimal and the energy return very limited under normal growth conditions.

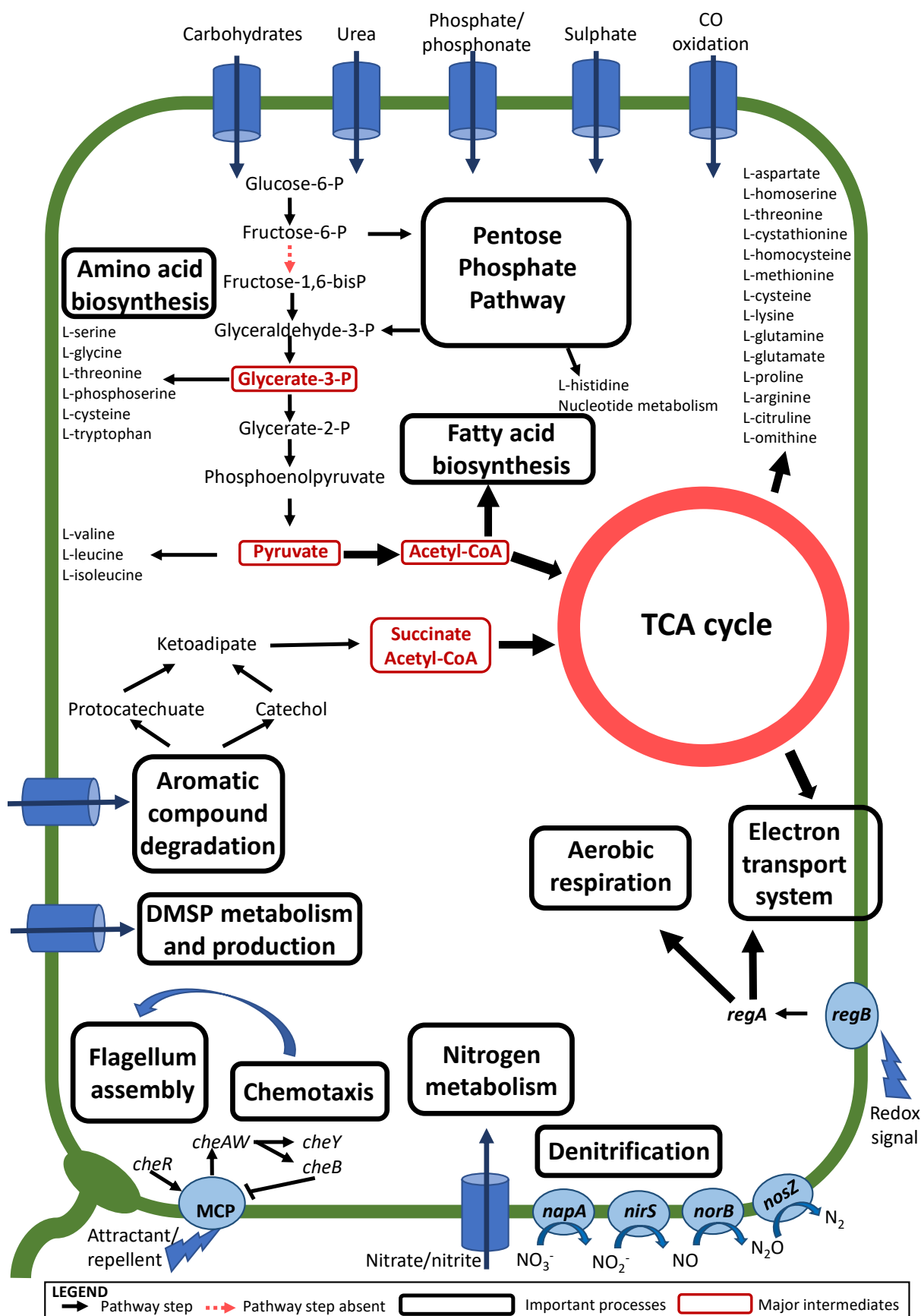


Figure 3.4. General depiction of the predicted central metabolic and selected other relevant pathways present in *Labrenzia* sp. strain 5N.

Aromatic compound degradation. Pathways involved in the metabolism of aromatic compounds were also reconstructed from the genome sequence data obtained for strain 5N. Aromatic compounds are highly stable hydrocarbons with a cyclic ring structure. Natural sources of aromatics include fossil fuels, plant degradation products, microbial remineralisation processes, and volcanism (Abdel-Shafy and Mansour, 2016). These aromatic compounds are derived naturally from sources such as the lignin produced by terrestrial and coastal plants and which constitutes ~30% of the global organic carbon reservoir (Martone *et al.*, 2009; Lu *et al.*, 2017). A major component of the plant cell wall, lignin is of particular industrial interest as a renewable, bio-based compound for the manufacture of polymers (Llevot *et al.*, 2016). The contribution of natural sources of aromatic compounds to the global inventory, however, is minor in comparison with anthropogenic emissions from automobiles, the petrochemical industry, and processes such as incineration (Zhang and Tao, 2009). Polycyclic aromatic hydrocarbons (PAH's) are significant environmental pollutants that are detrimental to human health and are particularly toxic to higher marine life due to their bioaccumulation up the food chain.

The transformation of aromatic compounds is an important aspect of global carbon cycling in which microbes play a central role during the remineralisation of organic matter. Furthermore, the capacity of some bacteria to degrade PAH's is of considerable applied interest for bioremediation purposes (Pieper and Reineke, 2000). *Labrenzia* sp. strain 5N harbours genes encoding a number of degradative pathways for aromatic compounds. It has genes involved in both the protocatechuate and catechol branches of the beta-ketoadipate pathway. In this pathway, protocatechuate and catechol, themselves generated from a number of different aromatic compounds, can be degraded ultimately to beta-ketoadipate (Harwood and Parales, 1996). Beta-ketoadipate can be further converted to TCA cycle intermediates and utilised as both a source of energy and carbon. Strain 5N harbours the *cat* genes necessary for the catechol branch of the beta-ketoadipate pathway (Shanley *et al.*, 1986; Kukor *et al.*, 1988), including *catA* (catechol 1,2-dioxygenase, EC:1.13.11.1), *catC* (muconolactone isomerase, EC 5.3.3.4), *catD*, (beta-ketoadipate enol-lactone hydrolase, EC 3.1.1.24), *catEA* and *catEB* (succinyl-CoA:3-ketoacid-coenzyme A transferase subunits A and B, EC 2.8.3.5; EC 2.8.3.5) and *catB* (muconate lactonizing enzyme family protein, EC 5.5.1.1). In addition, strain 5N has the potential to metabolise protocatechuate, a

derivative of phenolic compounds like 4-hydroxybenzoate and lignin (Harwood and Parales, 1996; MacLean *et al.*, 2006). The genes required for this pathway, which is well characterised in *Sinorhizobium meliloti* (MacLean *et al.*, 2006), are present in strain 5N. These include *pcaR* encoding the regulatory protein PcaR, *pcaG* and *pcaH* (protocatechuate 3,4-dioxygenase alpha and beta chains EC 1.13.11.3; EC 1.13.11.3), *pcaB* (3-carboxy-cis,cis-muconate cycloisomerase, EC 5.5.1.2), *pcaC* (4-carboxymuconolactone decarboxylase, EC 4.1.1.44), *pcaD* (beta-ketoadipate enol-lactone hydrolase, EC 3.1.1.24), *pcaI* and *pcaJ* (3-oxoadipate CoA-transferase subunits A and B, EC 2.8.3.6; EC 2.8.3.6) and *pcaI2* and *pcaJ2* (succinyl-CoA:3-ketoacid-coenzyme A transferase subunits A and B, EC 2.8.3.5; EC 2.8.3.5). The protocatechuate (*pca*) pathway for aromatic compound degradation has been detected in a number of *Roseobacter* members also (Newton *et al.*, 2010; Gulvik and Buchan, 2013) and highlights the metabolic versatility of this clade.

Strain 5N also has the genetic capacity to degrade 4-hydroxyphenylacetic acid, an intermediate of aromatic compound degradation and also present as metabolite in many animals (including humans) and plants. The pathway, which is well studied in *E. coli* (Prieto *et al.*, 1996; Díaz *et al.*, 2001; Carmona *et al.*, 2009) involves the *hpa* genes of which *hpaC* (4-hydroxyphenylacetate 3-monooxygenase, reductase component, EC 1.6.8), *hpaH* (2-oxo-hepta-3-ene-1,7-dioic acid hydratase, EC 4.2), *hpaF* (5-carboxymethyl-2-hydroxymuconate delta-isomerase, EC 5.3.3.10), *hpaD* (3,4-dihydroxyphenylacetate 2,3-dioxygenase, EC 1.13.11.15), *hpaE* (5-carboxymethyl-2-hydroxymuconate semialdehyde dehydrogenase, EC 1.2.1.60), *hpaG* (5-carboxymethyl-2-oxo-hex-3-ene-1,7-dioate decarboxylase, EC 4.1.1.68) and the homoprotocatechuate degradative operon repressor (*hpaR*) were detected in strain 5N.

Strain 5N possesses a complete homogentistic acid pathway for the degradation of the aromatic amino-acids L-phenylalanine, L-tyrosine, and the acetate derivative, 3-hydroxyphenylacetate (Arias-Barrau *et al.*, 2004). Amongst others, the genome harbours genes encoding homogentisate 1,2-dioxygenase (EC 1.13.11.5), 4-hydroxyphenylpyruvate dioxygenase (HPPD, EC 1.13.11.27), maleylacetoacetate isomerase (MAI, EC 5.2.1.2), fumarylacetoacetase (FAA, EC 3.7.1.2), and the IclR family transcription factor, HmgR.

In summary, strain 5N has the genetic potential to metabolise a range of naturally occurring, biogenic, aromatic compounds including those produced by plants. In addition it should be capable of metabolising several important classes of organic environmental contaminants which is a trait of biotechnological interest.

Iron acquisition and metabolism. There are numerous genes harboured within the genome of *Labrenzia* sp. strain 5N that are involved in iron uptake and transport. As well as those encoding transporters for ferrous and chelated forms of iron, genes specifying products involved in heme (Fe^{2+} complex of protoporphyrin IX) uptake are also present. These include *hmuTUV*, *exbBD*, and *tonB*, the components of the heme transport system identified recently in *Roseobacter* (Hogle *et al.*, 2017). A copy of the gene for the heme degradation enzyme, HmuS (Amarelle *et al.*, 2016) is also present within the genome of strain 5N. In addition, genes encoding probable siderophore transport system proteins, hemin transport/uptake proteins (*hemSP*), heme binding/exporter proteins (*hbpA*, *ccmBCD*) and an Fe^{3+} -hydroxamate transporter *fhuC* were also detected. Like a number of other members of the *Rhodobacterales*, these findings suggest that strain 5N is capable of using heme-type compounds as iron sources as well as more conventional inorganic forms of the element (Roe *et al.*, 2013). Indeed, some marine bacteria including the *Cytophagales* species, *Microscilla marina*, are capable of growth on heme as their sole iron source (Hopkinson *et al.*, 2008). About one fifth (~20%) of the total iron content of phytoplankton cells (and particulate organic material, generally) occurs in the form of heme-type compounds (Honey *et al.*, 2013). These include those required for the operation of major metabolic processes including photosynthesis, respiration, and the reduction of nitrate.

Like *Labrenzia* spp., the *Roseobacter* clade are commonly found living in association with marine phytoplankton and approximately half of them harbour putative heme transport systems (Roe *et al.*, 2013; Hogle *et al.*, 2017). These include the *Trichodesmium*-associated *Roseobacter* member, *Ruegeria* sp. strain TrichCH4B that is capable of growing exclusively on natural heme sources under iron-limitation (Roe *et al.*, 2013). In *Sulfitobacter* sp. strain SA11, a known coloniser of the diatom *Pseudo-nitzschia multiseriata* (Amin *et al.*, 2015), the heme transport system is downregulated when growing in association with its host (Hogle *et al.*, 2017). Extensive interaction and signalling between this *Roseobacter* strain and its diatom

host has been demonstrated (Amin *et al.*, 2015) and Hogle *et al.* (2017) suggest this drives heme uptake in phytoplankton-associated *Roseobacter* species more generally. Presumably, iron limitation is less severe in association with its host than it is in the free-living, planktonic state, or heme-type compounds only become readily available from moribund phytoplankton cells undergoing decomposition and remineralization (Strzepek and Harrison, 2004).

3.4.3 Nitrogen metabolism in *Labrenzia* sp. strain 5N

Denitrification is a plasmid encoded trait in *Labrenzia* sp. strain 5N. Like many other members of the genus, *Labrenzia* sp. strain 5N is a denitrifier capable of utilising nitrate as a terminal electron acceptor in support of growth (Chapter 2). All of the genes encoding the enzymes required for the complete denitrification pathway were identified within the genome of strain 5N. Interestingly, however, the localisation of the denitrification genes is split between the chromosome and plasmid 1 (410 kb). The chromosome itself harbours the genes whose products are required for the first step of denitrification: nitrate reduction. These include *napC,B,D,F,E* which are co-localised on the chromosome with *napA*, the gene encoding the catalytic subunit. Unlike *L. alba*, strain 5N does not appear to harbour *napG* or *napH* whose products are required for electron transport from ubiquinol (but not menaquinol) to NapAB (Brondijk *et al.*, 2002). It seems likely, therefore, that menaquinol is the sole electron donor utilised by the dissimilatory nitrate reductase present in strain 5N.

The genes encoding the remainder of the denitrification apparatus are located on plasmid 1 rather than the chromosome. This plasmid harbours single copies of the genes encoding products that are required for dissimilatory nitrite reduction; *nirS* (cytochrome cd1 nitrite reductase EC 1.7.2.1), *nirC* (cytochrome c55X precursor), *nirN* (nitrite reductase associated c-type cytochrome NirN) and the *nirCDFGHJL* operon encoding the heme d1 biosynthesis proteins (Figure 3.5). Plasmid 1 also contains the only copies of the genes (*nor* and *nos*) encoding nitric oxide reductase (Nor; EC 1.7.99.7) and nitrous oxide reductase (NosZ; EC 1.7.99.6), which, along with their maturation and activation proteins, are clustered either side of the *nir* operon (Figure 3.5). Consequently, the primary and latter steps of the denitrification pathway are

encoded by separate replicons, the combined products of which are required to complete this metabolic pathway.

Denitrification has been shown to be a plasmid-borne trait in many other bacteria, including *Rhodobacter sphaeroides* 2.4.1 and *Azospirillum brasilense* Sp245 (Schwintner *et al.*, 1998; Petrova *et al.*, 2010). The localisation of denitrification genes on the chromosome or on one or more plasmids is variable, and, like strain 5N, genes can be split between different replicons. In *Ralstonia eutropha*, *nirS* is found on the chromosome whereas *napA* and *nosZ* are both plasmid-borne (Zumft *et al.*, 1992; Warnecke-Eberz and Friedrich, 1993; Rees *et al.*, 1997). In *Paracoccus denitrificans* isolates the denitrification genes are usually harboured by the chromosome (Zumft, 1997a) whereas in the full denitrifier, *Sinorhizobium meliloti* strain JJ1c10, the *nos* genes are located on the *nod* (nodulation) megaplasmid (Holloway *et al.*, 1996).

Phylogenetic reconstructions based on the analysis of denitrification genes have highlighted differences in their evolutionary origins when compared to phylogenies based on 16S rRNA from the same organisms. These disparities might be indicative of the horizontal transfer of denitrification genes between unrelated hosts (Jones *et al.*, 2008; Jang *et al.*, 2019). Even when chromosomally located, entire clusters of denitrification genes might be transferred laterally between organisms, as has been demonstrated for *Thermus thermophilus* (Alvarez *et al.*, 2011).

Plasmid 1 of *Labrenzia* sp. strain 5N harbours a type II toxin-antitoxin maintenance system and so the loss of the entire plasmid would most likely to be lethal to the host. However, non-beneficial genes could still be lost selectively from the plasmid to counteract the high energetic costs associated with their maintenance and expression. The presence of intact denitrification operons on this plasmid, however, and the experimental demonstration that they are functional (Chapter 2), is indicative of the overall importance of denitrification for the success of this organism.

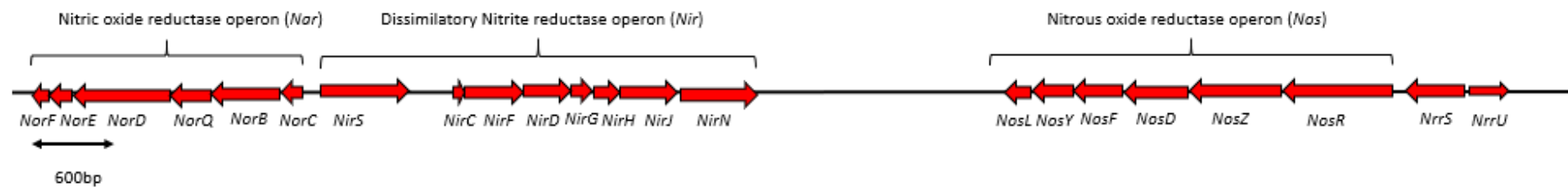


Figure 3.5. Organisation of the denitrification genes harboured by the ~410-kb plasmid in *Labrenzia* sp. strain 5N. Arrows indicate the orientation of the CDS.

Control of *Labrenzia* sp. strain 5N plasmid 1 replication. The copy number of *Labrenzia* sp. strain 5N plasmid 1 relative to that of the chromosome was estimated by qPCR assays targeting two genes; *narB*, located on the chromosome, and *nosZ*, located on plasmid 1. Since these genes are both found in single copy within the genome of strain 5N, the ratio of *narB:nosZ* gene copy numbers can be used directly to determine the relative plasmid copy number (PCN). The estimate is relative because the number of genome equivalents in *Labrenzia* sp. strain 5N cells is unknown. The qPCR assays were performed simultaneously on DNA extractions from three separate cultures of strain 5N grown under either aerobic or anaerobic, denitrifying conditions. Three technical replicates were performed in parallel for each of the cultures.

Amplification specificity was verified by melting curve analysis. A single melting peak was found for each gene, confirming the absence of non-specific PCR products and primer dimers (Appendix Figures 8.4 and 8.5). The melting peaks for the *narB* standards and samples were at around 82°C while for the *nosZ* primer set the melting point was a little higher at approximately 85°C. Additionally, several of the qPCR standards and sample products were examined by gel electrophoresis to confirm the presence of single bands of the expected sizes and relative intensities. Standard curves prepared for *narB* and *nosZ* genes ranged in concentration from 1×10^2 to 1×10^8 copies/μl. The standard curve gradients for *narB* and *nosZ* were -3.235 ($R^2 = 0.998$) and -3.106 ($R^2 = 0.997$) respectively (Appendix Figure 8.6). The amplification efficiency ($E=10^{(-1/\text{slope})}-1$; Rasmussen, 2001) for *narB* was 103.80% and that for *nosZ*, 109.87%. The standard curves were used for the absolute quantification of the gene copies in the DNA samples and were found by interpolation of their respective Ct values using the instrument's software package, MxPro.

The mean copy number of plasmid 1 when compared to the chromosome was 0.82 under aerobic growth and 1.33 under anaerobiosis (Table 3.3). Given the degree of resolution of the qPCR (1 cycle), it was concluded that this plasmid is present at a similar copy number to that of the chromosome, implying that their replication during the cell cycle is coordinated.

Table 3.3. Quantification of relative plasmid 1 copy number (PCN) in *Labrenzia* sp. strain 5N under aerobic and anaerobic conditions.

Culture	Ct ^a		Copies ^b (copies/ng dna)		PCN ^b
	<i>narB</i> (chromosome)	<i>nosZ</i> (plasmid)	<i>narB</i>	<i>nosZ</i>	
Aerobic	17.96 ± 0.41	18.51 ± 0.6	2.44E+04	1.99E+04	0.82
Anaerobic	18.22 ± 0.17	17.35 ± 0.45	8.26E+03	1.10E+04	1.33

^aAverage ± sd (n=3); ^bAverage (n=3); ^cAverage (n=3)

3.4.4 The scope for denitrification among *Labrenzia* spp.

Plasmid distribution within the *Labrenzia* genus. Following the detection of a plasmid harbouring denitrification genes in strain 5N, the occurrence of similar “denitrification plasmids” was investigated among all other *Labrenzia* strains for which whole genome sequencing data were available. The presence of the RepABC-type plasmid replication system and the *ccd* toxin-antitoxin system found in the strain 5N plasmid 1 were chosen as informative genetic markers to identify related plasmids in those genomes where their occurrence was not documented in the original annotation. Plasmids with RepABC-type replication systems have been detected among many alphaproteobacteria and are especially common among members of the *Rhodobacterales* and *Rhizobiales* (Cevallos *et al.*, 2008; Petersen *et al.*, 2009, 2011). The *ccd* (control of cell death) system is involved in plasmid maintenance (Jaffe *et al.*, 1985; Bahassi *et al.*, 1995). Both component genes (*ccdAB*) are encoded by strain 5N plasmid 1 and the presence of similar copies was searched for in all *rep*-containing replicons detected to inform plasmid identification.

Nine *Labrenzia* strains are known to harbour one or more plasmids, amounting to twenty three annotated plasmids in total between them (Appendix Table 8.5). Primarily, these identities were based on whole genome sequencing data rather than established by experimental investigation such as plasmid isolation and characterisation. BLAST searches were performed targetting both *repABC* and *ccdAB* to detect any additional putative plasmids within the genomes of all thirty four sequenced *Labrenzia* strains available at the start of the investigation (Appendix Table 8.5). *Rep* genes were detected thirty five times among eighteen distinct strains. The Rep system was identified within both named and possible plasmid sequences, as well as within sequence contigs from draft genomes. Twenty three of these thirty five *rep* positive hits also encoded the *ccd* typeII toxin/anti-toxin plasmid maintenance system and were classified as candidate plasmids by this criterion. Among these, ten represent newly identified plasmids that were not documented in the published genome annotation. These novel *rep* and *ccd* positive replicons are harboured by *Labrenzia* sp. PO1, *Labrenzia* sp. C1B10, *Labrenzia* sp. C1B70, *Labrenzia* sp. 011, *Labrenzia aggregata* IAM 12614, *Labrenzia aggregata* CECT4801, *Labrenzia* sp. PT13C1 and *Labrenzia alexandrii* CECT 5112.

Localisation of the denitrification machinery across *Labrenzia* genomes. All thirty four *Labrenzia* genome sequences (including those in which *rep*- and/or *ccd*-type replicons were not detected) were screened for the presence of *nap*, *nir*, *nor* and *nos* genes as markers for denitrification. Twenty-two strains harboured the genes required for either the complete or partial denitrification of nitrate (Appendix Table 8.5). *Nap* genes, encoding proteins responsible for the initial dissimilatory reduction of nitrate to nitrite, were detected in twenty of these strains and were located exclusively on the chromosome in all cases.

Twelve of the twenty-two known or predicted denitrifying strains also harboured chromosomal copies of genes encoding other components of the denitrification machinery. Of these, nine have the capacity to fully reduce nitrate as far as N_2 , one should be capable of reducing nitrite to N_2O , while the two remaining strains also lack *nap* but harbour the genes necessary to reduce nitrite further to N_2 . Interestingly, three of the twelve strains, *Labrenzia* sp. 011, *Labrenzia marina* DSM 17023 and *Labrenzia suaedae* DSM 22153, possess a copy of the nitrite reductase gene *nirK* rather than the more common *nirS*-encoded form found in most denitrifying *Labrenzia* spp.

Among the twenty-three potential *Labrenzia* plasmids (*Rep* and *ccd* positive), ten contained copies of *nirS*, *norBC* and *nosZ*. These were found in each of the remaining predicted denitrifiers identified where these genes were not present on the chromosome. Hence, these known (or candidate) plasmids provide all ten strains with the genetic capacity to reduce nitrite completely to N_2 . The same ten strains also harboured a copy of *nap* within the chromosome. This finding demonstrates that their predicted capacity to fully denitrify nitrate requires a combination of chromosomal and plasmid-borne gene products.

***Labrenzia* plasmids harbouring denitrification machinery demonstrate high levels of synteny.** Synteny between the ten predicted “denitrification plasmids” harboured by *Labrenzia* sp. strain 5N (plasmid 1), *Labrenzia aggregata* RMAR6-6 (plasmid 1), *Labrenzia* sp. THAF187 (pTHAF187b_a), *Labrenzia* sp. THAF35 (pTHAF35_a), *Labrenzia* sp. CECT4801 (scaffold 0004), *Labrenzia* sp. C1B10 (contig 4), *Labrenzia* sp. C1B70 (contig 11), *Labrenzia* sp. THAF191a (pTHAF191a_b), *Labrenzia* sp. THAF191b (pTHAF191b_a) and *Labrenzia* sp. PO1 (scaffold5_1) was

initially detected through tBLASTn analyses. Comparisons revealed large syntenic regions between the annotated or predicted plasmids of all of these strains. Long range orthologies between the plasmids were confirmed when analysed in more detail using MAUVE (Figure 3.6). The ten plasmids share large conserved regions, spanning almost the entirety of the plasmid in some instances. The plasmids ranged in size from ~315 to 540kb with orthologous regions sharing nucleotide sequence identities in excess of 93%. Genes present in the non-syntenic regions largely encoded hypothetical proteins. While some strains harboured plasmids that were clearly smaller than others, the missing, non-syntenic regions were not detected elsewhere in their genomes. This suggests that these fragments have either been incorporated into some of the largest plasmids identified or were originally harboured by all plasmids and have since been lost in the more compact plasmids. Without exception, this highly syntenic plasmid is shared by all the *Labrenzia* strains in which *nir*, *norBC* and *nos* are plasmid-encoded, which is perhaps indicative of the capacity for lateral gene transfer between different members of this genus.

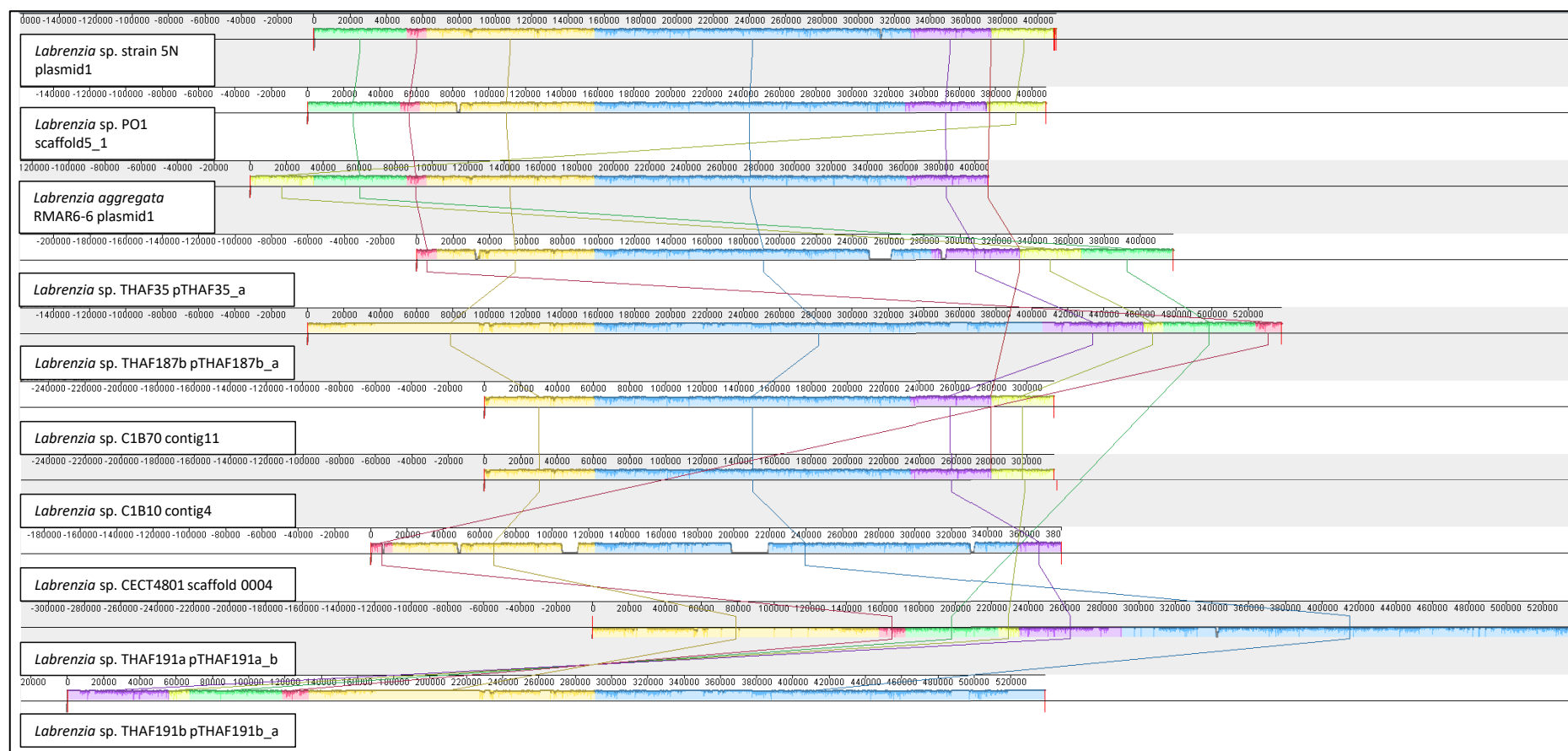


Figure 3.6. Multiple alignment of 10 putative denitrification plasmids detected in the genomes of ten *Labrenzia* strains and visualized using the Mauve interface. Large blocks identified with the same colour represent conserved, syntenic regions across the sequences. Within each block, coloured similarity plots indicate the degree of sequence identity. Rearrangements are shown with coloured lines connecting between plasmids. The sequences included, from top to bottom, are: *Labrenzia* sp. 5N plasmid1, *Labrenzia* sp. PO1 scaffold5_1, *Labrenzia aggregata* RMAR6-6 plasmid 1, *Labrenzia* sp. THAF35 plasmid pTHAF35_a, *Labrenzia* sp. THAF187b plasmid pTHAF187b_a, *Labrenzia* sp. C1B70 contig 11, *Labrenzia* sp. C1B10 contig 4, *Labrenzia* sp. CECT4801 scaffold 0004, *Labrenzia* sp. THAF191a pTHAF191a_b and *Labrenzia* sp. THAF191b pTHAF191b_a.

A comparative analysis of the organisation of denitrification genes between *Labrenzia* strains. A multiple whole genome alignment between the chromosomes and plasmids of the twenty two sequenced *Labrenzia* strains that encode denitrification (DN) gene clusters was performed using Mauve (Darling *et al.*, 2010). The alignment viewer in Geneious 11.0.2 was used to visualise the genomic regions containing DN genes in greater detail (Figure 3.7). The organization and orientation of the *norCBQDEF*, *nirSCFDGHJN* and *nosRZDFYL* operons in all ten plasmid-containing strains was highly conserved (Figure 3.7A). Located within the *nir* operon was *cobA* encoding uroporphyrinogen-III c-methyltransferase that is required for the biosynthesis of cobalamin for the heme d₁ component of NirS. All plasmids also contained a copy of *apbE* immediately downstream of the *nosRZDFYL* operon that encodes a thiamine biosynthesis protein necessary for NosR maturation (Zhang *et al.*, 2017). ApbE has been shown to perform a similar role to NosX in *Pseudomonas stutzeri* and can act as a functional substitute for this protein in nitrous oxide reduction (Zhang *et al.*, 2017). The plasmid-encoded ApbE proteins all shared ~50% amino-acid identity with the NosX protein found in other denitrifying *Labrenzia* strains.

The gene encoding ApbE is directly replaced by *nosX* in the same downstream location in most *Labrenzia* strains that harbour the *nos* operon on the chromosome. The occurrence of *nosX* amongst denitrifiers is variable but its organisation in *Labrenzia* is similar to that reported for other alphaproteobacterial species including *Paracoccus denitrificans*, *Rhizobium meliloti* and *Achromobacter cycloclastes* (Holloway *et al.*, 1996; McGuirl *et al.*, 1998; Saunders *et al.*, 2000). Disruption of *nosX* in *Sinorhizobium meliloti* produces a Nos⁻ phenotype (Chan *et al.*, 1997). Gene inactivation studies with *P. denitrificans*, by contrast, showed that *nosX* does not appear to be absolutely essential for nitrous oxide reductase activity in this organism (Saunders *et al.*, 2000). All activity is eliminated in *nosX* double deletion mutants, however, in which *nirX* (a gene unique to *P. denitrificans* to date) is also inactivated, leading Saunders *et al.* (2000) to suggest that these two proteins are functional homologues with interchangeable roles in this organism.

The core structure of the *nor*, *nir* and *nos* operons was also highly conserved among the twelve strains harbouring their DN genes exclusively on the chromosome although there are some notable variations in certain strains (Figure 3.7B). In *L. aggregata* strain IAM12614 and *L. aggregata* LZB033, the organisation of these regions is

identical to that found in the ten *Labrenzia* DN plasmids analysed, including the possession of *apbE* rather than *nosX*. *Labrenzia marina* DSM 17023 and *L. suaedae* DSM 22153 lack a recognisable copy of *napA* (Appendix Table 8.5) but might still be capable of denitrifying nitrite produced by assimilatory nitrate reduction if a more favourable N source is available (Vairinhos *et al.*, 1989). Both species have a copy of *nirK* rather than *nirS* while *L. suaedae* DSM 22153 also harbours *nirV*. These genes are located further upstream of the *nor* operons and their overall organisation does not share similarity with that of the other strains. While *L. suaedae* DSM 22153 has copies of both *nor* and *nos* and *L. marina* DSM 17023 encodes the *nor* operon only, their overall organisation is consistent with the other strains. Eight strains; *Labrenzia* sp. DG1229, *Labrenzia* sp. EL143, *Labrenzia* sp. PHM0005, *Labrenzia* sp. THAF82, *L. alba* CECT5094, *L. alba* CECT5095, *L. alba* CECT5096 and *L. alba* CECT7551 all harbour the gene encoding an additional NirT/NapC family c-type cytochrome, in a chromosomal location downstream of *nirS*. This tetraheme c-type cytochrome is a component of the periplasmic nitrite reductase and is involved in electron transfer (Jüngst *et al.*, 1991; J. Simon *et al.*, 2000). Of these eight strains, all except *Labrenzia alba* CECT7551 harbour a copy of *nosX*, downstream of *nosRZDFYL* while *Labrenzia alba* CECT7551, *Labrenzia aggregata* IAM12614, and *Labrenzia aggregata* LZB033, like the “denitrification plasmid” bearing strains, harbour *apbE* instead.

The chromosomal and plasmid encoded denitrification gene regions share high identity and synteny across *Labrenzia* strains (Figure 3.7). However, it cannot be established whether the those DN genes harboured be the chromosome might be in this location as the result of an earlier plasmid integration event or whether the “denitrification plasmid” has arisen as result of an excision and recombination with an existing plasmid. The “denitrification plasmid” of *Labrenzia* sp. strain CECT4801 has a transposase gene inserted directly upstream of the *nos* operon. It is not known whether this transposase is functional but it does suggest one potential mechanism by which the denitrification machinery might be mobilised. The coding regions directly surrounding the denitrification genes differ markedly between plasmids and chromosomes. In the plasmids, the gene complement both up and downstream of the denitrification genes remain highly conserved and syntenic whereas these regions are much more variable in the strains that harbour denitrification genes on their chromosomes exclusively (data not shown).

The evolutionary history of mobile phenotypes is complex but phototrophy amongst members of the marine *Roseobacter* clade is a well-studied example of this process. The chromosomally located photosynthesis gene cluster (PGC) in *Roseobacter denitrificans* and that of the equivalent plasmid-encoded region in *Roseobacter litoralis* are structurally very similar (Swingley *et al.*, 2007; Kalhoefer *et al.*, 2011). It has been proposed that the plasmid localisation of the PGC in the latter species (*R. litoralis*) and in *Sulfitobacter guttiformis* is the result of two independent cases of “chromosomal outsourcing” (Petersen *et al.*, 2012). A detailed recent examination of one hundred and five sequenced members of the Rhodobacteraceae further led to the proposal of a scenario in which the plasmid-located PGC has been reintegrated back into the chromosomes of some members of the group (Brinkmann *et al.*, 2018). Without reintegration, selective plasmid loss would lead to a patchy distribution of traits among this closely related group. Similar events might well explain the variation in denitrification capacity among different *Labrenzia* species (Appendix Table 8.5).

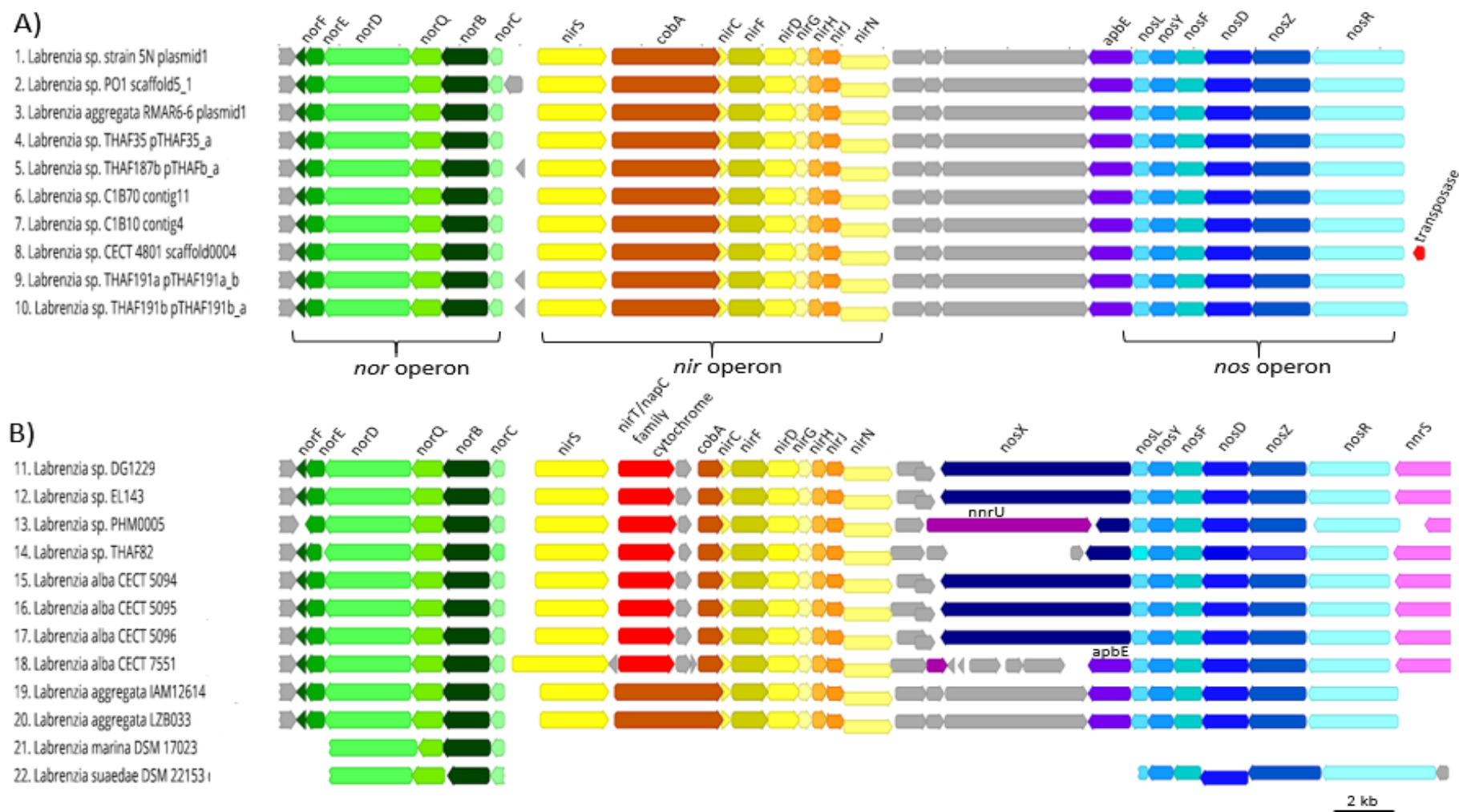


Figure 3.7. Organisation of the denitrification gene clusters located on plasmids (A) or on chromosomes (B) in 22 Labrenzia species. Individual genes are represented by the same colour for all strains that have them in common. Arrows indicate the orientation of each coding sequence. Hypothetical genes and genes not key to the denitrification process are shown in grey.

Co-occurrence of denitrification plasmids and a second, conjugative plasmid among *Labrenzia* spp.. Analyses revealed that five of the *Labrenzia* strains that harbour “denitrification plasmids” also have a second plasmid which displays high levels of mutual synteny (Figure 3.8). This second syntenic plasmid was also detected in *Labrenzia* sp. CP4 (pBCL1) although this strain lacks the “denitrification plasmid” and is incapable of complete denitrification (Appendix Table 8.5). The second plasmid present in the strains, *Labrenzia* sp. strain 5N (plasmid 2), *Labrenzia aggregata* RMAR6-6 (plasmid 2), *Labrenzia* sp. CP4 (pBCL1), *Labrenzia* sp. THAF187b (pTHAF187b_b), *Labrenzia* sp. THAF191a (pTHAF191a_a) and *Labrenzia* sp. THAF191b (pTHAF191b_b) range in size from ~94 to 135 kb and appear to be conjugative in nature. They present genes encoding transposases, conjugal transfer proteins and various mobile element proteins. The transfer elements include those often found in conjugative plasmids, like the *tra* operon encoding *traABFGH* and the *trb* operon (*trbBCDEFGHIJL*), and these are present in all.

The Type IV secretion system (T4SS) encoded by these elements is implicated not only in the conjugal transfer of DNA between bacterial cells but also in protein secretion and pathogenesis (Cascales and Christie, 2003; Christie *et al.*, 2005). The additional presence of a gene encoding VirD4 on these plasmids suggests the system is a type IVA T4SS, which has been well characterised in the plant pathogen, *Agrobacterium tumefaciens*, and is required for the transfer of the Ti plasmid to the infected host (Christie, 2004). T4SS's have additionally been linked with biofilm formation and the intracellular survival of symbionts (Masui *et al.*, 2000; Hubber *et al.*, 2004; Backert and Meyer, 2006). Highly specialised T4SS's are also used for the injection of virulence factors by both plant and animal pathogens (Backert and Meyer, 2006). The conjugation-mediating T4SS has been detected on plasmids belonging to many members of the *Roseobacter* clade including *Dinoroseobacter shibae* DFL12T and *Silicibacter* sp. TM1040 (Moran *et al.*, 2007; Wagner-Döbler *et al.*, 2010). The presence of genes encoding the type 2 toxin-antitoxin system *ParDE* and death on curing protein (*doc*) within the *Labrenzia* plasmids suggests that the stability of these conjugative plasmids is maintained via a toxin-antitoxin system (Kang *et al.*, 2018).

All six plasmids share large, conserved regions (ranging up to 13kb in length) that are interrupted by regions unique to each host strain. The syntenic regions shared high peptide sequence similarities, ranging from 77% to 99% amino acid identity. These

encode the *tra* and *trb* operons necessary for conjugative plasmid transfer, repABC operons, heavy metal resistance genes and those involved in phosphonate utilization. Their combined presence suggests a common origin for the plasmid in the six strains although they originated from distinct, geographically isolated regions. In addition to strain 5N from the Arabian Sea (Wyman *et al.*, 2013), the origin of the other strains were: a biocathode community enriched from seawater collected off the New Jersey, USA coastline; crude oil contaminated beach sands from the Gulf of Mexico, and the red alga, *Rhodospirillum rubrum*, from the Yellow Sea off South Korea (Overholt *et al.*, 2013; Wang *et al.*, 2016).

Petersen and Wagner-Döbler (2017) recently demonstrated the importance of conjugative plasmid transfer among marine Rhodobacterales. They showed that syntenic plasmids acquired independently by three distinct species of *Roseobacter* all originated from another unknown alphaproteobacterial donor. Evidence for the transfer of plasmids between unrelated species provides insight into the possible importance of conjugative plasmids in helping to shape the widespread success of Rhodobacterales in marine environments. Petersen and Wagner-Döbler (2017) suggest that gene transfer by conjugation may be especially prevalent among those species found within epibiotic micro-communities. Conjugal transfer genes, including the pilus assembly machinery, have been implicated also in adhesion and physical association thereby promoting biofilm formation and micro-community stability (Sauer *et al.*, 2000; Ghigo, 2001).

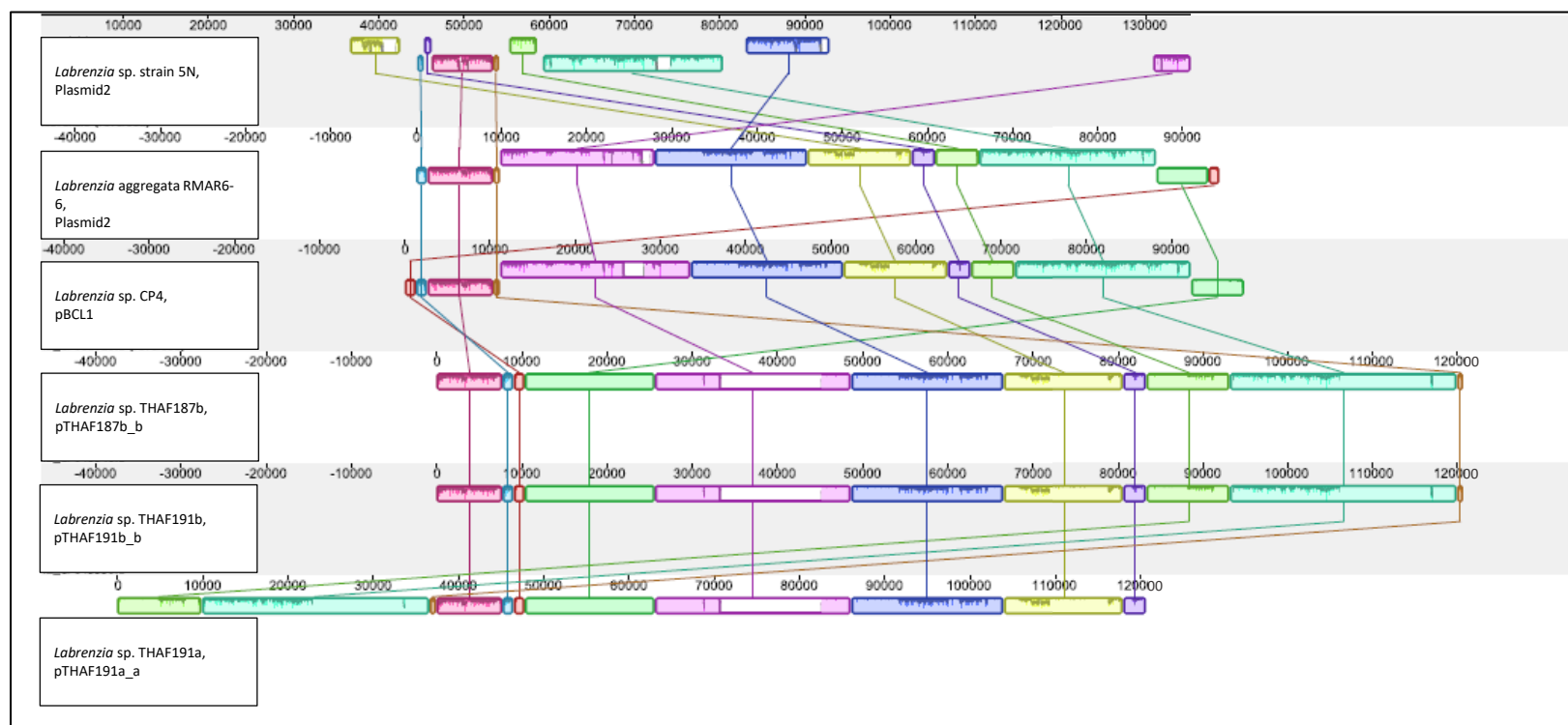


Figure 3.8. Mauve alignment of six conjugative plasmids harboured by *Labrenzia* sp. strain 5N (plasmid 2), *Labrenzia aggregata* RMAR6-6 (plasmid 2), *Labrenzia* sp. CP4 (pBCL1), *Labrenzia* sp. THAF187b (pTHAF187b_b), *Labrenzia* sp. THAF191a (pTHAF191a_a) and *Labrenzia* sp. THAF191b (pTHAF191b_b).

Hypothesized co-transfer of the denitrification and conjugative plasmids in *Labrenzia* spp. Genomic signatures such as GC content and codon usage bias are commonly used as indicators of horizontal gene transfer. This is because genomic regions or, indeed, entire replicons including plasmids, may exhibit different trends in these properties from those of their host (Wu *et al.*, 2012; Li and Du, 2014; Shintani *et al.*, 2015). Analyses of the *Labrenzia* replicons show that the GC content of the hosts chromosome is similar to that of the “denitrification plasmids” (t-test, p-value 0.2542) but significantly different in all strains from that of the conjugative plasmids (t-test, p-value 0.0002). Codon usage bias of the replicons present in strain 5N and its two closest relatives was also investigated. Relative Synonymous Codon Usage (RSCU) values were calculated for all 8 *Labrenzia* replicons belonging to *Labrenzia* sp. strain 5N, *Labrenzia aggregata* RMAR6-6 and *Labrenzia* sp. CP4 (Appendix Table 8.4). Results mirror that of the GC content analyses. The three conjugative plasmids (*Labrenzia* sp. strain 5N plasmid 2, *Labrenzia aggregata* RMAR6-6 plasmid 2 and *Labrenzia* sp. CP4 pBCL-1) display distinct codon usage patterns when compared with the hosts chromosomes (Appendix Figure 8.7).

The co-occurrence of the two plasmids in five *Labrenzia* strains might be a result of their sequential acquisition or may have arisen from their simultaneous transmission. The results of the foregoing analyses would suggest that the conjugative plasmid was acquired more recently than the “denitrification plasmid”, whose genomic signature has become more similar to that of the chromosome over time. However, it seems rather improbable that the co-occurrence of these two replicons should result from two independent plasmid-transfer events in five independent *Labrenzia* strains from three different environments and geographic locations. In addition, if the two plasmids were not co-transferred it would be expected that the conjugative plasmid should be found more frequently than it is, since it is more readily transmissible. Only in one strain (*Labrenzia* sp. CP4) has the conjugative plasmid been detected in the absence of the “denitrification plasmid” to date.

Considering that the “denitrification plasmid” doesn’t appear to be self-transmissible, it seems more probable that both plasmids were acquired through simultaneous transfer. Indeed, the conjugative plasmids may have a role in mediating the transfer of the second larger “denitrification plasmid” that encodes several phenotypic traits of potential benefit to the new host apart from the DN genes. Co-transfer of multiple

plasmids is known to result from mechanisms including synchronised conjugation, mobilization and co-integration (Dionisio *et al.*, 2019). The “denitrification plasmids” lack the genetic machinery required for conjugation or mobilization, though some harbour a number of transposases and mobile element proteins. The very large size (>400-Kb) of these megaplasms would militate against their transfer via transduction or transformation. Non-mobilizable plasmids (absence of *oriT* region and/or relaxases) may potentially transfer through co-integration, however, which relies on plasmid recombination (Dionisio *et al.*, 2019). If this mechanism was operative in the present case, the conjugative plasmid and “denitrification plasmid” would have been resolved following transfer since they now exist as separate replicons in all cases.

Interestingly, strain 5N harbours homologues of genes encoding the CinA, ComEC, and TfoX/Sxy family proteins as well as the DNA-protecting protein DprA involved in the uptake of large DNA fragments (including whole chromosomes) by natural transformation (Karudapuram *et al.*, 1995; Ando *et al.*, 1999; Dai *et al.*, 2018; Huang *et al.*, 2019). The presence of these genes suggests strain 5N has the capacity to develop natural competence at some point during its development. The ability of this strain to acquire plasmid DNA through *in vitro* conjugation was demonstrated experimentally in the present study and is described in detail elsewhere (Chapter 6). Strain 5N was not demonstrably competent for transformation under the experimental conditions tested. Nevertheless, the presence of these chromosomal elements in addition to the conjugal transfer systems harboured by plasmid 2 suggest the strain might become competent under certain natural conditions. Although natural competence can be constitutive, it is also known to be induced only under particular circumstances including stressors such as that caused by high cell density, antibiotic presence or starvation (Blokesch, 2016).

It remains that the means of transmission of these two *Labrenzia* plasmids is unclear. As Dionisio *et al.* (2019) speculate, when considering other similar evolutionary conundrums, many mechanisms of co-transfer of seemingly non-mobilizable plasmids likely remain to be discovered. If they were inherited by co-transfer, the differences in their genomic signatures suggest that the “denitrification plasmid” was subject to higher levels of codon usage selection in the host, perhaps because it carries particularly useful metabolic traits. This form of selection would lead to the pronounced

difference in the substitution rates for the two replicons observed because the evolutionary rate of the “denitrification plasmid” would appear somewhat faster (DiCenzo and Finan, 2017). For the same reason, it is possible that both plasmids were initially co-acquired by all ten strains harbouring the denitrification plasmid before, in some, the conjugative plasmid was lost owing to environmental selection and/or to reduce the metabolic costs associated with its maintenance. Even though the “denitrification megaplasmid” does not appear to have been acquired recently by the ten *Labrenzia* strains, the replicon remains independent and has not been integrated into the chromosome. Several studies suggest that multipartite genomes might even be advantageous and may increase fitness if replicons remain separate (DiCenzo and Finan, 2017).

3.5 Conclusions

This work, combined with characterisation performed in chapter 2, provides a comprehensive overview of the physiology, metabolism and functional potential of *Labrenzia* sp. strain 5N. When put into the context of a representative *Trichodesmium*-associated denitrifier, the study highlights the vast functional potential of these epibionts. Their metabolisms and nutrient cycling capacities could interact with other members of the consortium or indeed with *Trichodesmium* itself. The genetic capacity for pathways involved in N cycling represent a starting point for further studies N transfer between these denitrifiers and *Trichodesmium*, given the potential consequences of these processes on N losses.

Strain 5N is metabolically versatile, with 54% of all CDS being implicated in metabolic functions. It is a strain of potential climatic and environmental importance with the genetic potential to metabolise and synthesise DMSP, oxidise CO, degrade aromatic compounds and to produce NO and N₂O as by-products of denitrification. Genome analysis revealed that the denitrification genes, apart from those required for nitrate reduction, are located on a large repABC-type plasmid (~400 kb). Members of the *Labrenzia* genus are known colonisers of other organisms and frequently live in association with other microbes in biofilms (Pujalte *et al.*, 2005; Kim *et al.*, 2006; Bibi *et al.*, 2014; Blanchet *et al.*, 2017; Coates and Wyman, 2017). The plasmid localisation of the denitrification genes hints at their potential ease of horizontal transfer within

such micro-communities. Long-range synteny was detected both for the “denitrification plasmid” and independently for a second conjugative plasmid also harboured by certain *Labrenzia* strains. Given that these *Labrenzia* hosts are isolates from quite distinct environments that are also geographically separated, it was suggested that both plasmids may have been acquired simultaneously through conjugation-mediated events. This work, therefore, provides some hints of the potential for the active transfer of genetic material between members of the genus *Labrenzia* and perhaps more widely.

Chapter 4:

Proteomic analysis of adaptation to anaerobiosis in *Labrenzia* sp. strain 5N, a representative of the *Trichodesmium*-associated denitrifying community

4.1 Abstract

Labrenzia sp. strain 5N is a marine, heterotrophic, denitrifying bacterium isolated from the northwest Arabian Sea. It, and its close relatives, encounter a wide range of oxygen concentrations in their natural environment. These organisms are present in well-ventilated surface waters, throughout the extensive Oxygen Minimum Zone (OMZ) in this region, and within the epibiotic community of *Trichodesmium*. Previous studies demonstrated that transcription of structural components of the denitrification apparatus was upregulated by as much as 2000-fold when strain 5N was switched from aerobic to anaerobic growth conditions. The present study investigated how strain 5N adapts to anaerobic growth at the post-transcriptional level by comparing the proteomes of cells grown either aerobically or while denitrifying in the absence of oxygen. A shotgun proteomics approach revealed 634 proteins were changed in abundance between the two growth conditions, representing ~10% of the entire predicted protein pool. Of these, 333 proteins increased in abundance under anaerobic conditions, representing 52% of the differentially expressed proteome detected, while 301 decreased in abundance when compared to aerobic conditions. Cellular metabolism underwent major transitions under anoxia, most notably with the substantial upregulation of the reductases and associated proteins involved in denitrification. The electron transport chain shifted composition towards a dominance of *cbb₃*-type cytochrome oxidases, cytochrome *c* oxidase and cytochrome *c₂*. Central cellular processes including proteins involved in DNA replication, transcription and translation were downregulated when compared with the proteome of aerobically grown cells. The strain appears to enter a more sedentary lifestyle under denitrifying conditions. Flagellum biosynthesis is downregulated while proteins associated with biofilm formation increased in abundance suggesting a greater facility to attach to substrates and/or other organisms when oxygen is limited. These findings are of

interest in terms of N cycling, considering their known physical association with the N₂-fixer *Trichodesmium*.

4.2 Introduction

Proteomics, a term first introduced in the 1990's, designates the "PROTein complement of a genOME" (Wilkins *et al.*, 1996). It is the study of the array and quantity of proteins produced by an organism, tissue or system, at any given time. Of the total potential number of proteins (the theoretical proteome) only a certain proportion (the expressed proteome) are produced by the cell at any one time. Gene expression studies, using techniques such as microarray analysis and RTqPCR, enable changes in transcription to be determined through mRNA quantification. The post-transcriptional and post-translational regulation of protein synthesis, however, means that mRNA abundance alone is insufficient to confirm the successful production of a functional protein or pathway (Cox and Mann, 2007; Aslam *et al.*, 2017). Through proteomics, a wide range of cellular processes can be investigated including metabolic processes, protein-protein interactions, protein production and degradation rates, and the localisation of protein expression.

The proteome of an organism constantly changes and reflects the physiological and environmental conditions to which it is acclimated (Tomanek, 2010). Marine bacteria are subject to variable physical conditions and may encounter steep gradients in nutrient availabilities. Their capacity to adapt physiologically and metabolically to new conditions relies on their underlying genetic potential and the subsequent ability to alter cellular protein composition when required. Whether adopting a physiological/biochemical approach or studying the transcriptome or proteome, comparative analysis of cells grown under varying physiological states is a fundamental way to unveil biological processes. Protein expression can be studied quantitatively to compare the key differences in bacteria from different environments. Advanced mass spectrometry (MS)-based techniques have been developed for both qualitative and quantitative proteomics analyses over the past decades (Yates III, 1998; Ong and Mann, 2005). MS analysis uses the mass to charge (m/z) ratio to identify proteins. Shotgun proteomics identifies and quantifies proteins using a "bottom-up" strategy based on the detection of smaller peptides to then infer protein identity (Zhang *et al.*, 2013). In general, the peptides derived from proteolysis of the

sample are fractionated and analysed by liquid chromatography tandem mass spectrometry (LC-MS/MS), and proteins are then identified by comparison with protein (and/or nucleotide) databases. The use of peptides for LC-MS/MS is more straightforward than for intact proteins and this, alongside the increasing availability of whole genome sequences, has contributed to the popularity of the shotgun proteomics approach. The shotgun strategy has many applications from single cells to complex micro-community studies and is used in a wide range of sectors including environmental research, forensic investigation, agriculture and medicine (Corthals *et al.*, 2012; Zhang *et al.*, 2013; Mahalingam, 2017).

Metabolic plasticity underlies the success of marine microbes. When oxygen concentrations in the environment are limiting, facultative denitrifying bacteria can switch from respiring oxygen to using nitrate/nitrite as the terminal electron acceptor. This flexibility means denitrifiers can adapt to changing environmental conditions by modifying their respiratory machinery. *Labrenzia* sp. strain 5N is a marine heterotrophic bacterium with the genetic capacity to denitrify nitrate fully to dinitrogen gas (Coates and Wyman, 2017). The present study provides a comprehensive overview of the adaptation to anoxia in strain 5N; a representative of the *Trichodesmium*-associated denitrifying community. Previous work showed that transcripts encoding the terminal gene product in the denitrification pathway, *nosZ*, are expressed within *Trichodesmium* colonies (Coates and Wyman, 2017). Additionally, Coates and Wyman (2017) demonstrated that this gene is regulated by oxygen availability in strain 5N and is not expressed at significant levels under oxic conditions. To date, however, most of the work on denitrification in *Labrenzia*-like denitrifiers associated with *Trichodesmium* has been based on transcriptional analyses (Wyman *et al.*, 2013; Coates and Wyman, 2017).

Marine denitrification is a major process in marine nitrogen cycling and is responsible for the production and consumption of the greenhouse gas, nitrous oxide. Proteomics studies on denitrifying bacteria have been limited in their scope and have focussed on well-studied genera such as *Paracoccus*, and those of interest to the biotechnology industry (Zhang *et al.*, 2018). Here, a shotgun proteomics approach based on LC-MS/MS analysis was used to compare the proteome of strain 5N cells grown aerobically or anaerobically using nitrate as the terminal electron acceptor. Proteomics analysis was employed to provide a more complete understanding of the regulation of

the denitrification apparatus to complement earlier characterisation and genome analyses of *Labrenzia* sp. strain 5N (Chapters 2 and 3). The present study provides insights into the regulation of central metabolic pathways and other physiological changes that accompany a switch to growth under anoxia.

4.3 Materials and Methods

Protein extractions, LC-MS/MS and initial data manipulations were kindly performed by Dr Sabine Matallana-Surget at the University of Mons, Belgium. Analysis and interpretation of the primary data was performed as part of the present investigation.

4.3.1 Microorganisms, media and culture conditions

Labrenzia sp. strain 5N was isolated previously, and brought into culture, from sub-surface (120 m) water samples collected from the Arabian Sea in September 2001 (Wyman *et al.*, 2013). Experimental cultures (n=3) were grown at 33°C in 25 mL of 75% (vol:vol) ASW medium (Wyman *et al.*, 1985) amended with 2 g.L sodium acetate, 1 g.L yeast extract, 2 g.L sodium nitrate, and 1 g.L ammonium chloride (Coates and Wyman, 2017). Aerobic cultures were grown to mid-log phase in loosely-bunged, 250 mL conical flasks in an orbital incubator (200 rpm). Anaerobic cultures were grown in sealed 25 mL glass universal bottles in a static incubator. The stock medium was boiled until bubbling ceased and the starting volume was maintained by the addition of boiled, sterile distilled water to replace losses to evaporation. The experimental universals were filled to the brim with medium and sealed with screw caps lined with silicon rubber septa. Once cooled, a sodium dithionite solution was injected via the pierceable septum to a final concentration of 20 mg.L⁻¹ to remove any residual oxygen. An inoculum from an overnight anaerobic culture was then injected and cultures were grown for 48 hours to mid-log phase.

4.3.2 Protein extraction and LC-MS/MS

Protein extraction and Quantification. The cell suspensions were centrifuged at 8000 g for 5 minutes at 4°C and washed once in 25 mL sterile deionised water. The resulting cell pellets were transferred to a microcentrifuge tube and centrifuged once more at 16,000 g for 1 minute. The supernatant was removed and the concentrated pellets were dried for 15 minutes at room temperature in a Savant Speedvac DNA100 Vacuum Concentrator (Thermofisher, etc.) before freezing at -80°C until analysis.

The thawed cell pellets were resuspended in one pellet volume of Lysis Buffer (6M guanidine chloride), and mechanically lysed by sonication at 4°C (5 cycles of 10 s, amplitude 60%, 1 pulse rate). Lysed cells were centrifuged at 16,000 g at 4°C for 15 min and the supernatant fraction was used for analysis. Protein samples were reduced with 25 mM dithiothreitol (DTT) at 56°C for 30 min and alkylated with 50 mM iodoacetamide at room temperature for 30 min. Proteins were then precipitated with cold acetone overnight at -80°C, with an acetone/protein ratio of 4/1. The protein pellet was dissolved in 100 mM phosphate buffer (pH 8) containing 2 M urea. Total protein concentration was determined by the Bradford method (Bradford, 1976) according to the manufacturer's instructions, using bovine γ -globulin as a protein standard (Bio-Rad Protein Assay kit, Bio-Rad, Hertfordshire, United Kingdom). For LC-MS/MS analysis, a tryptic digestion (Sequencing grade modified trypsin, Promega) was performed overnight at 37°C, with an enzyme/substrate ratio of 1/25.

Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS). Protein identification and quantification were performed using a label-free strategy on an UHPLC-HRMS platform composed of an Eksigent 2D liquid chromatograph and an AB SCIEX Triple TOFTM 5600. Peptides were separated on a 25 cm C18 column (Acclaim pepmap 100, 3 μ m, Dionex) by a linear acetonitrile (ACN) gradient [5–35% (v/v), in 15 or 120 min] in water containing 0.1% (v/v) formic acid at a flow rate of 300 nL min⁻¹. Mass spectra (MS) were acquired across 400–1,500 m/z in high-resolution mode (resolution > 35000) with 500 ms accumulation time and MS/MS were acquired across 100–1,800 m/z. A long run procedure was used to acquire quantitative data, and a duty cycle of 3 s per cycle was used to ensure that high quality extracted ion chromatograms (XIC) could be obtained.

Protein searches were performed against the *Labrenzia* sp. strain 5N genome, using ProteinPilot Software v4.1. Search parameters included differential amino acid mass shifts for oxidized methionine (+15.9949 Da). The identification of the overall set of proteins was validated by manual inspection of the MS/MS ion spectra, ensuring that a series of consecutive sequence-specific b- and y-type ions was observed. For quantification, the quant application of PeakView was used to calculate extracted ion chromatograms (XIC) for all peptides identified with a confidence > 0.99 using ProteinPilot. A retention time window of 2 min and a mass tolerance of 0.015 m/z were used. The area under the curve was exported in MarkerView, in which they were

normalized based on the summed area of the entire run. MarkerView enabled an average intensity for both experimental conditions to be calculated, as well as the significance of the difference between conditions based on a student t-test. Quantified proteins were kept with a p-value < 0.005 and with at least two peptides quantified with a p-value < 0.005. The false discovery rate (FDR) was calculated at the peptide level for all experimental runs using the Decoy option in Mascot; this rate was estimated to be lower than 1% using the identity threshold as the scoring threshold system. In order to be considered as well-quantified, proteins had to meet two criteria: fold change within the cut-off thresholds for a significantly differential regulation (1.5- or below 0.66-fold change in the anaerobic samples relative to the aerobic samples) and their p-value had to be < 0.005.

4.3.3 Computational analysis of the proteome

Protein annotations were determined via a combination of EggNOG, Blast and manual annotations. The 634 proteins were sorted into functional categories based on Clusters of Orthologous Groups (COGs) using the functional classifications obtained by EggNOG (Tatusov *et al.*, 2000; Huerta-Cepas *et al.*, 2016). Proteins were classified into 20 functional groups and more broadly into 3 main categories (Information Processing and Storage, Cell Processes and Signalling, Metabolism).

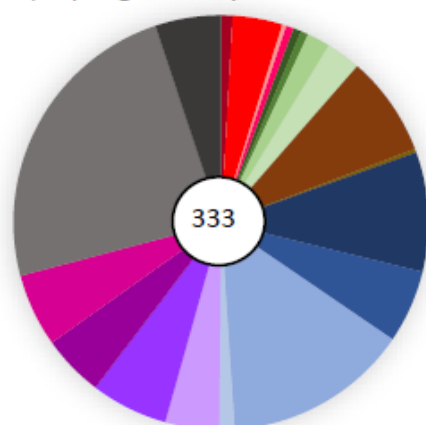
4.4 Results and Discussion

4.4.1 Response to anaerobiosis: shifts in central metabolism and slowed cell growth

Functional protein annotation. Six hundred and thirty-four proteins were identified as differentially abundant under aerobic or anaerobic growth conditions, implementing the commonly used cut-off thresholds of 0.66 and 1.5. This equates to ~10% of the theoretical protein-coding capacity of *Labrenzia* sp. strain 5N. Of these 634 proteins, 615 (97%) were categorised into Clusters of Orthologous Protein groups (COGs; Tatusov *et al.*, 2000) based on their function in cellular processes (Figure 4.1 and Appendix Table 8.7). Differential expression of metabolic functions dominated (51.4% of upregulated and 39.9% of downregulated proteins), highlighting a notable shift in global metabolic pathways under anaerobic conditions. Energy production and conversion (COG group C: 14.4%), amino acid transport and metabolism (COG group

E: 9.3%) and carbohydrate transport and metabolism (COG group G: 5.7%) were among the most highly upregulated COG groups. There was a decline in the abundance of proteins involved in information processing and storage, including cellular replication, transcription, ribosomal structure and translation which combined represented nearly 40% of the downregulated proteome (COG groups J: 29.2%, K: 6%, L: 4.3%).

A) Upregulated proteins



B) Downregulated proteins

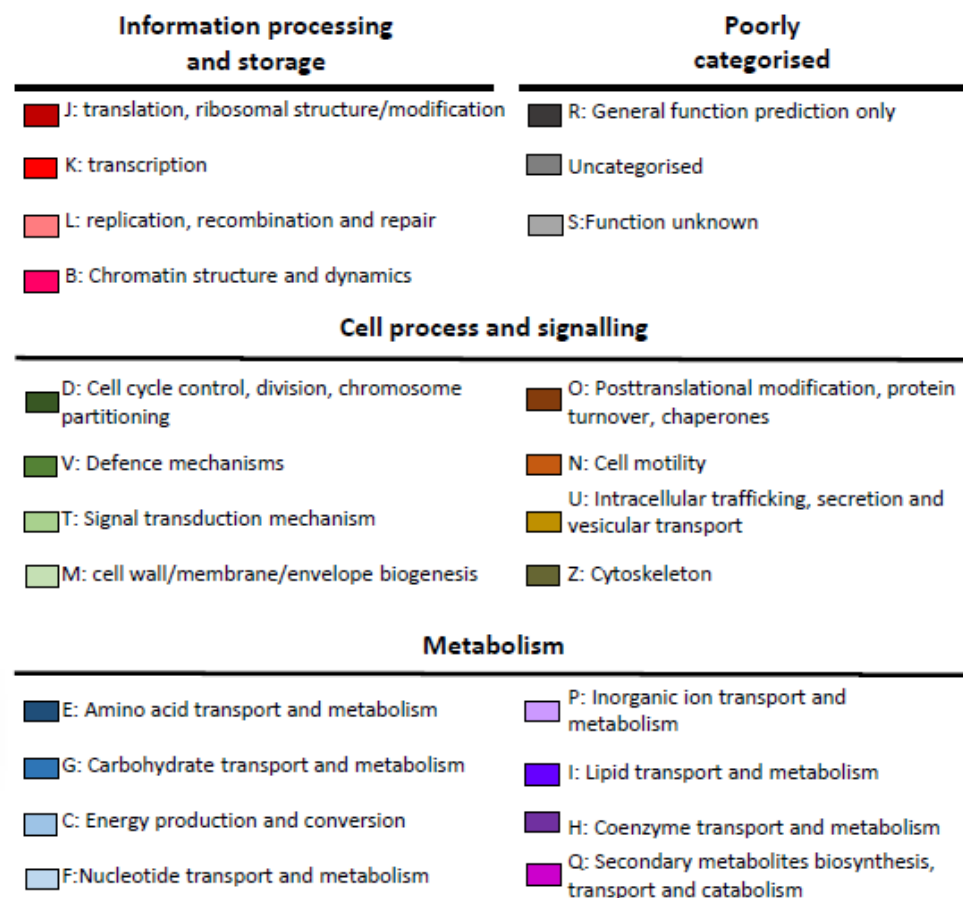
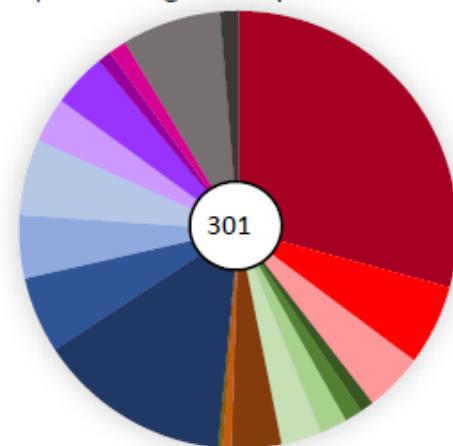


Figure 4.1. Functional classification of the 634 *Labrenzia* sp. strain 5N proteins with changed abundance under anaerobiosis. A) COG classification of the 333 proteins upregulated under anaerobic conditions. B) COG classification of the 301 proteins downregulated under anaerobic conditions.

Changes in central metabolism under anaerobiosis. Fundamental adaptations of central metabolism to growth under anaerobic conditions were readily apparent in the proteome. Components of the TRAP transport system, DctPQM, responsible for the uptake of C4-dicarboxylates such as malate and aspartate, were all upregulated (HEP89_13540, 5.09-fold; HEP89_03490, 3.78-fold; HEP89_25890, 2.79-fold; HEP89_15385, 1.76-fold) (Figure 4.2 and Appendix Table 8.7). During denitrification the DctPQM transport system is required to support the growth of *Wolinella succinogenes* on succinate (R. G. Simon *et al.*, 2000) and there is some evidence that C4-dicarboxylates may be preferentially used during nitrate respiration. As in strain 5N, C4-dicarboxylate transporters were upregulated during anaerobic denitrification in *Paracoccus denitrificans* PD1222 (Olaya-Abril *et al.*, 2018) and were shown to be essential for supporting anaerobic growth in *Dinoroseobacter shibae* (Ebert *et al.*, 2013).

All three carbon monoxide (CO) dehydrogenase subunits (CoxMSL) were also upregulated under anaerobiosis (HEP89_21400, 2.2; HEP89_21395, 2.19; HEP89_21405, 2.19) (Figure 4.2 and Appendix Table 8.7). At low CO concentrations (<100ppm), the type strain *Labrenzia aggregata* IAM12614 was shown to oxidise CO anaerobically only if nitrate is present (King, 2006). Although the rationale for this dependence on nitrate remains unclear, *L. aggregata* IAM12614 cannot use CO as a carbon substrate for growth. The oxidation of CO yields two reducing equivalents in addition to CO₂, however, and it seems likely that nitrate replaces oxygen as the terminal electron acceptor to support CO oxidation.

Components of the Entner-Doudoroff pathway (EDP), including phosphogluconate dehydratase (HEP89_17120) and keto-deoxy-phosphogluconate aldolase (HEP89_17115), declined in abundance by more than twofold in anoxic conditions. This response suggests underlying changes in the pathways used for glucose catabolism (Figure 4.2). The EDP represents an alternative route to glycolysis for the generation of the central metabolite, pyruvate, and is commonly found in aerobes (Conway, 1992) including members of the *Roseobacter* clade (Fürch *et al.*, 2009). The energy yield from the EDP is only 1 ATP per glucose molecule hydrolysed, however; half that generated via the Embden–Meyerhof–Parnas (EMP) glycolytic pathway (Flamholz *et al.*, 2013). Consequently, the EDP may be unfavourable when ATP

generation is already lower during anaerobic respiration and the resulting energy limitation may explain its downregulation.

The downregulation of the EDP and the upregulation of CO dehydrogenase and DctPQM suggest close control of carbon source utilisation under anaerobiosis. These changes in the proteome were accompanied by notable shifts in the abundance of several transcription factors. These included the LysR family transcription factor involved in carbon and nitrogen metabolism activation or repression (HEP89_11550, 0.28-fold), the LacI family transcriptional repressor involved in the control of carbon source utilisation (HEP89_28035, 2.42-fold) and the GntR family general metabolic repressor (HEP89_22610, 1.73-fold).

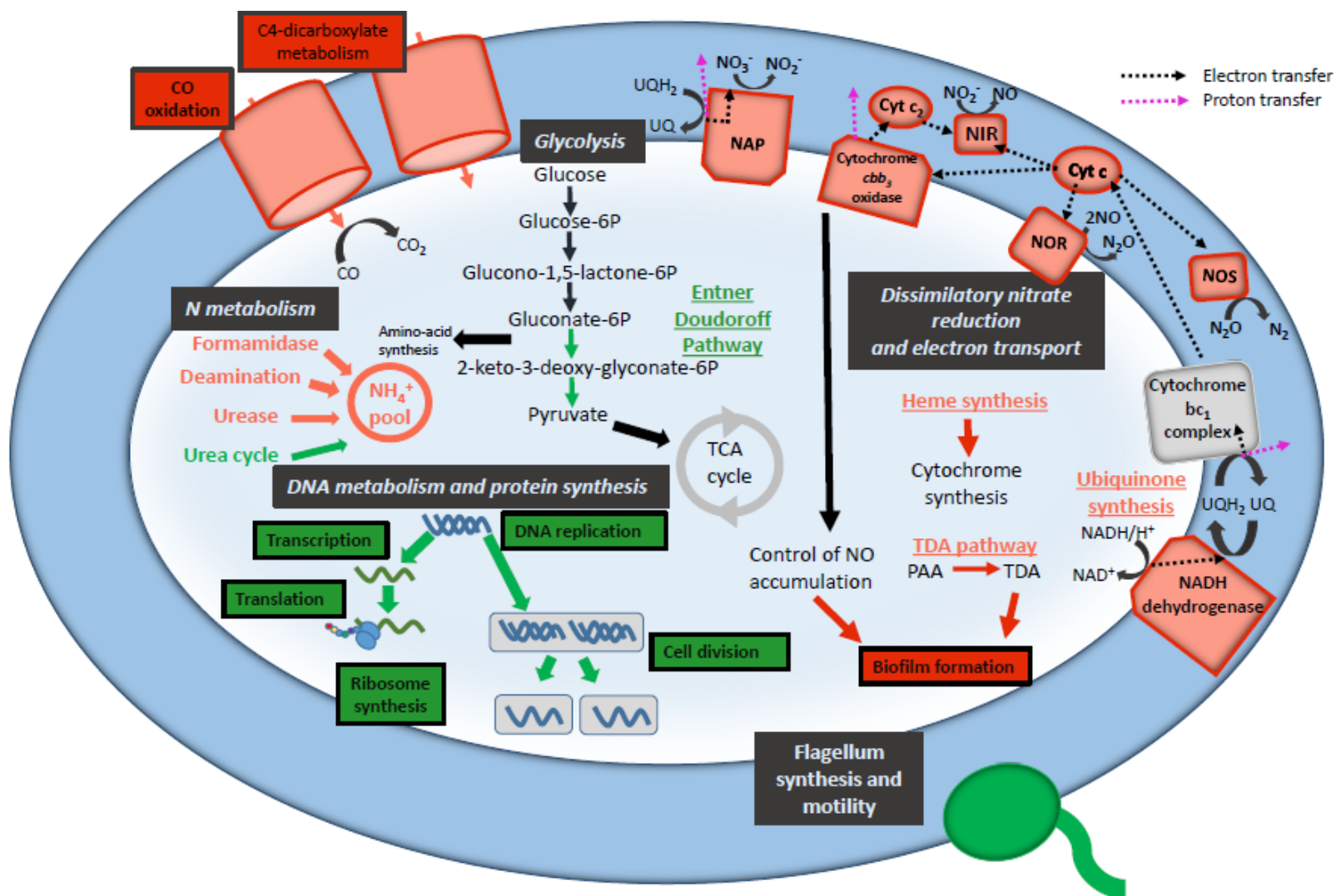


Figure 4.2. Diagram illustrating the major changes in cellular processes under anaerobiosis in *Labrenzia* sp. strain 5N. Up and downregulated processes and proteins are highlighted in red or green, respectively.

Cellular response to anaerobiosis: reduced cell growth and protein turnover.

Together, proteins involved in DNA replication and transcription represented over 10% of the downregulated proteome (COG groups L and K) (Figure 4.1). The downregulation of DNA topoisomerases, DNA helicases, DNA polymerases and chromosome replication proteins is indicative of a global decline in the rate of DNA replication under anaerobiosis (HEP89_24295, 0.61-fold; HEP89_25650, 0.39-fold; HEP89_06420, 0.62-fold; HEP89_22020, 0.59-fold; HEP89_24765, 0.43-fold; HEP89_06410, 0.63-fold; HEP89_06405, 0.2-fold) (Figure 4.2 and Appendix Table 8.7). Accordingly, the production of enzymes required for the synthesis of DNA/RNA precursors, including deoxyribonucleotide triphosphates (dNTPs), was also reduced (HEP89_24625, 0.5-fold; HEP89_20655, 0.38-fold; HEP89_21445, 0.61-fold). The abundance of cell division proteins decreased and those involved in DNA repair mechanisms were lower under anoxic conditions (HEP89_16540, 0.66-fold; HEP89_16525, 0.53-fold; HEP89_28565, 0.31-fold). The downregulation of RNA polymerase and related transcription termination factors suggested a general decline in the rates of transcription (Figure 4.2 and Appendix Table 8.7). All of these observations are consistent with the slower anaerobic growth rates observed experimentally in strain 5N (Chapters 2 and 5). Slower DNA metabolism accompanied by a reduction in the rate of cell division is to be expected as a result of the lower ATP yield from denitrification compared with that from aerobic respiration (Strohm *et al.*, 2007; Chen and Strous, 2013).

Reduced transcription ultimately leads to lower *de novo* protein production. Accordingly, 29.2% of the downregulated proteome was involved in translation and ribosome synthesis (COG group J) (Figure 4.1). The synthesis of core amino acids was reduced, with a downregulation of enzymes including acetolactate synthase (HEP89_16985, 0.27-fold) and isopropylmalate isomerase (HEP89_08320, 0.57-fold). Aminoacyl-tRNA synthesis was lower during denitrification and 20 tRNA ligases showed a decrease in abundance of at least 0.65-fold (Appendix Table 8.7). All of the 50S large ribosomal subunit proteins (L1-31) and the 30S small ribosomal subunit proteins (S1-21) were downregulated. Reduced protein biosynthesis was further apparent from the downregulation of translational elongation factors, initiation factors and peptide chain release factors. Heightened capacity for protease activity also suggests protein catabolism was increased, generating a pool of amino-acids that can

be recycled and used in the *de novo* synthesis of other proteins. These findings are entirely consistent with the reduction in the growth rate of cells grown anaerobically.

4.4.2 A clear switch to nitrate respiration under anaerobiosis

Nitrate reduction. The first step in denitrification, the reduction of NO_3^- to NO_2^- , is catalysed by the periplasmic dissimilatory nitrate reductase (NAP) encoded by the *napEFBAC* operon. Proteome analysis showed considerable upregulation of the catalytic subunit NapA as well as NapB and nitrate reductase cytochrome c550-type subunit (HEP89_19450, 10-fold; HEP89_19455, 22-fold; HEP89_19445, 6.2-fold). Although the increase in abundance of NAP enzymes reached 22-fold, this was still the smallest upregulation of all of the denitrification related reductases. This might indicate the constitutive expression of the NAP operon under aerobic conditions, as has been reported for other denitrifiers, to facilitate the smooth switch from oxygen to nitrate respiration when environmental conditions alter (Zumft, 1997b).

Nitrite reduction. NO_2^- is further reduced to nitric oxide (NO) by a dissimilatory periplasmic cytochrome *cd*₁-type nitrite reductase (NIR), encoded by *nirSCFDLGHJN*. There was pronounced upregulation of the catalytic subunit NirS, heme d₁ cofactor biosynthesis proteins NirFGHJ and the cytochrome NirN (HEP89_27870, 695-fold; HEP89_27885, 21-fold; HEP89_27895, 18.68; HEP89_27900, 16.79-fold; HEP89_27905, 7.5-fold; HEP89_27910, 35.7-fold). At 695-fold higher cell concentration, NirS was the single most highly upregulated protein detected under anaerobic conditions, which might relate to the toxicity of NO_2^- as well as the catalytic inefficiency of this enzyme (Richter *et al.*, 2002). The Rrf2 family transcriptional regulator NsrR, which controls NIR expression through nitrite sensing in *Nitrosomonas europaea* (Beaumont *et al.*, 2004), was also increased 10.18-fold in strain 5N (HEP89_03290). Considering that denitrification is a major biological route for both the production and consumption of nitric oxide and the potent greenhouse gas nitrous oxide, the regulation of this pathway is particularly pertinent.

Nitric oxide reduction. NO generated by NirS is further reduced to N_2O by the nitric oxide reductase enzyme complex NOR, which also plays a role in NO detoxification. In *Labrenzia* sp. strain 5N this cytochrome *bc* complex is encoded by the *norCBQDEF* operon. Proteome analysis showed that the structural subunit NorC and the activation

protein NorQ were both highly abundant under anaerobiosis (HEP89_27865, 73.5-fold; HEP89_27855, 12.6-fold).

Nitrous oxide reduction. The terminal reduction in the denitrification pathway, N_2O to N_2 , is encoded by the nitrous oxide reductase (NOS) complex *nosRZDFYL* operon in *Labrenzia* sp. strain 5N. This copper containing, periplasmic enzyme transfers electrons from NOR to cytochrome *c*. The catalytic subunit, NosZ, and maturation proteins, NosR, NosD and NosF, were strongly upregulated (HEP89_27955, 117-fold; HEP89_27950, 19.9-fold; HEP89_27960, 4.9-fold; HEP89_27945, 6.3-fold respectively). In addition, enzymes required for cobalamin biosynthesis were upregulated (HEP89_22560, 1.5-fold; HEP89_22575, 1.5-fold; HEP89_25240, 4.6-fold). N_2O is cytotoxic and is known to interact with and inactivate vitamin B_{12} with a negative effect on B_{12} -dependant processes including methionine synthesis and DNA replication (Drummond and Matthews, 1994a, 1994b). In *Paracoccus denitrificans* PD1222 it was shown that Cu limitation leads to decreased NOS activity, with a consequent increase in cellular N_2O , resulting in an up-regulation of B_{12} independent pathways (Sullivan *et al.*, 2013). The apparent upregulation of cobalamin biosynthesis in strain 5N might relate to a reliance on B_{12} -dependant biosynthetic pathways as N_2O barely accumulates under strictly anaerobic conditions in this strain (see Chapter 5).

These observations demonstrate that the four key denitrification reductases were all upregulated substantially, confirming that this pathway was active in its entirety, culminating in the complete reduction to N_2 under anaerobiosis. The findings were consistent with the 718-fold and >2000-fold respective increases in *nirS* and *nosZ* mRNA abundance previously reported under anaerobiosis (Coates and Wyman, 2017). The results confirm that the detection of *nosZ* transcripts from this strain, and likely from other closely related organisms, indicates the presence of a complete suite of denitrification reductases expressed not only at the transcriptional level but also, more importantly, at the protein level.

Nitrogen assimilation. Changes in the abundance of proteins involved in nitrogen assimilation were also detected, alongside an increase in the abundance of urease structural and accessory proteins and assorted amino acid transporters (Figure 4.2). There was a notable upregulation of quinoxinohemoprotein amine dehydrogenase

(QHNDH) (HEP89_17925, 69-fold; HEP89_17930, 35-fold; HEP89_17915, 12.4-fold) and the associated maturation protein (HEP89_17920). QHNDH is involved in the deamination of aliphatic and aromatic amines including butylamine and benzylamine. This enzyme cleaves the N containing amino group (-NH₂) which is then reduced to ammonia. Under anaerobiosis, any ammonia formed is less likely to enter the urea cycle than when oxygen is present since numerous enzymes involved in this cycle were downregulated (HEP89_15215, 0.484-fold; HEP89_15230, 0.38-fold; HEP89_08515, 0.631-fold; HEP89_01910, 0.516-fold). Urease (HEP89_08380, 1.8-fold; HEP89_08400, 2.68-fold; HEP89_08370, 1.9-fold; HEP89_08360, 1.7-fold), catalysing the hydrolysis of urea to ammonia and CO₂, and the abundance of formamidase also increased (HEP89_00760.1, 3.45-fold), further adding to the potential pool of ammonia that can be assimilated through deamination.

4.4.3 Energy production and the adaptation of the electron transport chain to anaerobiosis.

The electron transport system underwent major changes in response to anaerobiosis. Cytochrome *c* oxidase and associated biogenesis proteins were substantially upregulated (HEP89_26765, 8.7-fold; HEP89_26775, 7.58-fold; HEP89_26780, 5.8-fold). This cytochrome is required for the transfer of electrons to *cbb*₃-type cytochrome *c* oxidase and the NIR, NOR and NOS complexes (Chen and Strous, 2013) (Figure 4.2). The membrane bound *cbb*₃-type cytochrome *c* oxidase, encoded by the *ccoNOPQ* operon (*fixNOPQ*) (Buschmann *et al.*, 2010), also showed notable upregulation of the component subunits and accessory proteins (HEP89_27910, 35.7; HEP89_01565, 12.9; HEP89_01575, 5.9-fold; HEP89_01560, 25.2-fold; HEP89_01555, 10.6-fold). A single copy of the *cbb*₃ oxidase operon was found in the genome of *Labrenzia* sp. strain 5N whereas some other facultative denitrifiers, including *Pseudomonas aeruginosa*, harbour two copies. One copy is constitutively expressed while the expression of the second is fully induced only at low oxygen levels (Comolli and Donohue, 2004). *cbb*₃ oxidases reduce O₂ to H₂O and in many bacteria their high oxygen affinity means they dominate the cytochrome complement in low oxygen conditions. Being a facultative denitrifier, strain 5N is not absolutely reliant on O₂ for growth and so O₂ scavenging is not the most likely role for this *cbb*₃ oxidase under anaerobiosis. *cbb*₃ oxidase can act as an electron donor to cytochrome *c*₂, which

can then transfer electrons to NIR (Daldal *et al.*, 2001; Hopper *et al.*, 2013). Accordingly, the periplasmic cytochrome *c*₂ and accessory proteins were also upregulated (HEP89_15175, 22.9-fold; HEP89_18935, 14.8-fold). Cytochrome *bd*, known to play an important role in both oxidative and NO stresses, also increased in abundance (HEP89_24050, 2.1-fold) (Giuffrè *et al.*, 2012). Had sodium dithionite not been used in this experiment to remove residual oxygen from the medium, cytochrome *bd* may have shown an even more prominent abundance during denitrification.

Major shifts in the nature of the electron transport were also observed through the increased abundance of ubiquinone biosynthesis proteins and ferredoxins (Figure 4.2 and Appendix Table 8.7) (HEP89_02190, 26.3-fold; HEP89_27995, 21.9-fold; HEP89_28000, 19.5-fold; HEP89_11860, 7.5-fold; HEP89_21910, 3.18-fold; HEP89_06380, 1.54-fold; HEP89_28660, 4.5-fold; HEP89_18850, 2.1-fold). Enzymes involved in the biosynthesis of heme were also upregulated, including delta-aminolevulinic acid dehydratase (HEP89_25550, 2.47-fold), porphobilinogen deaminase (HEP89_10370, 3.38-fold), uroporphyrinogen decarboxylase (HEP89_08180, 3.85-fold), coproporphyrinogen III oxidase (HEP89_14400, 3.64-fold), protoporphyrinogen III oxidase (HEP89_10385, 2.98-fold) and protoporphyrin ferrochelatase (HEP89_11675, 2.13-fold). Well studied denitrifiers like *Pseudomonas aeruginosa* increase the synthesis of heme, which is essential for electron transfer, under anaerobic conditions (Schobert and Jahn, 2002). The SUF system, involved in Fe transport and FeS assembly, was also upregulated (Ayala-Castro *et al.*, 2008) (HEP89_22450, 6.7-fold; HEP89_22475, 2.36-fold; HEP89_22470, 2.53-fold; HEP89_22465, 2.22-fold; HEP89_05975, 1.86-fold). Iron-sulfur cluster proteins are involved in the regulation of gene expression, O₂ and NO sensing, and are integral components of electron transport system. The regulatory protein NosR which is necessary for the synthesis of nitrous oxide reductase is itself a Fe-S protein (Crack *et al.*, 2014).

It is proposed that anaerobic nitrate respiration in strain 5N proceeds as follows (Figure 4.2). Electrons are transferred from NADH dehydrogenase to cytochrome *bc*₁ via ubiquinol, pumping protons into the periplasm in the process. From cytochrome *bc*₁, electrons are transferred to cytochrome *c* oxidase followed by the periplasmic NIR and NOS complexes as well as the membrane bound NOR complex. The electrons supplied by cytochrome *c* oxidase are used in the sequential reduction of each NO₂⁻,

NO and N₂O by the NIR, NOR and NOS complexes respectively, culminating in the generation of the N₂ as the end product of the denitrification pathway. Electrons may also be transferred from *cbb*₃-type cytochrome c oxidase to cytochrome *c*₂ and then on to NIR, as has been proposed for *Neisseria gonorrhoeae* (Hopper *et al.*, 2013). The proton motive force generated through the electron transport chain is used to drive ATP generation via the transmembrane ATPase.

4.4.4 Host association: switch to a sedentary lifestyle linked with denitrification

The expressed proteome showed that strain 5N exhibited a reduction in the expression of proteins required for motility and an increased potential to form biofilms under denitrifying conditions (Figure 4.2 and Appendix Table 8.7). This connection between biofilm formation and anaerobic respiration has been made previously for other organisms where it has been shown that the initiation of biofilm formation is often a response to stressful environmental conditions. Studies on *transcription* (Falsetta *et al.*, 2009) and protein expression (Phillips *et al.*, 2012) in *Neisseria gonorrhoeae* demonstrated that anaerobic respiration promotes biofilm formation. Clearly, microbes growing at high cell density within biofilms are more likely to be subject to microaerobic conditions and so it is unsurprising that there is a link with denitrification (Falsetta *et al.*, 2009).

Flagellum biosynthesis was reduced under anaerobiosis in strain 5N, with the downregulation of the motor switch proteins FliG (HEP89_14285, 0.59) and FliN (HEP89_14295, 0.34-fold) as well as the upregulation of a negative regulator of flagellar synthesis (HEP89_09865, 1.58-fold). A LuxR family transcription factor involved in the induction of biofilm formation was also upregulated (HEP89_18855, 1.69-fold) while the YebC/PmpR family transcription factor, a negative regulator of quorum sensing in *Pseudomonas aeruginosa* (Liang *et al.*, 2008), was downregulated (HEP89_12075, 0.39-fold). In addition, there was a marked increase in the abundance of a MarR family transcriptional activator of exopolysaccharide II synthesis (HEP89_14085, 2.96-fold). This galactoglucan has been linked with the regulation of motility and biofilm formation in the establishment of the symbiosis between *Rhizobium meliloti* and its host plant, alfalfa (Lloret *et al.*, 1998).

Hamada *et al.* (2014) demonstrated that *cbb₃* oxidases also play a role in biofilm formation under anaerobic conditions in *Pseudomonas aeruginosa*. *cbb₃* oxidase influences the accumulation of NO which, in turn, induces cell elongation and promotes the formation of biofilms; a known cellular response to environmental stressors (Justice *et al.*, 2008; Yoon *et al.*, 2011; Hamada *et al.*, 2014). Thus in strain 5N, the upregulation of *cbb₃* oxidases under denitrifying conditions is also implicated in biofilm formation through the control of NO levels. Additionally, catabolism via the Entner-Doudoroff pathway has been shown to negatively impact biofilm formation in *Vibrio cholerae* (Patra *et al.*, 2012). The downregulated EDP observed in strain 5N during denitrification, therefore, might also be conducive to the establishment of biofilms and the development of host associations.

Recent work with a marine *Roseobacter* strain (*Silicibacter* sp. strain TM1040) known to form close associations with phytoplankton, has demonstrated that a signalling molecule, named Roseobacter Motility Inducer (RMI), is involved in the establishment of motility (Sule and Belas, 2013). RMI, synthesized from phenyl acetic acid (PAA), increases the production of flagellae via regulation of *fliC3* transcription. RMI also acts as an algicide, negatively impacting host motility and eventually lysing algal cells. In the non-motile sessile phase of *Silicibacter* sp. strain TM1040, by contrast, the quorum-signalling molecule tropodithietic acid (TDA) is synthesized from PAA instead and its synthesis promotes the formation of biofilms. Several proteins involved in phenylacetic acid catabolism and the TDA pathway were significantly upregulated in the absence of oxygen in the present study suggesting that the TDA pathway may also regulate biofilm formation in strain 5N (HEP89_22245, 8.6-fold; HEP89_22205, 10-fold; HEP89_22215, 8.3-fold; HEP89_22210, 6.3-fold; HEP89_22220, 4.45-fold; HEP89_24860, 4.35-fold; HEP89_01745, 4.53-fold) (Figure 4.2 and Appendix Table 8.7) (Timmermans *et al.*, 2017). Additionally, an increase in the production of a reddish brown pigment is observed routinely in mature cultures of strain 5N which is a known phenotype linked with TDA production (Gram *et al.*, 2010; Harrington *et al.*, 2014; Timmermans *et al.*, 2017). TDA itself acts also as an antibiotic that in epibiotic bacteria protects the host from infection by pathogens and also reduces the extent of colonisation by other bacteria. As a result, TDA-producing bacteria are likely to have a competitive advantage in mixed species biofilms such as those associated with *Trichodesmium* (Coates and Wyman, 2017) and many other phytoplankton.

4.5 Conclusions

This shotgun proteomics study aimed to identify the major pathways active in *Labrenzia* sp. strain 5N under anaerobic conditions. This approach addressed the outstanding concern that the presence of *Labrenzia* denitrification gene mRNA transcripts in natural samples may reflect their constitutive expression, rather than a functional pathway at the protein level. Based on results of this study, we can be confident that previous detections of *nosZ* mRNA in suboxic waters, and within *Trichodesmium* colonies, indicates that denitrification is indeed active.

This study provides insights into the metabolic changes that occur in *Labrenzia* sp. strain 5N during denitrification and how this organism, as well as other closely related *Labrenzia* spp., may adapt to anaerobiosis when in association with *Trichodesmium* colonies. Strain 5N showed a high level of metabolic plasticity that would contribute to its success within high density, competitive environments like biofilms. 634 proteins were differentially expressed under aerobic and anaerobic conditions, which equates to ~10% of the total genome. All key reductases involved in the denitrification pathway including the NAR, NIR, NOR and NOS enzyme complexes increased in abundance markedly. A major respiratory shift was apparent, with changes in electron transport chain components consistent with anaerobic respiration and oxygen scavenging. At the cellular level, growth rates were reduced under anaerobiosis. Proteins involved in DNA replication, transcription, translation, amino acid and protein synthesis, as well as ribosomal subunit proteins all showed a decrease in abundance. The shift to denitrification is likely to be under the control of transcriptional and translational regulators that are implicated in the control global cell processes as well as in nitrite sensing, carbon and nitrogen source utilisation, antibiotic resistance, and biofilm formation.

Asides from the key processes directly related to anaerobic respiration, the shotgun proteomics approach also gave an overview of other strategies employed by denitrifying cells in the absence of oxygen. In particular, strain 5N showed an increased tendency to become less motile and to form biofilms under anaerobiosis. This suggests that this strain enters a more sedentary lifestyle during denitrification and has a greater facility to attach to substrates and/or other organisms. This observation is of note in the context of the association of closely related *Labrenzia* spp. with the cyanobacterium *Trichodesmium*. It implies (i) that denitrification is only

possible, in the upper water column at least, when *Labrenzia* spp. are in close physical association with the trichomes and (ii), that this process may be confined to larger *Trichodesmium* colonies in which the capacity for respiratory oxygen depletion is greatest.

This study serves as a reference for future work on N cycling by *Labrenzia*-type *Trichodesmium* associates. The results obtained complement previous work on the regulation of *nosZ* gene expression in these organisms (Coates and Wyman, 2017). Under anaerobiosis, a complete denitrification pathway including NosZ is in place at the protein level. Hence the detection of *Labrenzia*-type denitrification gene transcripts within *Trichodesmium* colonies and in bulk water column samples is indicative of the expression of fully functional denitrification pathways. The extent to which *Labrenzia*-type denitrifiers rely on N fixed by *Trichodesmium* is still undetermined, though newly fixed N is liberated by this diazotroph and nitrified in the upper water column. Further studies are essential to determine the wider biogeochemical significance of this interaction and the transfer of N between *Trichodesmium*-associated denitrifiers and the overlying atmosphere.

Chapter 5:

Nitrogen transformations in *Labrenzia* sp. strain 5N

5.1 Abstract

The marine bacterium *Labrenzia* sp. strain 5N is known to inhabit low oxygen waters and has the genetic potential for a range of N transformation processes. N isotopes (^{15}N) were used to determine the transformations of ammonium and nitrate performed by *Labrenzia* sp. strain 5N under both aerobic and anaerobic conditions. ^{15}N -Nitrite resulting from the oxidation of ammonium was detected by IRMS under aerobic conditions, demonstrating that this strain is capable of heterotrophic nitrification. Furthermore, it was shown that strain 5N can perform simultaneous heterotrophic nitrification and aerobic denitrification. The denitrification products of *Labrenzia* sp. strain 5N included NO_2 , N_2O , and N_2 . The relative production of N_2O [$\text{N}_2\text{O}/(\text{N}_2\text{O}+\text{N}_2)$] varied greatly with oxygen availability, reaching $75.5 \pm 0.28\%$ in the aerobic cultures and just $0.13 \pm 0.0016\%$ under anoxia, highlighting the inhibitory effect of oxygen on nitrous oxide reductase activity. The high amounts of N_2O produced in the presence of O_2 , reaching $\sim 10\text{mmoles.L}^{-1}$ culture, suggest aerobic denitrification by this strain could be a significant source of this potent greenhouse gas. This finding is particularly relevant considering the variable oxygen environments where this strain and its close relatives have been found; both within suboxic waters and in association with *Trichodesmium* colonies. Strain 5N is the first reported member of the genus *Labrenzia* with the capacity for aerobic heterotrophic nitrification-denitrification. This finding has implications for small-scale N-cycling within the *Trichodesmium* microbiome. Although the potential for *Trichodesmium*-associated denitrification has been demonstrated previously, the source of nitrate/nitrite in the oligotrophic surface waters where it is found is unclear. This study shows nitrification of the ammonium released by *Trichodesmium* by epibiotic strains related to 5N could provide a supply of nitrite as a substrate for denitrification under suitable conditions. Hence, *Trichodesmium* colonies may represent not just important sources of fixed N but also extensive sites of N recycling and potential loss from the oceans.

5.2 Introduction

Marine nitrogen (N) cycling controls the availability and global distribution of N within the oceans. An essential nutrient for all organisms, N availability sets an upper limit on marine productivity for much of the global ocean. The balance between N fixation and denitrification is key to the oceanic distribution and abundance of bioavailable N, such as nitrate and ammonium. Recent findings, however, suggest that these two processes are imbalanced, with current N losses from marine systems exceeding inputs (Codispoti *et al.*, 2001; Gruber and Galloway, 2008). Climate change and anthropogenic activities greatly influence global N cycling and the future implications of these disruptions of nitrogen cycling in the oceans are unsettling (Diaz and Rosenberg, 2008; Duce *et al.*, 2008). The biogeochemical processes of N cycling are dominated by microorganisms, whose distribution and activity are in turn influenced by environmental variables including O₂ concentration, temperature and nutrient availability.

Denitrification, the reduction of nitrate to gaseous forms of N, accounts for major losses of fixed N from marine systems and is key to the balance of global N budgets (Gruber, 2008). Denitrification is linked to the other N cycling processes of ammonification and N fixation by nitrification; the oxidation of ammonia (NH₃) or ammonium (NH₄⁺) to nitrite/nitrate (NO₂⁻/NO₃⁻) via the intermediate product, hydroxylamine (NH₂OH). Traditionally, denitrification was thought to be strictly confined to anoxic waters owing to the oxygen sensitivity of the denitrification machinery and because O₂ competes with NO₃⁻ as an electron acceptor (Zumft, 1997). However, the simultaneous use of two terminal electron acceptors (i.e. oxygen and nitrate), termed aerobic denitrification, is now a widely reported phenomenon (Robertson and Kuenen, 1984; Robertson *et al.*, 1995). As such, the range of environments in which potential denitrification can occur is much greater than traditionally thought.

In those heterotrophic nitrifying bacteria that have been examined to date, all but one harbouring a hydroxylamine-oxidising enzyme are able to perform aerobic denitrification also (Stein, 2011). Indeed, the pathways of heterotrophic nitrification and aerobic denitrification are often tightly coupled (Figure 5.1). Deletion or mutation of denitrification genes also reduces or eliminates the nitrification capacity of many

organisms (Nemergut and Schmidt, 2002; Matsuzaka *et al.*, 2003). Active research into simultaneous nitrification and denitrification (SND) has grown in recent years, because of its application in wastewater treatment, in particular. By exploiting the potential of microbes to perform SND, sewage treatment processes no longer have to involve the traditional series of costly and time-consuming steps that were necessary to accommodate the different oxygen requirements of nitrifiers and anaerobic denitrifiers (Schmidt *et al.*, 2003).

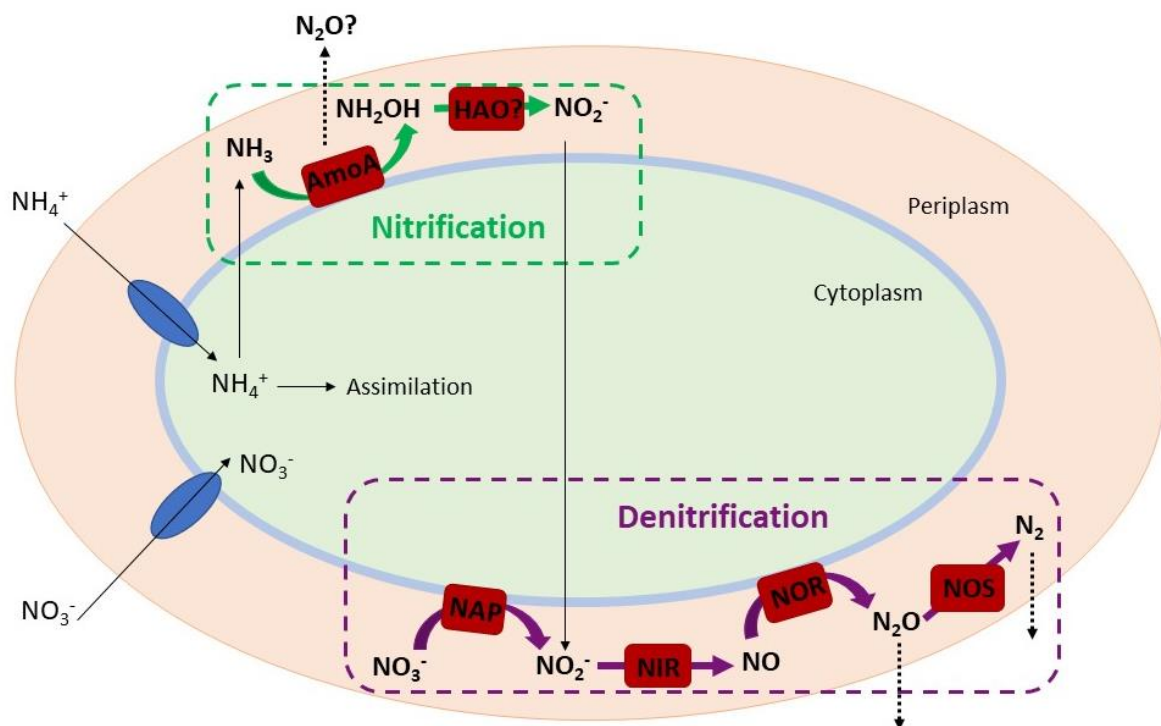


Figure 5.1. Hypothesized heterotrophic nitrification and denitrification pathways in *Labrenzia* sp. strain 5N based on current knowledge of these pathways in other organisms. AmoA, ammonia monooxygenase; HAO, presumed hydroxylamine oxidoreductase; NAP, nitrate reductase; NIR, nitrite reductase; NOR, nitric oxide reductase; NOS, nitrous oxide reductase.

Nitrification and denitrification are of particular environmental relevance because both processes can generate nitrous oxide (N₂O) (Figure 5.1). N₂O is the most potent greenhouse gas in terms of its warming potential (298 times that of CO₂) and has considerable ozone destruction potential also (Ravishankara *et al.*, 2009; Wuebbles, 2009). Since the industrial era began in the mid-eighteenth century N₂O emissions have risen to their highest atmospheric concentrations in 400,000 years (Codispoti, 2010). N₂O is produced through microbial processes and also by human activities including agriculture and industrial processes such as wastewater treatment. There is much current focus on the human sources of greenhouse gases like CO₂ and methane (CH₄) because their future production must be monitored and reduced to slow global warming. However, natural sources of N₂O should not be overlooked. Biological production and consumption rates are also shifting in response to climate change and as a consequence of human exploitation of natural resources (Codispoti *et al.*, 2001). Release of N₂O from marine systems is likely to accelerate as the oceans become more acidic, warmer and further enriched in nutrients through both natural and human inputs. The oceans are already responsible for an annual net production of approximately $\sim 1.4 \times 10^{11}$ mol of N₂O (Codispoti, 2010). Excess N₂O outgassed to the atmosphere has a positive feedback, further enhancing the effects of global warming.

In aerobic conditions, nitrification represents the primary biological source of N₂O in marine systems. N₂O and nitric oxide (NO) have been shown to be produced as by-products of the oxidation of hydroxylamine and nitrite reduction (Hooper and Terry, 1979; Hooper *et al.*, 1997). During denitrification N₂O may be released as an end product of the reduction of oxidised nitrogen, but can also be further reduced to dinitrogen gas (N₂) by many denitrifiers (Figure 5.1). Therefore, these organisms have the potential to both produce and consume N₂O. The balance between production and consumption processes relies on factors including temperature, pH and O₂ concentrations in the immediate environment (Sorai *et al.*, 2007). The extent of marine N₂O production via nitrification and denitrification is related to water oxygenation. When oxygen is scarce, production of N₂O through nitrification is increased (Ritchie and Nicholas, 1972; Schmidt *et al.*, 2004). Nitrous oxide can also accumulate through denitrification due to the oxygen sensitivity of NosZ, which can impede the complete reduction of N₂O to N₂ (Alefounder and Ferguson, 1982; Coyle *et al.*, 1985). As such,

N₂O production in suboxic (O₂, <1% saturation) and hypoxic (O₂, <30% saturation) waters (which together represent ~10% of the ocean volume) is of particular climatical importance (Canfield *et al.*, 2010).

This study focussed on N transformation processes in the marine bacterium *Labrenzia* sp. strain 5N. This strain was originally isolated from the suboxic waters of the Arabian Sea (Wyman *et al.*, 2013) and is closely related to *Labrenzia*-like denitrifiers known to associate with the marine, N₂-fixing cyanobacterium, *Trichodesmium* (Coates and Wyman, 2017). *Trichodesmium* spp. are responsible for up to 50% of marine N fixation (Mahaffey *et al.*, 2005). The colonies formed by this diazotroph exhibit strong oxygen gradients, ranging from anoxia to oxygen supersaturation (Paerl and Bebout, 1992; Eichner *et al.*, 2017, 2018). Bacteria epiphytic with *Trichodesmium*, therefore, will clearly encounter varying O₂ concentrations when growing in association with the host. Thus, strain 5N represents an interesting model in which to study the capacity for N transformations under varying oxygen conditions. In addition, the potential production of N₂O by this strain also requires investigation given its capacity to occupy suboxic environments (Wyman *et al.* 2013).

It has been shown both experimentally and through genome analyses that *Labrenzia* sp. strain 5N is capable of full denitrification and the potential to nitrify is a possibility also (See Chapters 2 and 3). However, the overall N transformation capacity of this strain remains to be studied in detail. Here we used N isotopes (¹⁵N) to determine the ability of strain 5N to nitrify and denitrify under both aerobic and anaerobic conditions. NO₂⁻ was analysed by GCMS whereas N₂O and N₂ were analysed by isotope ratio mass spectrometry (IRMS) to quantify the production of the intermediates and end-products of these N transformation processes.

5.3 Methods

5.3.1 Colorimetric determination of ammonium and nitrite in initial pilot experiments

Nitrification culture conditions. Experimental replicate cultures ($n = 3$) were grown in 30ml nitrate-free ASW (Wyman *et al.*, 1985) amended with 0.5 g.L^{-1} yeast extract, 2 g.L^{-1} sodium acetate and 50 mg/L $\text{NH}_4\text{-N}$. Allylthiourea (ATU), an inhibitor of nitrification, was added at a concentration of $100 \text{ }\mu\text{M}$ to control cultures. Replicates were inoculated from an overnight culture to an OD_{600} of 0.01 and grown at 33°C in an orbital incubator (200 r.p.m). At each timepoint, 3 ml culture was removed for growth (OD_{600}), nitrite and ammonium determinations.

Nitrite analysis. At each time point following inoculation, a 1ml sample was centrifuged at $16,000g$ for 1.5 min and the supernatant was used for nitrite analysis. $50 \text{ }\mu\text{L.ml}^{-1}$ 0.2% sulphanilamide and $20 \text{ }\mu\text{L.ml}^{-1}$ 6M HCl was added to the supernatant and mixed by inversion. After 3 minutes at room temperature $10 \text{ }\mu\text{L.ml}^{-1}$ 0.1% NEDA was added and mixed by inversion. Colour was left to develop in the dark for 15 minutes before measuring the absorbance at 539 nm. Standards were prepared using KNO_2^- solutions diluted in growth medium and standard curves were generated to determine nitrite concentrations.

Ammonium analysis. As described above, 1 ml samples were harvested and the supernatant used for ammonium detection using the indophenol blue method (Tzollas *et al.*, 2010). $40 \text{ }\mu\text{l}$ phenol solution (11.1% phenol taken up in 95% ethanol to a final volume of 100 ml), $40 \text{ }\mu\text{l}$ of 0.5% w/v sodium nitroprusside solution (dissolved in deionised water) and $100 \text{ }\mu\text{l}$ oxidising solution were added to 1 ml supernatant. The oxidising solution consists of a 4:1 ratio of alkaline citrate solution and 5% sodium hypochlorite. The alkaline citrate solution was prepared by adding 200 g trisodium citrate and 10 g sodium hydroxide to a final volume of 1 L deionised water. Solutions were freshly prepared as required. Standards were generated from a NH_4Cl stock and calibration curves used to determine sample concentrations. Following the addition of reagents, samples and standards were incubated in the dark at room temperature for 1 hour and absorbance was read at 640 nm.

5.3.2 Culture conditions for ^{15}N tracer experiments involving *Labrenzia* sp. strain 5N

Aerobic growth medium for *Labrenzia* sp. strain 5N consisted of ASW:LB (3:1); a modification of N-free Artificial Seawater Medium (ASW) (Wyman *et al.*, 1985) amended with 25 % (vol/vol) Luria-Bertani medium (10 g.L⁻¹ tryptone, 5 g.L⁻¹ yeast extract, 10 g.L⁻¹ NaCl), 1 ml.L⁻¹ A5 trace metal solution (Stanier *et al.*, 1971) and 0.5 ml L⁻¹ f/2 vitamin solution (Guillard and Ryther, 1962). Anaerobic growth medium for *Labrenzia* sp. strain 5N consisted of N-free ASW amended with 1 g.L⁻¹ yeast extract, 2 g.L⁻¹ sodium acetate, 0.5 mg.L⁻¹ resazurin sodium, 0.1 g.L⁻¹ sodium thioglycolate, 1 ml.L⁻¹ A5 trace metal solution (Stanier *et al.*, 1971) and 0.5 ml L⁻¹ f/2 vitamin solution (Guillard and Ryther, 1962). *Labrenzia* sp. strain 5N cultures were grown at 33°C in an orbital incubator at 200 rpm. Combinations of labelled and unlabelled N substrates were also supplied, to target different cellular processes. The substrates $^{14}\text{NO}_3^-$, $^{15}\text{NO}_3^-$, $^{14}\text{NH}_4^+$ and $^{15}\text{NH}_4^+$ were added to all cultures at T_0 at a 100% enrichment with final nitrate nitrogen and ammonium nitrogen concentrations of 3mM.

The growth of *Labrenzia* sp. strain 5N under both aerobic and anaerobic conditions was assessed using cultures grown in 600ml glass flasks sealed with a SubaSeal rubber septum. A culture to headspace ratio of 1:3 (i.e. 150 mL medium: 450 mL headspace) was used. When anaerobic conditions were required, the headspace was flushed for 3 min with Argon whilst shaking. Pre-warmed flasks were inoculated (10% v/v) with an overnight culture suspension to an initial OD₆₀₀ of ~0.04. Each growth experiment was performed in triplicate and both unlabelled controls and uninoculated media were included to confirm the absence of abiotic production of end-products.

5.3.3 Sampling procedures

At each timepoint, gas was sampled via the SubaSeal stopper directly into 6ml pre-evacuated exetainers (Labco) using a two-way needle with a stopper valve. Using a gas tight syringe with luer lock (Hamilton), 1ml liquid culture was removed for optical density measurements and 10ml was collected for nitrite analysis. For the determination of nitrite concentrations, the 10ml culture suspensions were filtered immediately using a 0.22µm syringe filter (Whatman, Maidstone, UK) and the filtrates frozen at -18°C in plastic falcon tubes (Sigma) until analysis (described below). The volume of gas and liquid removed at each timepoint was replaced with argon or air,

as appropriate, and subsequent gas measurements were adjusted to take these dilutions into account. All experiments were performed in triplicate.

5.3.4 Gas analysis by gas chromatography-isotope ratio mass spectrometry (GC-IRMS)

A dual method was developed for the simultaneous detection of N₂O and N₂ using a Sercon 20-22 Continuous-flow Isotope Ratio Mass Spectrometer which uses electron-impact ionization. A chemical sodium hydroxide based CO₂ trap was used to remove any atmospheric or respiratory CO₂ which would interfere with the detection of N₂O at the same molar mass. The 6ml exetainers (Labco) filled with sample headspace were used directly for analysis of N₂O and N₂. The GC column temperature was set at 90°C and the trap current was set at 200 µAmps for the N₂O source and 25 µAmps for N₂. Helium was used as the carrier gas. Dummy samples containing 100% argon were run between samples in different experimental treatments to ensure flushing of any residual gases from the system. Quantitative analysis was carried out by recording ions m/z 28, 29 and 30 for N₂ and m/z 44, 45 and 46 for N₂O.

5.3.5 ¹⁵N-Nitrite analysis

Conversion of Nitrite to Sudan-1. Nitrite was first converted to the Sudan-1 dye and solid phase extractions were performed. Sudan-1 was then derivatized and analysed for ¹⁵N enrichment by GCMS. Sudan-1 conversion and extraction were performed following the procedure described by Preston *et al.*, 1998. 200µL of the diazotisation reagent (400 mg aniline sulphate in 100 ml 3M HCl) was added to each 10ml filtrate sample, mixed and a pH of ~2 was then confirmed by broad range pH paper. After 5 minutes, 205µL of the coupling reagent (104 mg 2-naphthol in 50ml 3M NaOH) was added, mixed and a pH of ~8 confirmed as before. The samples were left at room temperature for 10 minutes to allow for dye development. An aliquot (65µL) of 1M citric acid was added to lower the pH to 5-6 and protonate the azo dye in readiness for its extraction. External standards were prepared in a similar manner. A standard curve was generated by adding known concentrations of sodium nitrite to 10ml sterile culture medium. Sudan-1 conversion, extraction and derivatisation stages were performed for the standards as described for the experimental samples.

Sudan-1 extraction. Extraction of the Sudan-1 dye was performed using 3 ml 100 mg C-18 cartridges attached to a solid phase extraction manifold. The solid phase was first conditioned with 3 ml ethyl acetate by wetting for 2/3 minutes before applying a brief vacuum to initiate the passage of the ethyl acetate, without allowing the phase to dry. A washing step was then performed using 3 ml 0.9% NaCl. Reservoirs (70 ml) were connected to the cartridges via an adaptor. The 10ml samples were then loaded into the reservoirs, a vacuum was applied initially to encourage samples to pass and then samples were left to pass without vacuum. The solid phase was washed with 20 ml deionised water and dried using the vacuum. Sudan-1 was eluted into 5 ml glass receiving tubes with 2 x 1ml ethyl acetate. An internal standard (5 nmoles) 4-phenylazophenol (PAP; 0.2 mmol/L stock in ethyl acetate) was added to each sample. Samples were dried in a vacuum concentrator at room temperature. Once all ethyl acetate had evaporated from the samples the temperature was increased to 45°C for a brief period to remove any residual water. Once this stage was reached, samples could be stored at room temperature prior to the derivatisation step. A test standard was also prepared by drying 2 ml ethyl acetate containing 5 nmoles PAP and Sudan-1 and proceeding to derivatization.

Derivatization and GC/MS ^{15}N analysis. To each dried sample, 10 μL derivatization reagent N-tert-Butyldimethylsilyl-N-methyltrifluoroacetamide (MTBSTFA; Sigma, UK) and 90 μL ethyl acetate was added. Derivatization was performed for 30 minutes at 75°C. 100 μL derivatized sample was then transferred to crimp top wineglass vials for GC/MS analysis. GC/MS analysis of $^{15}\text{NO}_2^-$ was performed on an Agilent 5975C inert XL MSD with Triple-Axis Detector paired with an Agilent 7890A GC system. The GC column and temperature programme were as described previously (Preston *et al.*, 1998). The MS was operated with electron impact ionisation in selected ion monitoring mode. The intensity peaks at m/z 306.2 and 305.2 were monitored to measure the ^{15}N and ^{14}N species respectively, reflecting the loss of a butyl fragment (M-57) from the derivatised molecule.

5.3.6 Isotope analysis

Standard curves were used to calculate gas concentrations based on 1 mole of any gas occupying 24.4 L at standard ambient temperature and pressure (25°C and 100

kPa; Avogadro's hypothesis). The nitrogen-15 content of *air* nitrogen of 0.3663 atom % was used for Atom % ^{15}N excess calculations. Isotope abundances for $^{15}\text{N}_2\text{O}$ and $^{15}\text{N}_2$ were calculated as follows.

The IRMS measures molecular species from which atomic ratios are derived from the ion beam intensity of each measured mass:

$$\text{For N}_2, \text{ Atom percent (\%)}^{15}\text{N} = 100 \times (29/2 + 30)/(28+29+30)$$

$$\text{For N}_2\text{O, Atom percent (\%)}^{15}\text{N} = 100 \times (45/2+46)/(44+45+46)$$

$$\text{Atom \% }^{15}\text{N excess} = (\text{Atom \% }^{15}\text{N sample}) - ^{15}\text{N natural abundance}$$

$$\text{Atom fraction excess }^{15}\text{N} = (\text{atom \% }^{15}\text{N excess}) / 100$$

$$\text{Molar excess }^{15}\text{N} = (\text{atom fraction }^{15}\text{N}) \times (\text{moles gas})$$

^{15}N enrichment of nitrite was calculated as follows:

$$\text{Atom \% excess }^{15}\text{N} = 100 \times ((R_{\text{sample}} - R_{\text{natural}}) / (1 - (R_{\text{sample}} - R_{\text{natural}})))$$

where, R_{sample} is the m/z 306:305 ratio of the sample as measured by GCMS and R_{natural} is the m/z 306:305 ratio of a natural abundance standard.

5.3.7. Dissolved oxygen modelling

For the aerobic treatments, C and O budget modelling (Appendix Tables 8.8 and 8.9) was kindly performed by Dr Michael Wyman using the following parameters. The dissolved oxygen concentration is a function of temperature (33 °C), salinity (27 psu) and the rate of exchange with the overlying atmosphere and was set at $6.24 \times 10^{-3} \text{ g.L}^{-1}$ for fully saturated medium. A Respiratory Quotient (RQ; the molar ratio of CO_2 evolved to O_2 consumed in cellular respiration) of 0.35 for growth on acetate in exponential phase was assumed (Romero-Kutzner *et al.*, 2015). Optical density measurements were converted to cell densities ($1\text{OD}_{600} = 1.98 \times 10^8 \text{ cells.mL}^{-1}$) based on the mean determined for related marine bacteria of a similar size (Ichikawa *et al.*,

2015). The rate of exchange of oxygen between the medium surface and the overlying headspace was determined by Ficks First Law:

$$J = -D \cdot A \cdot d\phi/dx$$

where J = diffusion flux in mass (mol)/s; D = diffusion coefficient of oxygen ($2.86 \times 10^{-5} \text{ cm}^2 \cdot \text{s}^{-1}$ at 33°C and a salinity of 27 psu); A = area of the medium surface of $\sim 80 \text{ cm}^2$; $d\phi$ = change in oxygen concentration; dx = change in length in cm (i.e. depth) of the boundary layer for well-mixed aqueous solutions (0.015 cm; Broecker and Peng, 1974). For each treatment the growth curve was assumed to be a simple transition from the initial exponential period to stationary phase once the carbon source had been exhausted. This rapid entry from exponential to stationary phase during the growth cycle of *Labrenzia* sp. strain 5N in batch culture is illustrated for a different experiment in Figure 5.2.

5.4 Results and Discussion

5.4.1 Heterotrophic nitrification capacity of *Labrenzia* sp. strain 5N

To assess the capacity of strain 5N to nitrify, aerobically grown cultures were supplied with ammonium ($\text{NH}_4^+\text{-N}$) and growth (OD_{600}), NH_4^+ and nitrite (NO_2^-) concentrations were monitored over time. Control cultures were amended with the nitrification inhibitor, Allylthiourea (ATU), which targets ammonia monooxygenase, the enzyme responsible for the initial oxidation of $\text{NH}_4^+\text{-N}$ to hydroxylamine. In test cultures, ammonium levels started to decrease appreciably after 21 hours and by 48 hours, approximately 72 % of ammonium nitrogen was removed (50 mg/L initial $\text{NH}_4^+\text{-N}$) (Figure 5.2A). Nitrite first accumulated in the medium towards the end of exponential growth, when biomass was high ($\text{OD}_{600} > 2.03$). At 28 hours there was a rapid appearance of nitrite in the medium and concentrations reached a peak of $4.45(\pm 0.17) \text{ mg/L}$ by 44 hours. Cultures showed similar growth trends whether they contained ATU or not (mean growth rates $\mu = 0.310 \text{ hour}^{-1}$ and 0.309 hour^{-1} for cultures supplied with $\text{NH}_4^+\text{-N}$ and $\text{NH}_4^+\text{-N} + \text{ATU}$, respectively) (Figure 5.2B). However, in the +ATU cultures only $\sim 9.4 \%$ $\text{NH}_4^+\text{-N}$ was removed from the medium after forty-eight hours, which likely reflects assimilatory processes. Nitrite remained undetectable in the +ATU cultures throughout the experiment, demonstrating that the pathway of NO_2^- generation may be via heterotrophic nitrification.

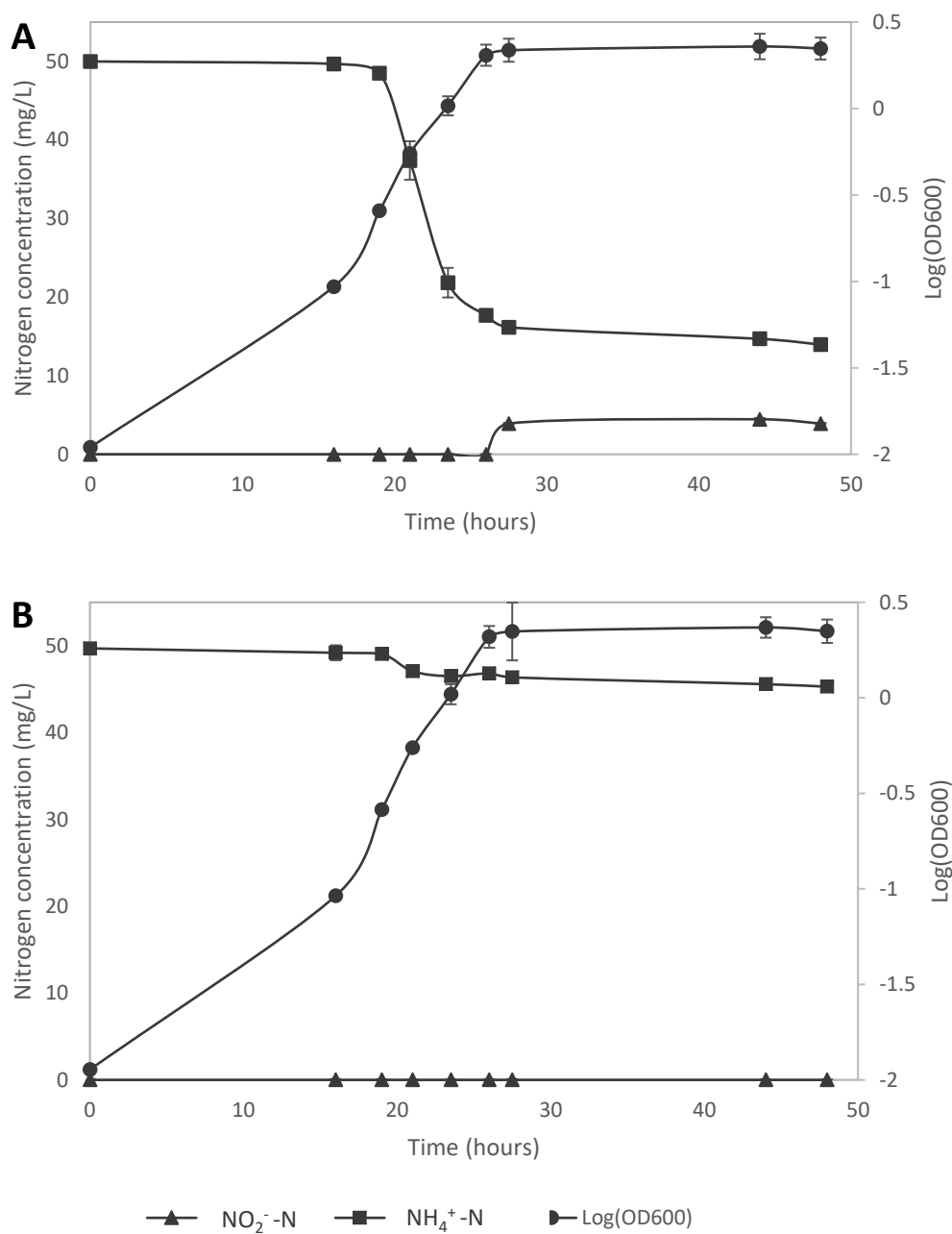


Figure 5.2. Ammonium oxidation capacity of *Labrenzia* sp. strain 5N grown aerobically at 33°C with (A) 50mg/L NH_4^+-N (A) and (B) 50mg/L NH_4^+-N and 10mM ATU. Error bars represent one SD (n=3).

Whilst autotrophic nitrifiers generate ATP from the oxidation of ammonia and use this energy for CO₂ fixation, heterotrophic nitrification does not result in any energetic gain (Robertson and Kuenen, 1990; Bock and Wagner, 2006; Stein, 2011). It has been shown, experimentally and through genome analyses (Chapters 2 and 3) that strain 5N is a heterotrophic bacterium incapable of CO₂ fixation via the Calvin cycle. Ammonia oxidation performed by this strain, therefore, was attributed to heterotrophic nitrification. By contrast with autotrophic nitrifiers, few studies of the ammonia- and hydroxylamine- oxidizing enzymes of heterotrophic nitrifiers have been published. There are known to be at least four types of hydroxylamine-oxidising enzymes, including HAO and cytochrome P460, but most remain uncharacterised (Stein, 2011). A putative ammonia monooxygenase gene (*amoA*), a functional marker of nitrification responsible for the conversion of NH₄⁺ to NH₂OH, has been detected in the genome of strain 5N (HEP89_RS01600) and other *Labrenzia* spp. The strain 5N protein shares 100% amino-acid identity with the peptide sequence from its closest relative, *Labrenzia aggregata* RMAR6-6, and 98.2% at the nucleotide level also (AQQ03046.1).

The subsequent step in the nitrification pathway is the oxidation of NH₂OH to NO₂⁻. Hydroxylamine oxidoreductase (HAO), the enzyme responsible for this step in autotrophic nitrifiers, has not been identified in strain 5N. However, an enzyme capable of oxidising hydroxylamine is presumed to be present in strain 5N because of the observed production of NO₂⁻ from ammonium oxidation (Figure 5.2A). The accumulation of NO₂⁻ during ammonium oxidation was low (3.91±0.09mg.L⁻¹ by the end of the experimental period) considering the amount of ammonium removed (36.03±0.35mg.L⁻¹ by 48 hours). Since the yield of nitrite was only about ten percent of the ammonium consumed, some NO₂⁻ generated by strain 5N might be further metabolised. One avenue might be through simultaneous heterotrophic nitrification and aerobic denitrification. The removal of any accumulated nitrite by assimilation may also be prioritised owing to its toxicity to the cells. High NO₂⁻ concentrations can impact on an organism's growth rate and, in mixed communities, it can exclude species that have low tolerance (Philips *et al.*, 2002).

Since these initial experiments unveiled unexplained N cycling dynamics in *Labrenzia* sp. strain 5N, this was further investigated using Nitrogen-15 tracer experiments. Enrichments of ¹⁵N are expressed as mole percent excess, i.e. the relative molar concentration of ¹⁵N to ¹⁴N. When strain 5N was grown aerobically and supplied with

$^{15}\text{NH}_4^+$ as the sole N source, the $^{15}\text{N-NO}_2^-$ excess rose gradually to a maximum after 48 hours (Figure 5.3A). Paired-sample t-tests were conducted to compare changes in $^{15}\text{N-N}$ excess throughout experiments. There was a significant difference between the $^{15}\text{N-NO}_2^-$ excess at the start ($0.0007[\pm 0.0023]$ pmoles.L $^{-1}$) and the end ($55.5[\pm 6.2]$ pmoles.L $^{-1}$) of the experiment; $t(3)=17.8$, $p=0.0004$. All of the NO_2^- detected was labelled. This demonstrates that this organism has the capacity to oxidise NH_4^+ to NO_2^- , the only possible route being nitrification. No N_2O or N_2 was produced in this treatment (Figure 5.3B), confirming the NO_2^- generated is not in fact further metabolised. The accumulation of nitrite towards the end of the experiment, therefore, suggests a potential switch to ammonium oxidation as the energy source (acetate) fell in concentration and the culture entered stationary phase. As heterotrophic nitrification is not linked to cell growth, this observation is not uncommon (Gunner, 1963). Towards the end of the experiment cell densities are high and available O_2 levels within the culture medium may have reduced. When conditions are hypoxic ($\text{O}_2 < 30\%$ saturation) heterotrophic nitrification has been linked to NAD(P)H reoxidation should Redox imbalances be encountered (Robertson and Kuenen, 1990; Stein, 2011).

With a starting concentration of 3mM $^{15}\text{NH}_4^+$, the production of labelled nitrite is modest ($\sim 10\%$). However, the ideal conditions required for nitrification were likely not met by the experimental setup adopted. Many factors, such as the C:N ratio, influence rates and yields of nitrification (Strauss and Lamberti, 2000). In this experiment a 10:1 C:N ratio, known to encourage nitrification in certain circumstances, was tested (Joo *et al.*, 2005; Ji *et al.*, 2015). It is highly probable that these conditions were not optimal and that nitrification rates of strain 5N may be higher under different environmental conditions or when supplied with other carbon sources.

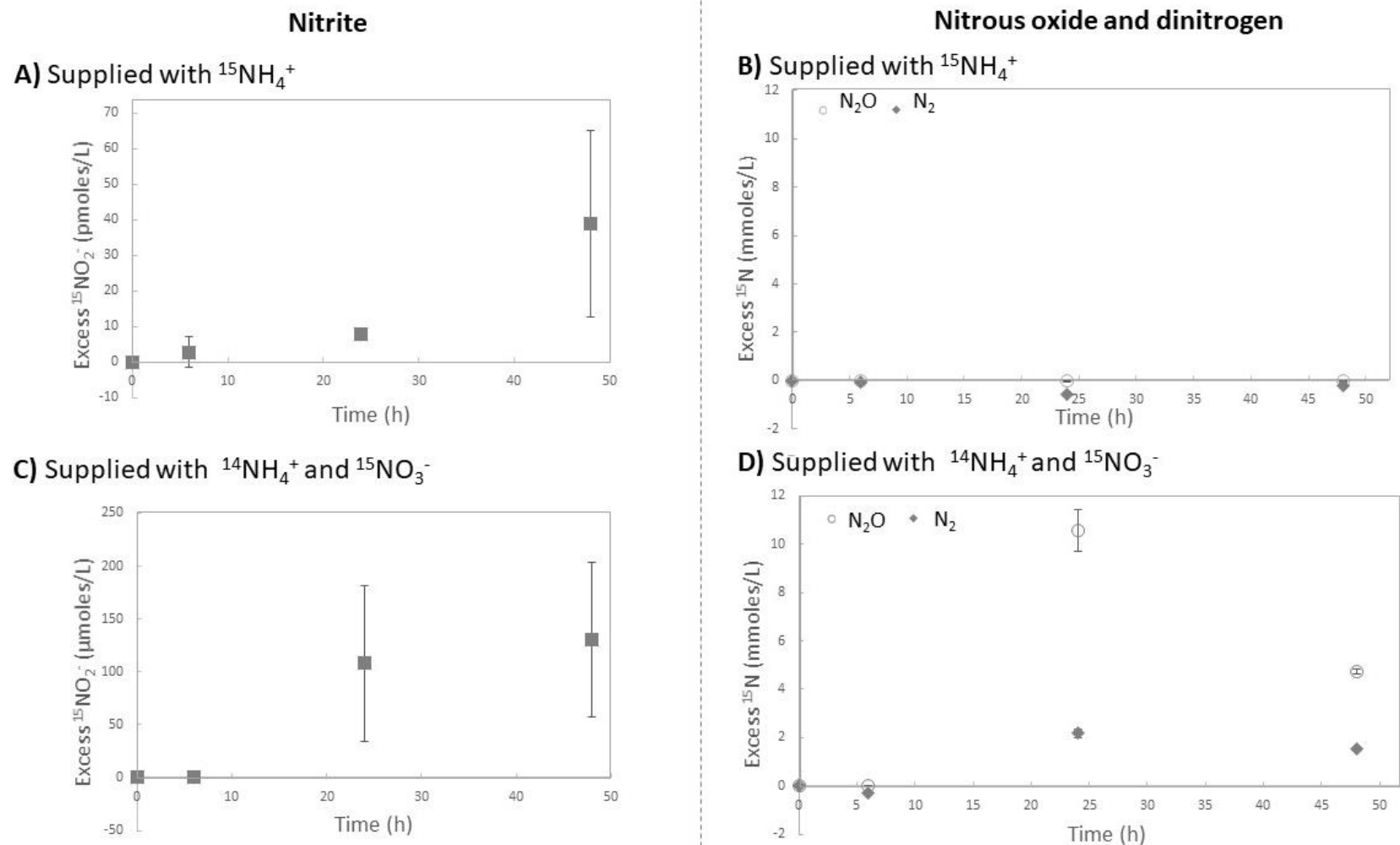


Figure 5.3. ^{15}N excess of NO_2^- (■), N_2O (○) and N_2 (◆) produced by *Labrenzia* sp. strain 5N in aerobic treatments. A) ^{15}N - NO_2^- production when supplied with $^{15}\text{NO}_3^-$; B) ^{15}N - N_2O and ^{15}N - N_2O production when supplied with $^{15}\text{NO}_3^-$; C) ^{15}N - NO_2^- production when supplied with $^{14}\text{NH}_4^+$ and $^{15}\text{NO}_3^-$; D) ^{15}N - N_2O and ^{15}N - N_2O production when supplied with $^{14}\text{NH}_4^+$ and $^{15}\text{NO}_3^-$. N substrates were added at 0 hours. Values represent mean $\pm$ sd. Note the differences in scale between treatments.

5.4.2 Strain 5N is capable of simultaneous heterotrophic nitrification and aerobic denitrification

To investigate the capacity of strain 5N for aerobic denitrification or for heterotrophic nitrification-aerobic denitrification, cultures were supplied with a combination of $^{14}\text{NH}_4^+$ and $^{15}\text{NO}_3^-$ alongside acetate as the C source. Aerobic cultures supplied with NO_3^- in addition to NH_4^+ produced considerably more nitrite than when supplied with NH_4^+ alone (Figure 5.3C). When grown aerobically with $^{14}\text{NH}_4^+$ and $^{15}\text{NO}_3^-$, $^{15}\text{N-NO}_2^-$ excess increased from $0.002(\pm 0.004) \mu\text{moles.L}^{-1}$ to $174(\pm 34) \mu\text{moles.L}^{-1}$ ($\sim 10^6$ -fold higher than with $^{15}\text{NH}_4^+$ alone) by 48 hours; $t(3)=10.10$, $p=0.0021$. In this treatment an increase in unlabelled NO_2^- , originating from the oxidation of the $^{14}\text{NH}_4^+$, that reached $4.04(\pm 0.8) \mu\text{moles.L}^{-1}$ by 48 hours was also observed. Although the contribution of $^{14}\text{N-NO}_2^-$ to the total production of NO_2^- is small ($\sim 2\%$ of the overall NO_2^- pool), this is consistent with the previously detected low levels of NO_2^- attributed to nitrification in the aerobic treatment supplied with $^{15}\text{NH}_4^+$ (Figure 5.3A). The majority ($\sim 98\%$) of the NO_2^- produced by the system is $^{15}\text{N-NO}_2^-$, however, which can only result from the reduction of the added $^{15}\text{NO}_3^-$.

The NO_2^- produced in experiment described above is further reduced to N_2O . $^{15}\text{N-N}_2\text{O}$ excess increases sharply to $10.57(\pm 0.84) \text{mmoles.L}^{-1}$ at 24 hours before decreasing to $4.75(\pm 0.1) \text{mmoles.L}^{-1}$ by 48 hours as it is further metabolised (Figure 5.3D). An increase in both $^{45}\text{N}_2\text{O}$ and $^{46}\text{N}_2\text{O}$ was detected. By the end of the experimental period, $\sim 99.5\%$ of the N_2O detected was $^{46}\text{N}_2\text{O}$. This demonstrates that the predominant source of N for aerobic denitrification was the added $^{15}\text{NO}_3^-$, although a small amount of $^{14}\text{NO}_2^-$ derived from nitrification was also reduced (accounting for $\sim 0.5\%$ of total N_2O). This ratio of $^{45}\text{N}_2\text{O}:^{46}\text{N}_2\text{O}$ is not surprising considering the amount of $^{15}\text{N-nitrate}$ added was considerably higher than the potential supply of nitrite from nitrification. $^{15}\text{N-N}_2$ levels followed a similar trend, with $^{15}\text{N-N}_2$ excess reaching $2.2(\pm 0.19) \text{mmoles.L}^{-1}$ by 24 hours before decreasing to $1.5(\pm 0.05) \text{mmoles.L}^{-1}$ at 48 hours (Figure 5.3D). Combined, these results demonstrate that the NO_2^- produced via nitrification can be used as a substrate for denitrification and therefore this strain is capable of simultaneous nitrification and aerobic denitrification.

The ability of strain 5N for aerobic denitrification is consistent with recent studies showing *Labrenzia* spp. (and closely related *Stappia* spp.) are prominent within communities of aerobic denitrifiers (Du *et al.*, 2017; Yuan *et al.*, 2020). Experiments

by Du *et al.* (2017) on the community composition of aerobic denitrification bioreactors showed that *Labrenzia* represented ~18% of the total non-biofilm bacterial community and *Stappia* represented ~23%. *Labrenzia* formed a smaller proportion of the overall denitrifying community within the biofilm itself (~9%), which would exhibit lower oxygen levels. This might suggest this genus of facultative anaerobes is more successful in conditions where oxygen can be used simultaneously with nitrate as electron acceptors. Yuan *et al.* (2020) looked at the effect of aeration on the composition of a mixed consortia of aerobic denitrifying bacteria. They also detected both *Labrenzia* and *Stappia* at all aeration flow rates tested (0.0, 0.25, 0.63, and 1.25 L/(L·min)), confirming the likely role of *Labrenzia* and close relatives in aerobic denitrification.

5.4.3 Anaerobic nitrate respiration

Cultures grown anaerobically with $^{15}\text{NO}_3^-$ as the sole N source showed a sharp peak in $^{15}\text{N-NO}_2^-$ excess of $29.4(\pm 14.5)$ nmoles.L $^{-1}$ after just 6 hours growth, before it's disappearance by 24 hours (Figure 5.4A). This rapid turnover demonstrates that the system is dynamic and denitrification pathway intermediates are quickly reduced further. As such, N_2O production reached its maximum following the NO_2^- peak, with a $0.24(\pm 0.06)$ mmole.L $^{-1}$ $^{15}\text{N-N}_2\text{O}$ excess at 24 hours before declining to $0.1(\pm 0.004)$ mmoles.L $^{-1}$ $^{15}\text{N-N}_2\text{O}$ excess by 48 hours (Figure 5.4B). N_2O was only detected as $^{46}\text{N}_2\text{O}$, consistent with the supply of $^{15}\text{NO}_3^-$ as the only useable source of N for denitrification in the absence of oxygen. $^{15}\text{N-N}_2$ excess rose to $10.3(\pm 0.56)$ mmoles.L $^{-1}$ by 6h, reaching $143(\pm 8.7)$ mmoles.L $^{-1}$ by 24 hours and $127(\pm 6.1)$ mmoles.L $^{-1}$ by the end of the experiment (Figure 5.4B).

When strain 5N was supplied with $^{14}\text{NH}_4^+$ and $^{15}\text{NO}_3^-$ under anaerobiosis, the production of NO_2^- , N_2O and N_2 followed similar patterns to that when grown in the presence of $^{15}\text{NO}_3^-$ only (Figures 5.4B and 5.4D). In the initial 6 hours, $^{15}\text{N-NO}_2^-$ excess rose from $-0.23(\pm 0.26)$ nmoles.L $^{-1}$ to a peak of $287(\pm 195)$ nmoles.L $^{-1}$; $t(8)=3.73$, $p=0.0057$. This was followed by peaks in both $^{15}\text{N-N}_2\text{O}$ excess ($0.26(\pm 0.029)$ mmoles.L $^{-1}$) and $^{15}\text{N-N}_2$ excess ($109.7(\pm 1.1)$ mmoles.L $^{-1}$ $^{15}\text{N-N}_2$ excess) at 24 hours. As found for the $^{15}\text{NO}_3^-$ anaerobic experiments discussed above, N_2O was only detected as $^{46}\text{N}_2\text{O}$ and N_2 as $^{30}\text{N}_2$, confirming that, as expected, the only substrate being used for denitrification was $^{15}\text{NO}_3^-$. The very high level of N_2 compared with N_2O (over 400-fold) shows that, under anaerobic conditions, the majority of NO_3^- is fully

reduced to N_2 . However, small amounts of N_2O were still released from the system as an intermediate product during the course of the experiment.

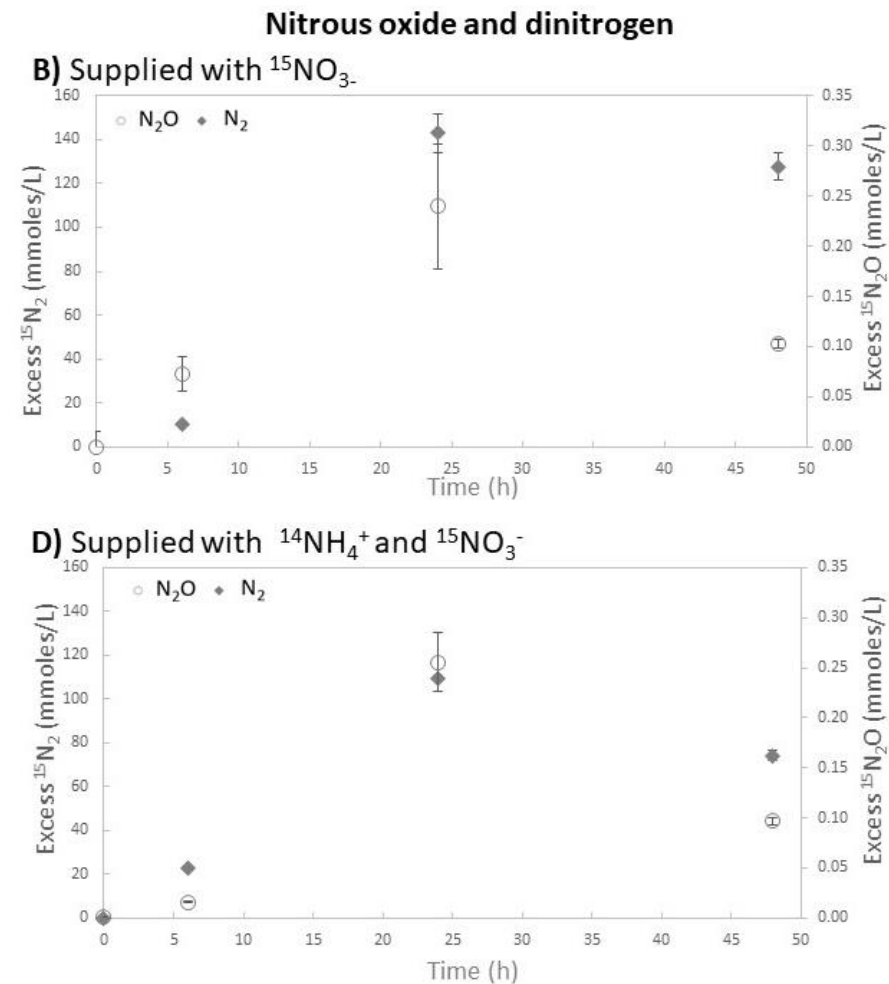
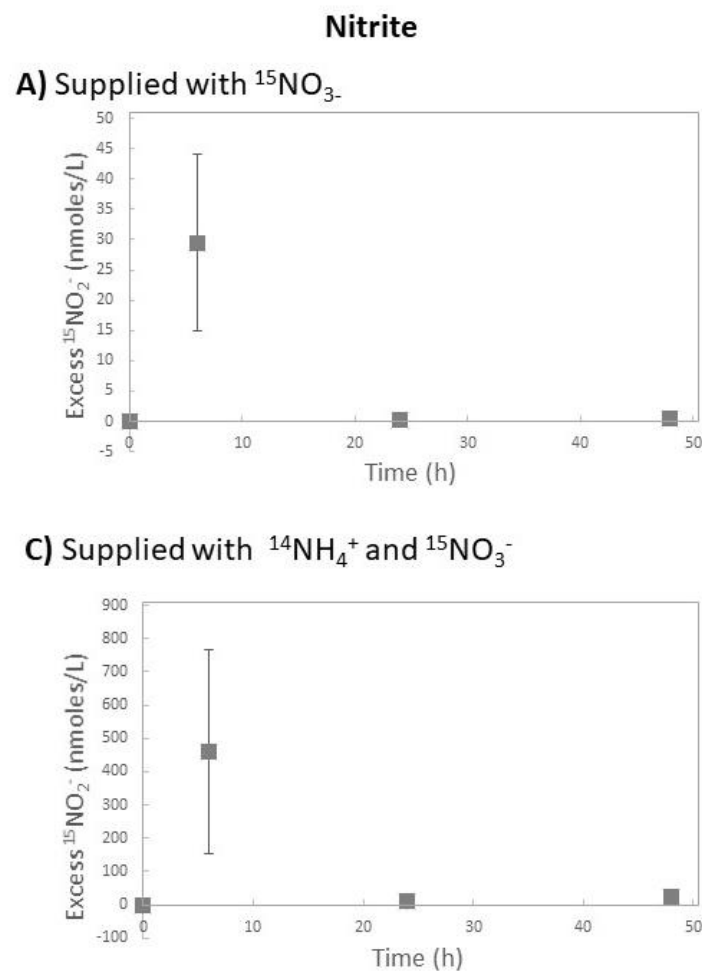


Figure 5.4. ^{15}N excess of NO_2^- (■), N_2O (○) and N_2 (◆) produced by *Labrenzia* sp. strain 5N in anaerobic treatments. A) ^{15}N - NO_2^- production when supplied with $^{15}\text{NO}_3^-$; B) ^{15}N - N_2O and ^{15}N - N_2O production when supplied with $^{15}\text{NO}_3^-$; C) ^{15}N - NO_2^- production when supplied with $^{14}\text{NH}_4^+$ and $^{15}\text{NO}_3^-$; D) ^{15}N - N_2O and ^{15}N - N_2O production when supplied with $^{14}\text{NH}_4^+$ and $^{15}\text{NO}_3^-$. N substrates were added at 0 hours. Values represent mean $\pm$ sd. Note the differences in scales between the treatments.

5.4.4 Oxygen sensitivity of denitrification in strain 5N and implications for nitrate reduction in the natural environment

The degree of oxygen sensitivity of the denitrification machinery varies significantly; some organisms can denitrify in 100% air, others require complete anoxia while others can denitrify in the presence of oxygen below a certain concentration threshold (Robertson and Kuenen, 1984). It would appear that strain 5N is among the latter class of aerobic denitrifiers. The onset of denitrification, which corresponds to the initial appearance of NO_2^- and subsequent gas production was slower in the aerobic cultures (Figures 5.3C and D) than in the corresponding anaerobic cultures (Figures 5.4C and D). This suggests that strain 5N does not denitrify under fully oxic conditions. Its denitrification machinery is sensitive to or repressed by high O_2 levels, as is the case for some other aerobic denitrifiers (Takaya *et al.*, 2003). The delayed timing of the onset of denitrification may result from the increase in biomass (and consequent reduction in available O_2 , despite vigorous shaking) during incubation which provides suitable conditions for the denitrification pathway to operate in this strain.

In the aerobic treatments, flasks were sealed at the start of the experiment with an air headspace ($\sim 21\% \text{ O}_2$) and a culture to headspace ratio of 1:3. Although the experimental setup did not allow the monitoring of oxygen consumption in the test cultures themselves, headspace analysis of temporal changes in O_2 concentrations was performed in a parallel control culture (growth rates comparable) using an oxygen electrode. By the end of the 48 h experimental period O_2 concentrations in the control headspace fell from 20.8% to $\sim 10\%$ (i.e. a decline of $\sim 50\%$ in O_2 availability). For the test cultures 35ml of air was reinjected at each sampling time (to replace the volume of medium and headspace removed) and so O_2 availability would have been somewhat higher by the end of the experimental period. The reduction in O_2 further confirms the co-respiration of O_2 and NO_3^- that satisfies the definition of aerobic denitrification (Robertson and Kuenen, 1984). A different experimental setup would be necessary to investigate the effects of oxygen on denitrification in this strain. However, the present experimental results confirm that strain 5N does not require full anoxia to denitrify.

To verify this point, C and O budgets were kindly modelled by Dr Michael Wyman for both aerobic treatments (Appendix Tables 8.8 and 8.9). The initial total available O_2 per 600 mL flask was ~ 0.135936 g including that dissolved in 150 mL of saturated

modified ASW medium at 27 psu at 33 °C ($0.000936\text{g}\cdot 150\text{mL}^{-1}$). Carbon concentrations (2mM acetate) at the start of the experiment were $0.0072\text{ gC}\cdot 150\text{mL}^{-1}$ which at an RQ = 0.35 and an estimated biomass of $100\text{ fgC}\cdot \text{cell}^{-1}$ should be exhausted by ~13-15 h of exponential growth at the mean growth rate ($\mu = 0.307\text{ h}^{-1}$; $g = 0.443\text{ h}^{-1}$) observed for the experiment. The biomass estimate is based on a cell volume of $117.8\text{ fgC}\cdot \mu\text{m}^{-3}$ which is above the midpoint range of values ($32 - 160\text{ fgC}\cdot \mu\text{m}^{-3}$) reported by Fagerbakke *et al.* (1996) for aquatic bacteria and at the low end of the range ($106 - 214\text{ fgC}\cdot \mu\text{m}^{-3}$) determined for marine bacteria growing in enrichment cultures (Bratbak, 1985). The use of a higher biomass estimate would have exhausted the C-supply before the end of exponential phase at the growth rates observed for the experiment. It would also require a lower estimate of the relation between OD and cell number although the final C and O₂ demands to support growth would be unaffected. The model, therefore, is insensitive to the $\text{C}\cdot \text{cell}^{-1}$ estimate used if one allows cell division to continue in the absence of an exogenous C source for the later parts of the growth cycle when C demand is greatest. This was thought most unlikely and so the biomass estimate modelled ($100\text{ fg}\cdot \text{cell}^{-1}$) was selected for this reason.

The total oxygen consumed in support of growth at an RQ = 0.35 leads to a medium deficit of ~0.014 g O₂ if no exchange with the headspace air is assumed. This estimate is a minimum because it does not include the O₂ required for cell maintenance during stationary phase. The O₂ demand would be higher if the RQ was lower than that applied but it could not be lower than 0.3 for growth on acetate and is likely to be much higher during stationary phase; i.e. the amount of CO₂ produced by starving cells would exceed that of O₂ consumed by a large margin (Romero-Kutzner *et al.*, 2015). Growth alone, therefore, can account for a decline in headspace O₂ of ~11% while cell maintenance (and nitrification) during stationary phase consumes an unknown additional amount.

To determine whether O₂ concentrations within the medium might become anoxic during the experiment, the rate of O₂ exchange with the headspace was calculated using Ficks Law and a diffusion coefficient of $2.86\times 10^{-5}\cdot \text{cm}^2\cdot \text{s}^{-1}$ (Broecker and Peng, 1974). At the most extreme theoretical values for the O₂ gradient within the boundary layer (surface = in equilibrium with the overlying air; base = anoxic) and at 100 % air saturation, full equilibration takes 6.56 s for the boundary layer and 13.67 min for 150 mL of medium. In other words, the maximum amount of O₂ that could be replaced in

one hour is 0.000936 g (the O₂ content of saturated medium) multiplied by 60/13.67; equivalent to 0.00411 g.h⁻¹. This is more than sufficient to maintain the medium in equilibrium with the headspace even during the last hour of exponential growth when the modelled rate of O₂ consumption (0.00379 g.h⁻¹) was close to the equilibration rate determined by Ficks Law with a boundary layer of 0.015 cm.

The rate of O₂ diffusion across the boundary layer is directly proportional to its thickness; the thinner the layer the more rapid the rate of transfer to the underlying medium. A thickness of 0.015 cm was used in the equilibration calculations and is taken from Broecker and Peng (1974). This depth (z) was determined for the equilibration of oxygen in seawater under quietly agitated conditions. In the ¹⁵N stable isotope experiments the culture medium was mixed at 200 rpm and so the actual degree of mixing was considerably more vigorous than that which could be described as “quietly agitated”. Hence the thickness of the boundary layer (z) in the current experiments would have been considerably thinner than 0.015cm and the actual O₂ equilibration time far shorter. The rate of oxygen absorption by culture medium agitated at a speed of ~200 rpm has been determined directly using simultaneous measurements of oxygen concentrations in the medium and overlying atmosphere (McDaniel and Bailey, 1969). The authors measured an absorption rate of ~0.2 mmoles O₂.L⁻¹.min⁻¹ for 300 mL flasks filled with 140 mL medium. The depth of the medium in this experimental setup would have been greater than that used in the present study (hence equilibration times would have been longer) but, even so, the absorption rate measured is equivalent to 8.96x10⁻⁴gO₂.flask.min⁻¹ or 0.05376 gO₂.flask.h⁻¹. It can be concluded, therefore, that at no time in the present study should the culture medium have been at anything other than equilibrium. The demand for O₂ would have to be at least 10-20 times higher than the modelled rate in the last hour of growth for any deficit to occur.

Had anoxia developed toward the end of the experiment it would have been expected that the biomass yield in the treatment containing nitrate would exceed that of the cultures provided with ammonium only. The final OD₆₀₀ achieved (2.13-2.16) was all but identical for both treatments despite the availability of additional oxidant in the cultures supplied with nitrate plus ammonium. This militates against any O₂ limitation in either treatment and is full agreement with the calculations presented above.

When placed in the field context, these experimental findings are particularly pertinent considering the nature of strain 5N's natural environment. This strain was originally isolated from the suboxic waters of the Arabian Sea (Wyman *et al.*, 2013). Wyman *et al.* detected *Labrenzia*-like denitrifiers (closely related to strain 5N) to be actively expressing *nosZ* in Oxygen Minimum Zone (OMZ) suboxic waters and also in shallower oxic waters. They later demonstrated the physical association of these denitrifiers with colonies of the cyanobacterium *Trichodesmium*, suggesting that their capacity to denitrify in seemingly oxic surface waters is a result of their localisation within suboxic microsites of the colonies (Coates and Wyman, 2017). Strain 5N clearly encounters varying O₂ conditions, whether it be when free-living or in association with other organisms, and is not restricted to “conventional” anoxic conditions for denitrification. This flexible metabolism considerably increases their potential impact on marine N cycling in these regions.

Denitrification is the main process responsible for losses of nitrate from the oceans (Codispoti *et al.*, 2001; Galloway *et al.*, 2004; Gruber, 2004, 2008). Reduction of nitrate limits the bioavailability of fixed N that could be assimilated by phytoplankton thereby potentially restricting marine productivity. The marine N cycle is tightly interlinked with oxygen, phosphorus and carbon cycling. As such, fixed N levels are strongly correlated with atmospheric CO₂ and further N losses from the oceans would contribute to increasing concentrations (Gruber, 2004). Hypoxic zones have already and, in line with future predictions, will continue to expand with ocean surface water warming (Diaz and Rosenberg, 2008; Keeling *et al.*, 2010). The increased denitrification rates that this will permit will influence the balance of global marine N budgets, with potential related knock-on effects.

5.4.5 Nitrous oxide emissions resulting from N transformations in strain 5N

The atmospheric release of N₂O as a by-product of both nitrification and denitrification is of considerable environmental importance. For both processes, the N₂O production is increased under hypoxic and suboxic conditions (Suntharalingam *et al.*, 2000; Canfield *et al.*, 2010). No N₂O or N₂ was produced in the aerobic treatment supplied solely with NH₄⁺-N as a N source (Figure 5.3B). The absence of gas production confirms that, under the experimental conditions tested, N₂O was not a by-product of

nitrification. However, under both aerobic (Figure 5.3D) and anaerobic conditions (Figures 5.4B and D), N₂O was produced as a by-product of denitrification. During heterotrophic nitrification-aerobic denitrification, N₂O accumulated throughout the experiment and ¹⁵N-N₂O excess reached a maximum of 10.57(±0.84) mmoles.L⁻¹ by 24 hours (Figure 5.3D). In the same treatment but under anaerobiosis, N₂O reached considerably lower maximum levels (0.26(±0.029) mmoles.L⁻¹ ¹⁵N-N₂O excess; Figure 5.4D). By the end of the experimental period, N₂O levels represented ~76% of the total gases analysed (N₂O + N₂) in the aerobic denitrification treatment though just ~0.1% in the anaerobic treatment, where the majority was fully reduced to N₂.

These results are consistent with the known oxygen sensitivity of NosZ, the enzyme responsible for the reduction of N₂O to N₂. It is common that under aerobic conditions the majority of the N₂O produced does not undergo complete reduction to N₂ (Ferguson, 1994). As such, many aerobic denitrifiers produce larger amounts of N₂O when O₂ is present than when under complete anoxia (Takaya *et al.*, 2003). NosZ is the most O₂ sensitive of all the denitrification reductases, though the degree of O₂ sensitivity is entirely organism dependent. Whilst some strains require complete anoxia for NosZ activity, others such as *Pseudomonas stutzeri* TR2 produce significant amounts of N₂ despite very high oxygen levels (Takaya *et al.*, 2003). We know that in alphaproteobacterium strain 4N, a close relative isolated simultaneously with strain 5N, *nosZ* mRNAs were negligible under fully aerobic conditions (Coates and Wyman, 2017). This indicates that, as is also suspected in strain 5N, NosZ cannot function under fully aerobic conditions, though its expression under intermediate O₂ levels was not assessed. It is worth noting, however, that even different strains of the same species can differ greatly in terms of the oxygen sensitivity of NosZ. For example, *Pseudomonas stutzeri* ZoBell produces large amounts of N₂O and no N₂ under aerobic conditions, whereas as *P. stutzeri* TR2 can denitrify fully to N₂ (Takaya *et al.*, 2003). The significantly increased accumulation of N₂O in aerobic conditions compared with anaerobic suggests that N₂ production in strain 5N shows a certain level of sensitivity to O₂. However, N₂ is still produced under such conditions, confirming that strain 5N does have some resistance to O₂. NosZ is still expressed, and functional, in the presence of oxygen even though its activity is clearly lower than in anoxia (Figures 5.3D and 5.4D). The degree of O₂ sensitivity of strain 5N would be an interesting focus for future studies.

These findings are of environmental significance as any N₂O produced during denitrification by strain 5N and its close relatives would be released into the atmosphere. *Paracoccus denitrificans* (formerly *Thiosphaera pantotropha*), the first described and best studied aerobic denitrifier, produces significant levels of N₂O during aerobic denitrification (0.33 mmol.L⁻¹ of culture) whereas others produce very little (within the μ M range) (Robertson and Kuenen, 1984; Takaya *et al.*, 2003). In the presence of O₂, strain 5N produces large amounts of N₂O (~10 mmol.L⁻¹ culture) at levels of potential climatic importance. Considering the suboxic conditions strain 5N is known to inhabit, N₂O production under this organism's natural environment could be important. Indeed, suboxia can induce some of the highest levels of N₂O production by aerobic denitrifiers. In *Alcaligenes faecalis* TUD, higher N₂O production was seen when dissolved oxygen concentrations were at 5% air saturation compared with both anoxia and fully aerobic conditions (Otte *et al.*, 1996). The environments in which strain 5N and close relatives have been detected are likely subject to variable O₂ concentrations, thus increasing the likelihood of by-production of N₂O. Oxygen gradients found within *Trichodesmium* colonies can be extreme and studies detected concentrations ranging from anaerobic (Paerl and Bebout, 1992) to between 61% and 203% compared to well oxygenated seawater (Eichner *et al.*, 2017). Internal O₂ conditions have also been shown to be related to colony size, with anoxic conditions occurring in the dark for the largest colonies (Eichner *et al.*, 2018). In addition, colony O₂ levels likely fluctuate diurnally due to photosynthetic oxygen evolution by *Trichodesmium*. As such, colonies have the potential to support denitrification under a wide variety of oxygen levels. In addition, the suboxic waters of the Arabian Sea, from where strain 5N was originally isolated, already represent a major source of oceanic N₂O (Naqvi *et al.*, 2005) with ideal low O₂ conditions for the production of N₂O via nitrification and denitrification. As such, the growth of hypoxic regions, combined with ocean acidification, will inevitably result in increased N₂O emissions from the marine environment (Diaz and Rosenberg, 2008; Keeling *et al.*, 2010).

5.5 Conclusions

Given that strain 5N inhabits regions of key importance to marine N-cycling, this study aimed to determine its potential contribution to such processes. Stable isotope tracers were used to provide a sound overview of the N-cycling transformations this organism

is capable of under strictly aerobic and anaerobic conditions. Alongside this, the production of the greenhouse gas N₂O was assessed under these varying oxygen conditions.

The strain can oxidise small amounts of ammonium to nitrite under aerobic conditions, in the absence of the by-production of N₂O. In the absence of added nitrate, the strain cannot further metabolise the nitrite generated from nitrification. However, when nitrate is supplied in addition to ammonium, strain 5N demonstrated both heterotrophic nitrification and aerobic denitrification capacity. Although the substrate used for aerobic denitrification was primarily the added nitrate, ¹⁵N analyses confirmed the nitrite produced from ammonium oxidation was also reduced to gaseous end-products. In addition, the production of N₂O from aerobic denitrification was considerable, reaching ~10mmoles.L⁻¹. Denitrification under anaerobic conditions primarily produced N₂, though very small amounts of N₂O still accumulated, confirming NosZ is sensitive (though still functional) to O₂ in this strain. These in vitro experiments demonstrate the potential for aerobic denitrification in this strain. In the natural environment, however, nitrification/denitrification rates and N₂O emissions are also influenced by O₂ levels, C:N ratios, temperature and pH (Ji *et al.*, 2015).

This is the first detailed study of aerobic/anaerobic denitrification in *Labrenzia*, and to our knowledge even within the wider *Roseobacter* clade, despite many members of the genus harbouring denitrification genes (See Chapter 3). *Labrenzia* spp. have a wide geographical range and inhabit a diversity of environments, including a biocathode community and in associations with other organisms (Wang *et al.*, 2016), in which varying O₂ conditions will likely be encountered. In light of predicted expansions of oceanic hypoxic waters greater knowledge of biological sources and sinks of N₂O, as well as the organisms controlling these processes, is of pressing importance. This strain shows considerable potential for N₂O release under such natural conditions that are not strictly anoxic and would serve as a useful model for further studies on N losses in suboxic waters.

Chapter 6:

Development of a genetic transformation system for *Labrenzia* sp. strain 5N

6.1 Abstract

The development of genetic manipulation systems opens the door to *in vivo* studies of many model microbes and potentially to those of significant environmental importance. *Labrenzia* sp. strain 5N harbours genes involved in a range of processes of environmental and climatic interest including DMSP metabolism and production, CO oxidation, denitrification, nitrification and the degradation of aromatic compounds. Here, a genetic manipulation system for this strain was developed to enable these processes to be studied under environmentally relevant conditions. A recombinant plasmid that replicates in *Labrenzia* sp. strain 5N and based on the broad host range vector, pBBR1MCS-5, harbouring the fluorescent marker *gfp* was constructed. The efficiency with which this construct could be introduced into strain 5N via chemical transformation, electroporation or conjugation techniques was investigated. While chemical and electro-transformation were unsuccessful, it was shown that strain 5N is capable of plasmid uptake via bi-partite conjugation with a promiscuous mating strain of *E.coli*. The recombinant plasmid was stably maintained in strain 5N alongside its two natural plasmids. A fluorescent signal was readily detectable by fluorescence microscopy in the strain 5N transformant, confirming that the *gfp* reporter gene was expressed. Thus, a successful protocol for the genetic manipulation of *Labrenzia* sp. strain 5N was developed with potential future uses in studies of *Trichodesmium*-associated N cycling and other biogeochemically important transformations.

6.2 Introduction

Genetic manipulation of marine bacteria. Marine microorganisms have large biotechnological potential for applications in the industrial, environmental, pharmaceutical and agricultural sectors (Dionisi *et al.*, 2012). For example, the discovery of marine microbial natural products with anti-viral, anti-cancer and anti-

microbial properties can lead to the development of new drugs (Xiong *et al.*, 2013). Marine bacteria represent a vast genetic diversity but this richness is little exploited owing to difficulties in laboratory culture and limited knowledge of how to genetically manipulate marine strains (Joint *et al.*, 2010; Zeaiter *et al.*, 2018).

Wider genetic manipulation methods would contribute vastly to research in the field of marine microbiology and open doors to a range of *in vivo* studies. The possibility of introducing, modifying or deleting specific genes expands the potential for studying environmental and biological systems at the molecular level. While functional metagenomic approaches can help to capture genes of potential biotechnological application from complex natural samples, the frequent reliance on *E. coli* vectors is not without its pitfalls. Notably, the successful expression of cloned genes originally isolated from phylogenetically distant marine bacteria can be problematic (Gabor *et al.*, 2004; Lam *et al.*, 2015). As this approach cannot fully substitute for studies with the marine strains of interest themselves, protocols for improving the culturability and for the direct genetic manipulation of marine bacteria must continue to be developed. Method development is time consuming and laborious, however, and final protocols are often species dependant, meaning they might need to be further adapted for studies on close relatives. Though genetic tools have been developed for a number of important marine bacteria including *Synechococcus*, *Prochlorococcus*, *Pseudoalteramonas* and *Vibrio* spp., further advances in this field are critical to enable the manipulation of a wider range of genera (Sawabe *et al.*, 2006; Tolonen *et al.*, 2006; Zhao *et al.*, 2011; Luo *et al.*, 2015).

Laboratory transformation of bacteria. Some bacteria have a natural genetic competence that enables them to take up naked DNA from the environment. Individual genes, entire plasmids, and larger DNA fragments can be naturally acquired by bacterial cells through transformation, transduction or conjugation (Hermansson and Linberg, 1994). Organisms capable of this horizontal transfer of genes can acquire new genetic material continuously and by increasing their gene pool may rapidly adapt to new conditions. The principles underlying these natural processes of genetic exchange have been exploited to develop laboratory methods for DNA transfer that act as a starting point for genetic manipulation.

DNA can be introduced artificially into bacteria by chemical transformation, electroporation and conjugation. Chemical transformations were first performed in the 1970's (Mandel and Higa, 1970; Cohen *et al.*, 1972). Protocols generally involve the use of cold CaCl₂ followed by heat-shocking to generate pores in the cell membrane and assist the uptake of foreign DNA into the recipient. By contrast, electroporation approaches use a high voltage electrical pulse to permeabilize the membrane to enable DNA uptake. Chemical and electro-transformation techniques are usually followed by an incubation step, to allow time for cell repair and gene expression, before the growth of potential transformants in appropriate selective medium.

Through conjugation, two bacterial cells in direct contact with each other may exchange genetic material. This might be between different strains of the same or closely related species or between quite distantly related bacteria. The standard laboratory method first involves the introduction of the target DNA into the chosen donor cell, through either a chemical transformation or by electroporation. Then, the plasmid containing the DNA can be transferred via conjugation from the donor to recipient cell, which might be unreceptive to chemical and electro- transformations itself. Conjugal transfer genes, located, for example, on a conjugative plasmid, encode the machinery necessary for all steps of the conjugation process including pili formation, cell attachment, fusion and DNA transfer. Marker genes, such as *gfp* encoding green fluorescent protein, provide an easily identifiable phenotype during manipulation and are commonly used to assist the detection and screening of successful transformants during method development.

DNA introduction into *Labrenzia* sp. strain 5N using the pBBR1MCS plasmid vector series. Plasmids are frequently used as vectors to introduce genes of interest into the target host cell. The genome of the bacterium being studied, *Labrenzia* sp. strain 5N, consists of a circular chromosome and two plasmids (~410-kb and ~135-kb). Plasmids are grouped into incompatibility groups based on their mode of replication. A plasmid vector that is incompatible with the host's natural plasmids may either not be able to be transformed into the host cells or may be quickly lost from the cells following transformation. Plasmids in the pBBR1MCS vector series are broad host range vectors, with a pBBR1 origin of replication derived from *Bordetella*

bronchiseptica (Antoine and Loch, 1992a). pBBR1MCS and its derivatives have been successfully introduced into a range of Gram-negative bacteria including *Escherichia coli*, *Vibrio* spp., *Rhizobium* spp. and *Rhodobacter* spp. (Antoine and Loch, 1992b; Kovach *et al.*, 1995). One of the vector series, pBBR1MCS-5, harbours a gentamycin resistance gene, to which *Labrenzia* sp. strain 5N was found to be sensitive during preliminary trials. Therefore, this antibiotic resistance trait was useful as a selective marker and the pBBR1MCS derivative pBBR1MCS-5 was chosen for use in this study.

Development of a genetic transformation system for *Labrenzia* sp. strain 5N. The genome of *Labrenzia* sp. strain 5N encodes genes involved in several environmentally and climatically important processes. These include DMSP metabolism and production, CO oxidation, denitrification, nitrification and aromatic compound degradation (Chapter 3) that, with an appropriate genetic manipulation system, could all be the targets for gene-specific studies.

The present study investigated how best to introduce plasmid DNA into *Labrenzia* sp. strain 5N by testing chemical transformation, electroporation and bi-partite conjugation methods. The potential to introduce plasmid DNA into *Labrenzia* sp. strain 5N opens the door to genetically manipulate this strain. The work reported acts as a starting point for a variety of future molecular studies that require the introduction, modification or deletion of genes, operons or even entire plasmids in strain 5N. The ability to take up plasmid DNA is a trait that could be exploited for the introduction of suicide vectors for gene inactivation via insertional mutagenesis, for example. The generation of deletion mutants would allow for functional pathways to be studied more easily. The availability of the genome sequencing data for strain 5N extends the current potential for studying gene function, mechanisms and pathways. Furthermore, the capacity to genetically manipulate *Labrenzia* sp. strain 5N has exciting experimental potential for studying denitrification and nutrient cycling within *Trichodesmium* colonies.

6.3 Methods

6.3.1 General experimental conditions

Unless otherwise stated, *Labrenzia* sp. 5N was grown in 50 ml medium at 33°C in an orbital incubator (200 rpm) in ASW:LB (3:1); a modification of artificial seawater (ASW) medium (Wyman *et al.*, 1985) amended with 25 % (vol/vol) Luria-Bertani

medium (10 g.L⁻¹ tryptone, 5 g.L⁻¹ yeast extract, 10 g.L⁻¹ NaCl). Denitrification medium consisted of ASW medium amended with 1 g.L⁻¹ ammonium chloride, 1 g.L⁻¹ yeast extract, 2 g.L⁻¹ sodium acetate and 2 g.L⁻¹ sodium nitrate. Bacto Agar at a concentration of 10 g.L⁻¹ was added to media when required. Antibiotics for the selection of recombinant cells were used at the following working concentrations: 100 µg.ml⁻¹ Gentamycin (Gm), 100 µg.ml⁻¹ Ampicillin (Ap) and 10 µg.ml⁻¹ Chloramphenicol (Cm). Competent cells were prepared as described in the following sections, dispensed into ice cold Eppendorf tubes, and either used immediately or stored at -80°C. The viability of each batch of competent cells was confirmed by growth on ASW:LB plates overnight at 33°C, with or without antibiotics as required.

6.3.2 Construction of pBBR1MCS-5-gfp plasmid vector for transformation of *Labrenzia* sp. strain 5N

The construct pBBR1MCS-5-gfp is a derivative of the broad host range vector pBBR1MCS-5 (Kovach *et al.*, 1994, 1995). It harbours a Gm resistance cassette and the *gfp* gene from pGFP (Clontech Laboratories Inc, CA, USA) under the control of the promoter *P_{lac}*. The plasmid was constructed by excising the *gfp* coding sequence from pGFP and ligating it in frame into pBBR1MCS-5, downstream of the promoter. The host plasmid, pBBR1MCS-5, (supplied by Dr. Vittorio Venturi; ICGEB, Trieste, Italy), and pGFP were digested with HindIII and EcoRI. Digests were resolved on a 1% (w/v) agarose gel and the desired linear fragments were recovered from the gel and purified. The reporter *gfp* was cloned into the HindIII/EcoRI sites of pBBR1MCS-5 using the Rapid DNA Dephos & Ligation Kit (Roche, Germany). The resulting plasmid, pBBR1MCS-5-gfp, was transformed into One Shot® TOP10 chemically competent *E. coli* cells (Invitrogen) and recombinant transformants were selected by alpha-complementation on Gm-amended agar plates. The construct, pBBR1MCS-5-gfp, was purified from a clonal sub-culture and the presence of the insert was verified by restriction digestion followed by analytical agarose gel electrophoresis.

6.3.3 Preparation of chemically competent *Labrenzia* sp. strain 5N cells

Labrenzia sp. strain 5N was grown to an OD₆₀₀ of 0.375 and fifty millilitres of the culture was harvested by centrifugation for 7 min (1600 g, 4°C). The supernatant was

discarded and the cell pellet was resuspended in 10 ml ice cold CaCl₂ solution (100mM CaCl₂, 60mM PIPES, 15% glycerol). Cells were centrifuged at 1100 x g at 4°C for 5 min, the supernatant discarded, and the cell pellet resuspended in 10 ml ice cold CaCl₂ solution before storage on ice for 30 min. The cell suspension was then centrifuged at 1100 x g at 4°C for 5 min and the supernatant discarded. The cell pellet was taken up in 2 ml ice cold CaCl₂ solution and 50 µl aliquots were dispensed into ice-cold 1.6 mL microcentrifuge tubes.

6.3.4 Chemical transformation of plasmid DNA into *Labrenzia* sp. strain 5N

A 50µl aliquot of CaCl₂-competent *Labrenzia* sp. 5N cells was thawed on ice and mixed with 10 ng of plasmid DNA. Cells were kept on ice for 30 min and then heat-shocked at 42°C for 30 s. One millilitre of ASW:LB medium (3:1) was added and the cells were incubated at 33°C for 2 hours at 200 rpm to induce Gm resistance gene expression. Cells were serially diluted and incubated overnight on ASW:LB (3:1) plates containing Gm.

6.3.5 Preparation of electro-competent *Labrenzia* sp. strain 5N cells

Cultures of *Labrenzia* sp. 5N were grown to an OD₆₀₀ of 0.375 and 10ml of each culture was pelleted at 10,000g at 4°C for 3 min. The supernatant was discarded and the cell pellet was washed twice in either ice cold 300mM sucrose, deionized H₂O or 10% (v/v) aqueous glycerol. The supernatant was discarded and the cell pellet resuspended in 200 µl of either 300mM sucrose, deionized H₂O or 10% (v/v) glycerol. 50µl aliquots of competent cells were dispensed into ice cold microcentrifuge tubes and either used immediately or stored at -80°C.

6.3.6 Electrotransformation of *Labrenzia* sp. strain 5N

Fifty microlitres of electro-competent *Labrenzia* sp. 5N cells were thawed on ice. Plasmid DNA (25 ng) was added to the competent cells, mixed gently, and then transferred to a 2mm-gap, pulser cuvette (Bio-Rad, Munich, Germany). Electrotransformations were performed with a MicroPulser™ (Bio-Rad, CA, USA) at a

range of voltages between 1.5 and 3kV, at 200 Ω resistance and 25 μ F capacitance. Immediately following the pulse, 1ml ASW:LB (3:1) was added to the cuvette, and cells were transferred to a 10ml Sterilin tube and incubated at 33°C in a 200rpm orbital incubator for between 4 and 24 hours. Following the incubation period, cells were serially diluted in ASW:LB (3:1) medium and grown for 2 days at 33°C on ASW:LB (3:1) agar plates containing Gm. Controls were also set up with non-transformed electroporated cells grown on ASW:LB (3:1) plates, to confirm cell viability, as well as on ASW:LB (3:1) plates containing Gm, to confirm adequate selection by the antibiotic (i.e., absence of growth).

6.3.7 Generation of a conjugal donor *E. coli* MC1061 strain containing pBBR1MCS-5-gfp

CaCl₂-competent *E. coli* MC1061 (pRK24, pRL542) cells were prepared as described for *Labrenzia* sp. strain 5N cells above. A stock of *E. coli* MC1061 (pRK24, pRL542) was streaked onto an LB agar plate containing Ap and Cm and grown overnight at 37°C. A single colony was inoculated into LB broth containing Ap and Cm and 50 ml of culture was harvested by centrifugation at 4°C at 6000g, once an OD₅₉₀ of 0.375 had been reached, washed in CaCl₂, and stored at -80 °C.

To construct the donor, a 50 μ l aliquot of CaCl₂-competent *E. coli* MC1061 (pRK24, pRL542) cells was thawed on ice and mixed with 10ng of pBBR1MCS-5-gfp. Cells were kept on ice for 30 min and then heat shocked at 42°C for 2min. 1ml LB was added and cells were incubated at 33°C for 1 hour at 200 rpm. Cells were inoculated onto LB agar plates containing Ap, Cm and Gm and incubated overnight at 33°C. Transformants were screened for the presence of pBBR1MCS-5-gfp by plasmid purification followed by PCR using primers targeting the Gm^r gene harboured by the vector (Appendix Table 8.1).

6.3.8 Conjugal transfer of plasmid vectors into *Labrenzia* sp. strain 5N

Conjugation of *Labrenzia* sp. strain 5N with the donor strain *E. coli* MC1061 (pRK24, pRL542, pBBR1MCS-5-gfp) was performed using a modified version of the protocol described by Thoma and Schobert (2009). The donor and recipient strains were both grown to an OD₅₉₀ of 0.5 and mixed at ratios of 1:1, 5:1 and 10:1 (donor:recipient). The

mixed cell suspensions were pelleted via centrifugation for 5 min at 6000g and resuspended in 1 ml ASW. A spot of the mixed cell suspension was plated onto ASW:LB plates (1:4 vol:vol) containing the lowest salt concentration tolerated by *Labrenzia* sp. strain 5N in order to maintain the viability of the *E. coli* donor. After 24 hours at 33°C, cells were scraped from the plate with a sterile glass spreader and resuspended in ASW medium. Cell suspensions were serially diluted and plated onto selective denitrification medium containing Gm and incubated anaerobically at 33°C for 72 hours. A combination of the high salt concentration of this medium and the requirement to respire nitrate anaerobically using acetate as the sole, non-fermentable, carbon source was employed to limit the overgrowth of the *E. coli* donor strain. A selection of the resulting *Labrenzia* sp. strain 5N exconjugants were then streaked onto fresh denitrification medium to further isolate colonies from any contaminating *E. coli*.

6.3 9 Screening of *Labrenzia* sp. strain 5N pBBR1MCS-5-gfp transformants by PCR

Putative transformants were screened to confirm the presence of pBBR1MCS-5-gfp by colony PCR by targeting the M13 forward and reverse priming sites (Appendix Table 8.1), that flank the *gfp* gene present in the vector. To exclude *E. coli* donors carrying the construct, the identity of the host was confirmed using *Labrenzia* sp. strain 5N nitrous oxide reductase (*nosZ*) specific primers (StanosF and StanosR) (Appendix Table 8.1). Total DNA (chromosome plus plasmid DNA) was extracted by boiling a subsample of the colony in 10µl dH₂O at 98°C for 10 min. The 10µl DNA extraction was then added to 12.5 µl of 2 × MyTaq™ Red Mix (Bioline reagents, London, UK) amended with 50pmol forward and reverse primer mix and diluted to a final 25µl volume with sterile dH₂O. PCR thermocycling conditions were as follows: initial denaturation at 95 °C for 2 min followed by 25 amplification cycles of a 94 °C denaturation step for 30 s, a 30 s annealing step and 72°C extension for 30 s and a final extension step for 10 min at 72°C. Annealing temperatures were 55°C for M13 and 52°C for *nosZ*. Following amplification, the 996bp PCR products were resolved on a 2% (w/vol) agarose gel stained with 0.5 µg.mL⁻¹ ethidium bromide. Positive and negative controls were performed containing DNA from wild-type, non-transformed *Labrenzia* sp. strain 5N with (positive) or without (negative) the addition of 10ng pBBR1MCS-5-gfp in the reaction mix.

Screening of Gm-resistant colonies by PCR amplification of *gfp* using M13 primers was unsuccessful for the electrotransformations and so potential transformants were additionally screened by PCR targeting the Gm resistance gene. This procedure was adopted to establish whether these Gm resistant colonies had in fact acquired a copy of the Gm^r cassette or if they were resistant, spontaneous mutants. 50pmol GenRFor and GenRRev primer mix was used in the reactions (Appendix Table 8.1). PCR conditions were as follows: initial denaturation at 95°C for 2 min followed by 30 amplification cycles of a 94°C denaturation step for 30 s, a 30 s annealing step at 55°C and 72°C extension for 30 s and a final extension step for 10 min at 72°C. Following amplification, the expected 155bp product was resolved on a 2% (w/v) agarose gel.

6.3.9 Screening of potential *Labrenzia* sp. strain 5N pBBR1MCS-5-gfp transformants by plasmid isolation

Isolation of pBBR1MCS-5-gfp from *Labrenzia* sp. strain 5N transformants was performed using a range of extraction and purification methods. Negative controls were included for all isolations, consisting of DNA from a wild-type, non-transformed *Labrenzia* sp. strain 5N culture. Putative transformants were grown in ASW:LB (3:1) containing Gm in an orbital incubator at 33°C, 200 rpm overnight. Plasmid purification was carried out using the NucleoSpin® Plasmid kit (MACHEREY-NAGEL, Germany), following the suppliers' recommended protocols. This was the preferred method for screening *Labrenzia* sp. strain 5N transconjugant colonies. Purified plasmids were then digested with EcoR1 at 37°C for 2 hours to linearize the plasmids (0.5 units EcoR1 [New England BioLabs, MA, USA], 5µl pBBR1MCS-5-gfp, 10x buffer). Plasmid purifications were resolved on a 1% (w/v) agarose gel.

Gm-resistant colonies resulting from the electroporation experiments were screened by two additional methods of plasmid isolation, as the NucleoSpin® Plasmid kit failed to isolate the target plasmid despite Gm resistance of the host. A 'boiling method' and the ZymoPURE Plasmid midiprep kit (Zymo research, USA) were also tested but the negative results obtained were consistent with those with the NucleoSpin® kit.

6.3.10 Fluorescent imaging

Labrenzia sp. strain 5N cells harbouring pBBR1MCS-5-gfp were examined by fluorescence microscopy to confirm expression of the *gfp* reporter gene. Fluorescence was visualised using a Zeiss Axiovert 135 microscope using a x63 1.2 NA oil immersion objective and the Filter Set 38 HE (Excitation 470 nm, Beamsplitter 495 nm, Emission 525 nm). Images were captured with an AxioCam MRc camera system and analysed using Zen (Zeiss) software.

6.4 Results and Discussion

6.4.1 Construction of a *gfp* vector capable of replication in *Labrenzia* sp. strain 5N

To generate a fluorescent reporter plasmid with the potential to replicate in *Labrenzia* sp. strain 5N, *gfp* was inserted into the Multiple Cloning Site of pBBR1MCS-5 (Figure 6.1). The successful construction of this recombinant reporter, pBBR1MCS-5-gfp, was confirmed by plasmid isolation and restriction digestion. This showed that the plasmid isolated from the transformant selected carried an insert of the expected size (Figure 6.2). Colony PCR screening was also used to verify the presence of the Gm resistance cassette (Figure 6.3). The positive results of both these preliminary screens were corroborated by DNA sequencing.

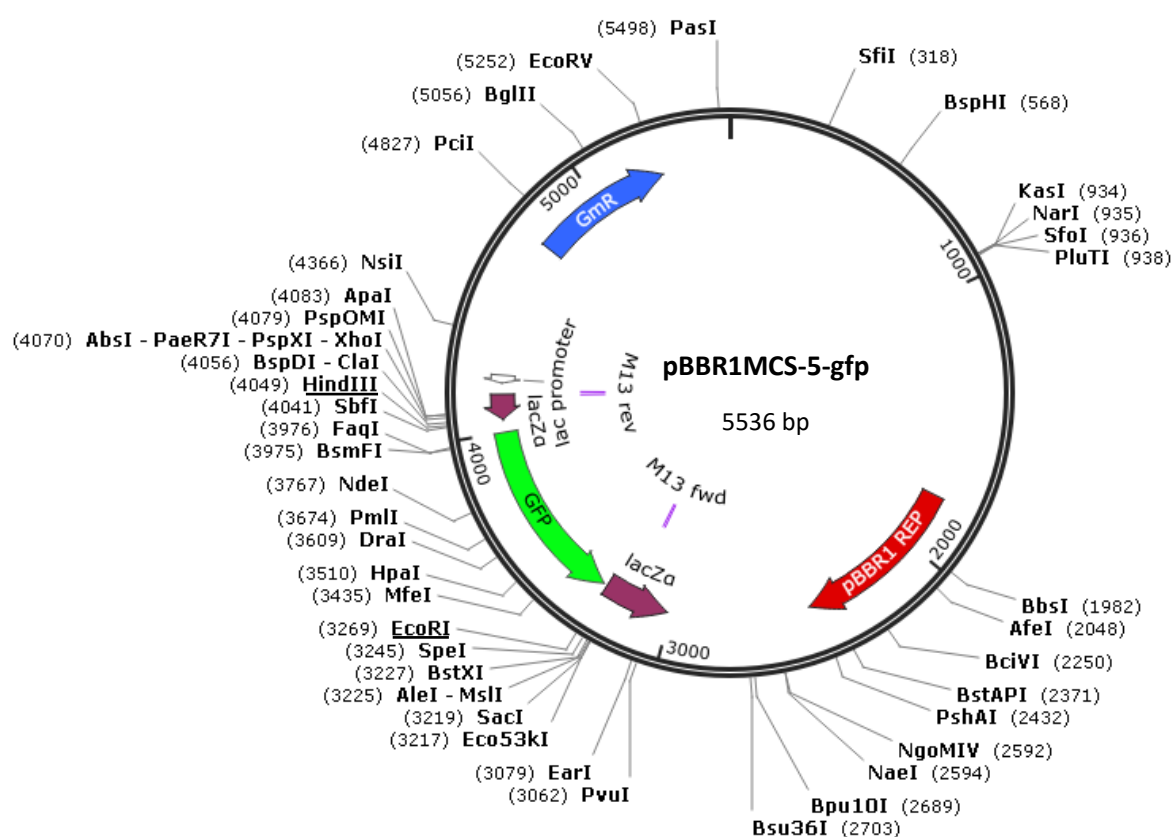


Figure 6.1. Map of the plasmid vector pBBR1MCS-5-gfp. The vector was constructed by cloning the *gfp* gene into the HindIII and EcoRI restriction sites (underlined). Locations of the main ORFs, unique restriction enzyme sites, M13 forward and reverse primer regions and promoters are displayed. GmR, gentamycin resistance cassette; pBBR1 REP, origin of replication.

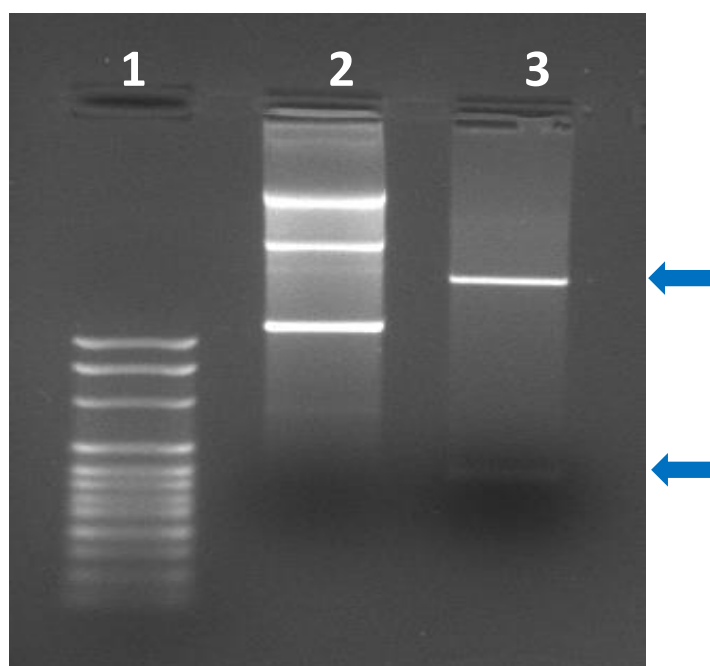


Figure 6.2. Analytical agarose gel analysis of the purified pBBR1MCS-5-gfp construct purified after transformation of One Shot® TOP10 chemically competent *E. coli*. Lane 1, PCR markers; Lane 2, un-digested pBBR1MCS-5-gfp; Lane 3, pBBR1MCS-5-gfp digested with EcoRI and HindIII to release the *gfp* insert. Arrows indicate the expected sizes and locations of the digested pBBR1MCS-5-gfp backbone (4756bp) and *gfp* insert (780bp).

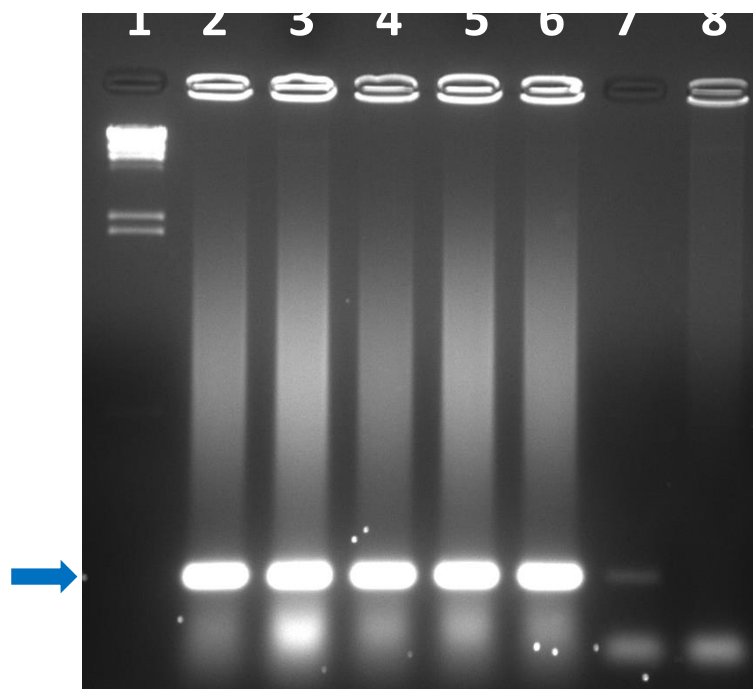


Figure 6.3. Analytical agarose gel analysis of putative Gm products amplified from pBBR1MCS-5-gfp transformant *E. coli* colonies. Lane 1, Lambda HindIII marker; Lanes 2-6, test colonies 1-5 respectively; Lane 7, positive control; Lane 8, negative control. Arrow indicates the expected size (155bp) and location of the target PCR product.

6.4.2 *Labrenzia* sp. strain 5N is unreceptive to chemical transformation and electroporation techniques

Several chemical transformation, electroporation and bi-partite conjugation methods were tested to investigate how best to introduce plasmid DNA into *Labrenzia* sp. strain 5N. First, a chemical transformation approach was taken but no Gm-resistant *Labrenzia* colonies were recovered. This negative result was a consistent outcome despite varying the heat shock period between 30 – 120 s and the length of time used for expression between 2 and 6 hours (to account for the relatively slow growth rate in *Labrenzia* sp. strain 5N). It was concluded that *Labrenzia* sp. strain 5N cells cannot be made chemically competent and transformed under the conditions investigated. These results are in agreement with studies by Piekarski *et al.* (2009) who investigated the potential for chemical transformation in several *Roseobacter* strains.

Next, the introduction of plasmid DNA into strain 5N by electroporation was attempted. Cells were prepared in ice cold deionized water, 300mM sucrose or 10% (v/v) glycerol following the method described by Miller and Belas (Miller and Belas, 2006); a preparation technique found to be successful with *Roseobacter* strains (Miller and Belas, 2006; Piekarski *et al.*, 2009; Sebastian and Ammerman, 2009). Pulse intensity was varied between 1 and 3kV (5 msec length, capacitance of 20 μ F) and the expression period varied from 2 to 8 hours. Gentamycin resistant colonies were obtained and screened for the plasmid by Nucleospin purification and also by colony PCR. PCR amplifications targeting the M13 primer regions that flank the MCS were consistently negative (Figure 6.4). Likewise, plasmid DNA could not be isolated from the putative transformants, despite their ability to grow on plates containing Gm. It was concluded that these potential clones were either spontaneous mutants or may have incorporated the gentamycin resistance gene into the chromosome since they exhibit Gm resistance, though appear not to contain the resistance plasmid. Selection due to recombination of the Gm^r gene into the chromosome may be a sign of plasmid incompatibility with the two natural plasmids harboured by *Labrenzia* sp. strain 5N. Since this phenomenon would impede the further use of pBBR1MCS-5-gfp in this study, primers specific to the Gm^r gene were synthesized to screen for the presence of the resistance cassette within the genome (Appendix Table 8.1). Colony PCRs using the Gm^r primers were all negative (Figure 6.5). Therefore, it was concluded that

the resistant colonies isolated were spontaneous Gm-insensitive mutants, rather than genuine recombinants or cryptic pBBR1MCS-5-gfp transformants.

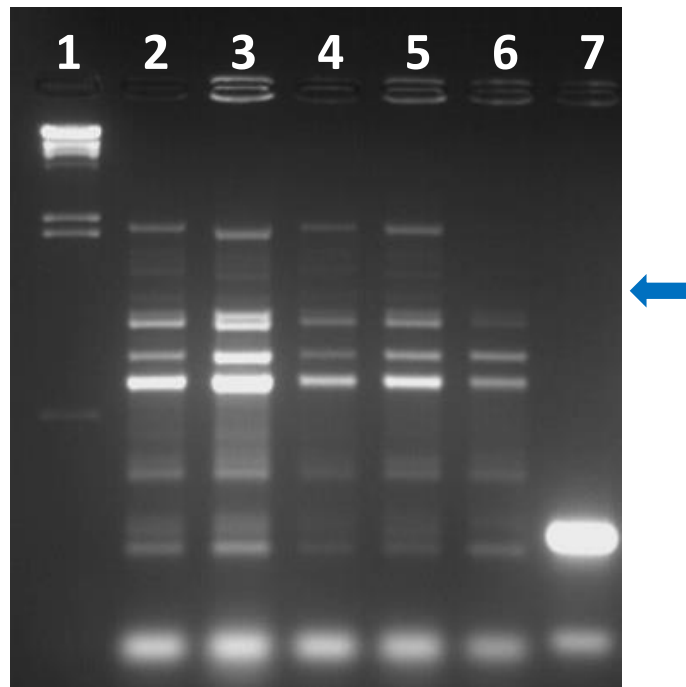


Figure 6.4. Analytical agarose gel separation of M13-primed PCR products from putative *Labrenzia* sp. strain 5N electro-transformants. Lane 1, Lambda HindIII ladder; Lanes 2-7, test colonies 1-6 respectively. The expected size (996bp) and position of the missing gfp-containing product) is indicated by an arrow.

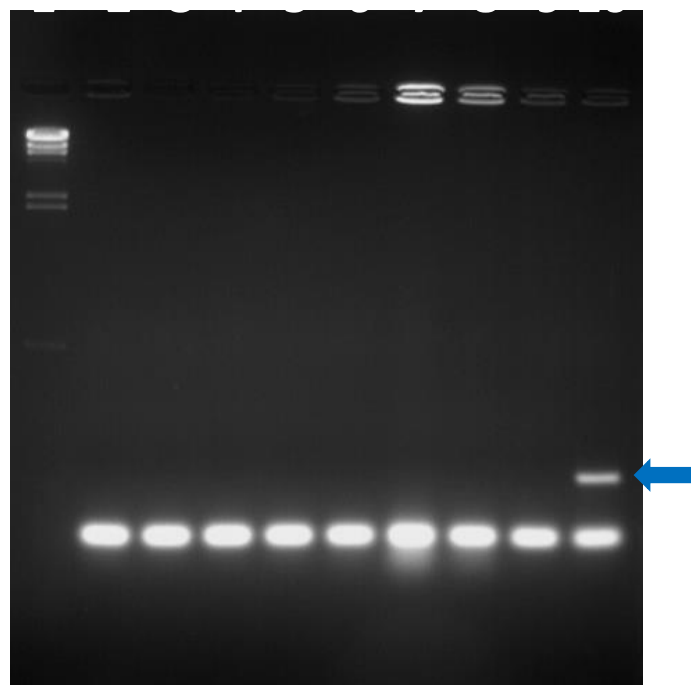


Figure 6.5. Analytical agarose gel PCR screen for the gentamycin resistance gene in putative *Labrenzia* sp. strain 5N electro-transformants. Lane 1, Lambda HindIII ladder; Lanes 2-8, colonies 1-7; Lane 9, negative control; Lane 10, positive control. The arrow indicates the position of the target PCR product (155bp).

6.4.3 Successful transformation of *Labrenzia* sp. strain 5N via conjugation

Conjugations were carried out between a donor strain *E. coli* MC1061 (pRK24, pRL542, pBBR1MCS-5-gfp) and the recipient *Labrenzia* sp. strain 5N (Figure 6.6 and Appendix Table 8.10). The donor strain contains the plasmid of interest as well as pRK24 (Figurski and Helinski, 1979), which encodes conjugative machinery, and the helper plasmid pRL542 (Elhai *et al.*, 1997), harbouring mobilization (*mob*) genes. Strain 5N has an obligate requirement for salt, but the high NaCl levels typically used in its growth medium (2.5% w/v) inhibit the growth of *E. coli*. Therefore, the growth of both the donor and recipient was tested at a range of salt concentrations from 0 to 2.5% (w/v). An ASW:LB ratio of 1:4, corresponding to an NaCl concentration of 0.5% (w/v), was the only salt level that allowed the growth of both organisms, albeit at slower growth rates than when in their preferred media. Therefore, this diluted medium was selected for use in mating experiments. Different ratios of donor to recipient cells were tested, of which a 1:1 ratio was found to be the most effective. Conjugations were performed 33°C and after 24 hours incubation on solid medium, the cells were scraped from the plate with a sterile glass spreader and resuspended in full strength ASW medium (i.e. unamended with LB).

The cell suspensions were serially diluted and plated onto selective denitrification medium containing Gm. After 72 hours of anaerobic growth at 33°C, numerous individual colonies of *Labrenzia* sp. strain 5N were visible. These putative recombinants were of a larger size and also possessed the distinct brown/red colouring of the *Labrenzia* host among much smaller *E. coli* colonies. The putative transconjugants were subcultured onto individual fresh denitrification media plates to further eliminate any donor *E. coli* contamination. PCR detection of the (i) the pBBR1MCS-5 Gm^r gene and (ii) *nosZ* (using *Labrenzia*-specific primers, Appendix Table 8.10) was used to confirm successful transformation of pBBR1MCS-5-gfp into strain 5N (Figures 6.7 and 6.8). Plasmids of the expected size were also isolated from transconjugant colonies and visualised by agarose gel electrophoresis (Figure 6.9).

pBBR1MCS-5 was stably maintained in *Labrenzia* sp. strain 5N after passage through several subcultures on selective medium followed by confirmation by plasmid isolation. Findings demonstrate it was compatible with the strain's natural plasmids and a suitable choice of vector. This is consistent with recent studies that have confirmed

the pBBR1MCS vector series is suitable for the stable transformation of many marine Alphaproteobacteria, including members of the marine *Roseobacter* clade that is closely affiliated with *Labrenzia* species (Piekarski *et al.*, 2009; Hördt *et al.*, 2020).

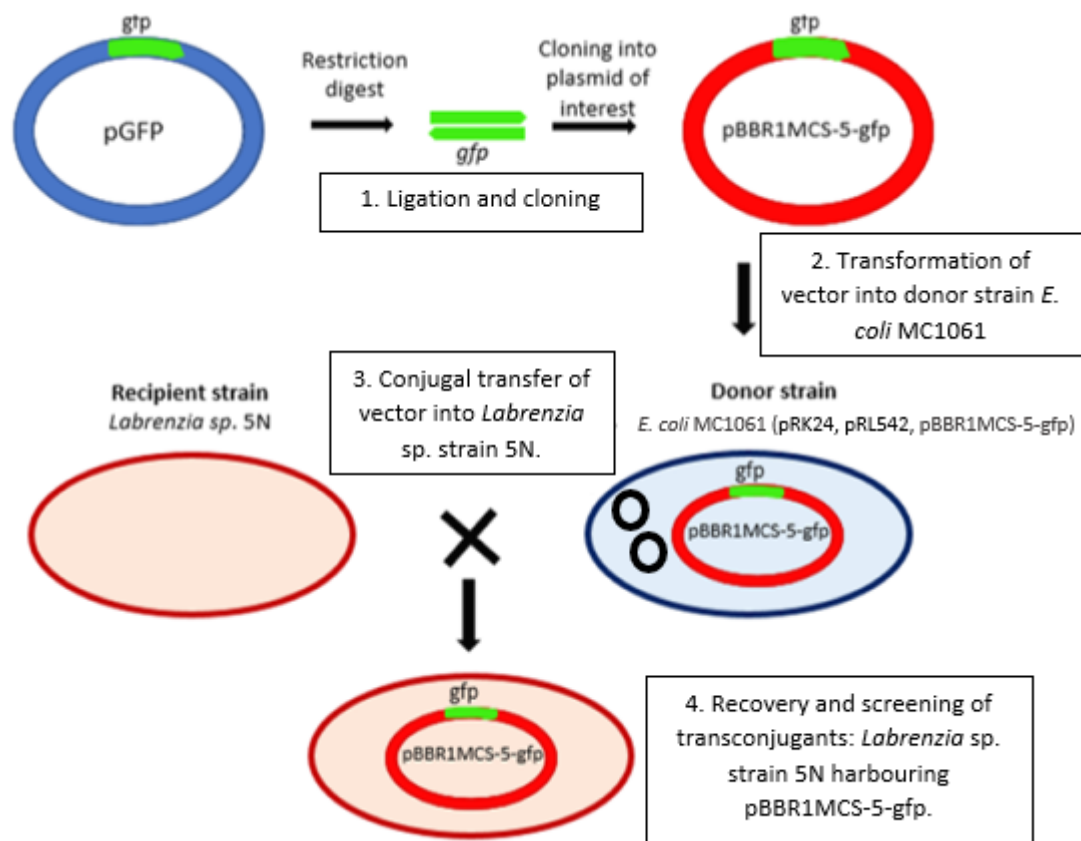


Figure 6.6. Generation of a GFP labelled *Labrenzia* sp. strain 5N via conjugation.

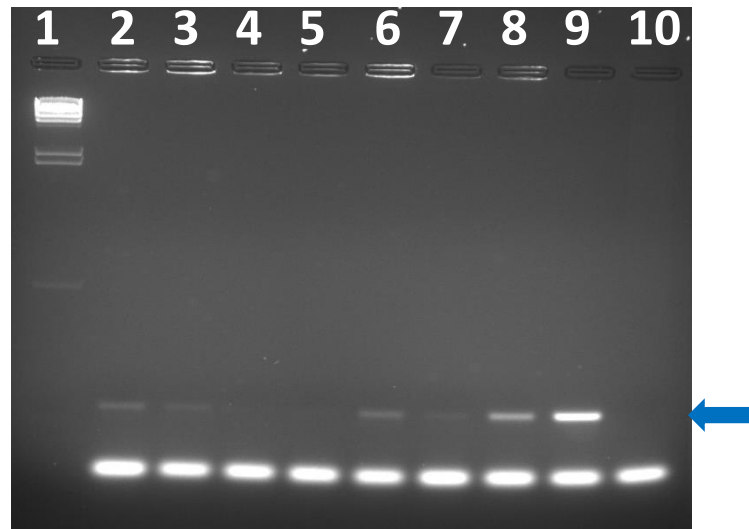


Figure 6.7. Analytical agarose gel of colony PCR screens for Gm in *Labrenzia* sp. 5N pBBR1MCS-5-gfp transconjugants. Lane 1, Lambda HindIII marker; Lanes 2-8, transconjugant colonies 1-7 respectively; lane 9, positive control (pBBR1MCS-5-gfp); lane 10, negative control (wild-type *Labrenzia* sp. strain 5N). The arrow indicates the expected position of the 155 bp target PCR product.

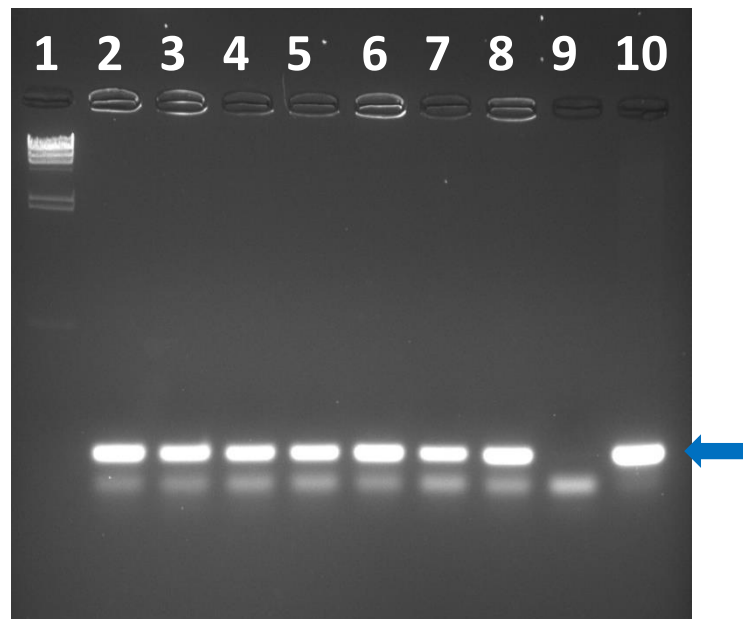


Figure 6.8. Analytical agarose gel of a PCR screen for *nosZ* in *Labrenzia* sp. strain 5N pBBR1MCS-5-gfp transconjugants. Lane 1, Lambda HindIII ladder, Lanes 2-8, transconjugant colonies 1-7; Lane 9, negative control (pBBR1MCS-5-gfp); Lane 10, positive control (wild-type *Labrenzia* sp. strain 5N). The arrow indicates the expected position of the 252 bp target PCR product.

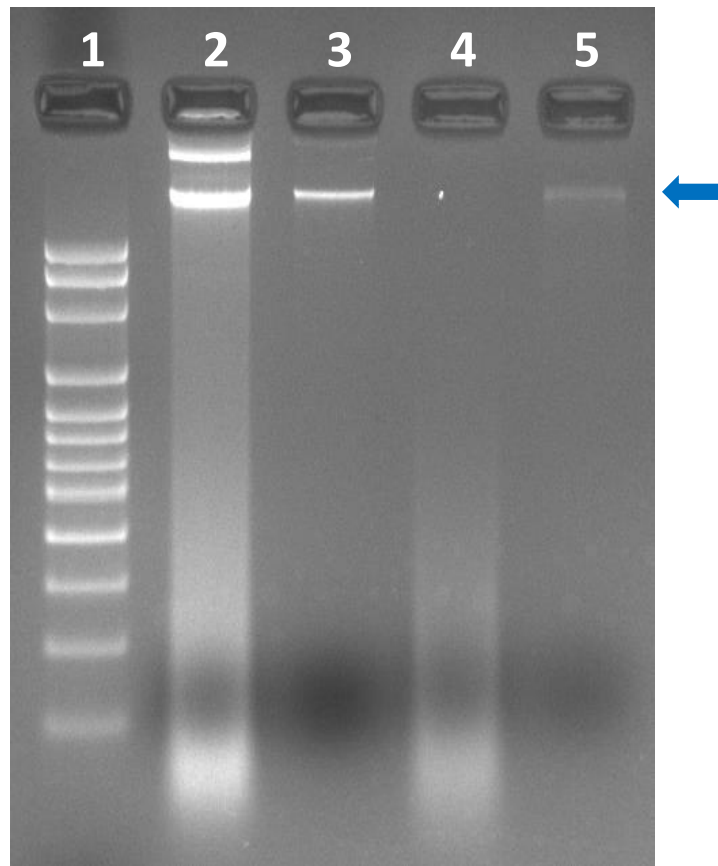


Figure 6.9. Analytical agarose gel of pBBR1MCS-5-gfp plasmid isolations. Lane 1, PCR markers; Lane 2, *E. coli* MC1061 (pRK24, pRL542, pBBR1MCS-5-gfp); Lane 3, *Labrenzia* sp. strain 5N (pBBR1MCS-5-gfp) transconjugant colony number 8; Lane 4, EcoRI restriction digest of *E. coli* MC1061 (pRK24, pRL542, pBBR1MCS-5-gfp) plasmid isolation; Lane 5, EcoRI restriction digest of *Labrenzia* sp. strain 5N (pBBR1MCS-5-gfp) transconjugant colony number 8. The arrow indicates the position of the ~5.5kb plasmid pBBR1MCS-5-gfp.

When examined by fluorescence microscopy (Figure 6.10) pBBR1MCS-5-gfp transformed exconjugant *Labrenzia* sp. strain 5N cells emit a bright fluorescent signal that appears to be localised to the cytoplasm surrounding the unstained nucleoid. Although the fluorescence intensity appears somewhat variable between individual cells, this phenomenon is the result of a deep specimen layer. Cells in the same focal plane are comparable in the intensity of their fluorescent signals. It was concluded, therefore, that *Labrenzia* sp. strain 5N cells transformed with pBBR1MCS-5-gfp by conjugation expresss gfp and generate an easily detectable and reasonably stable fluorescent signal.

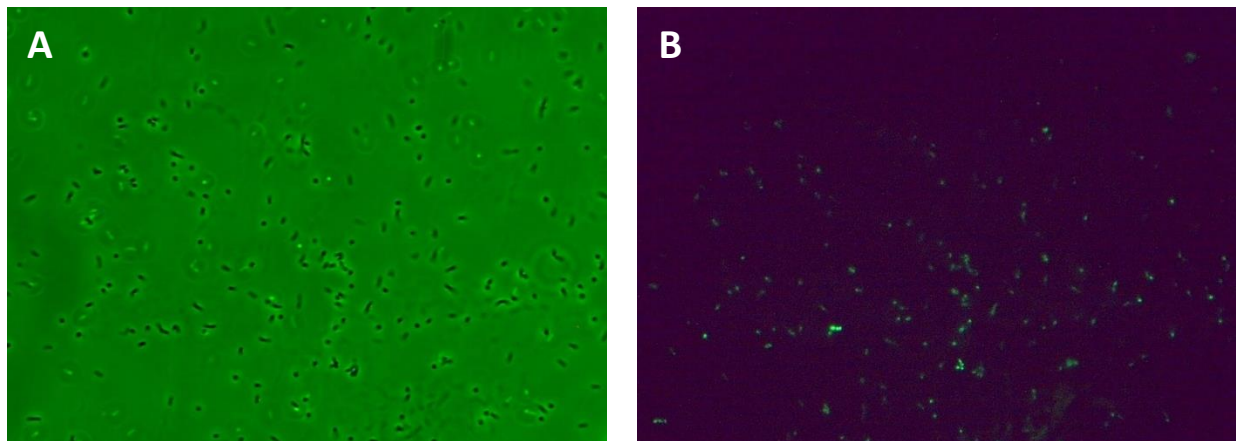


Figure 6.10. (A) Bright Field and (B) fluorescence microscopy images of *Labrenzia* sp. strain 5N transformant harbouring pBBR1MCS-5-gfp at 64x magnification.

6.4.4 Potential future avenues of genetic manipulation of *Labrenzia* sp. strain 5N

The protocol for the introduction of DNA into *Labrenzia* sp. strain 5N by conjugation that is reported in this study opens the door to genetically manipulate this strain. The combination of a successful protocol for genetic manipulation and access to genome sequencing information for strain 5N (Chapter 3), means that the function of specific genes can now be studied easily. Also, the genetic control and regulation of specific pathways of interest at both the transcriptional and translational levels can also be investigated readily. Aside from the genes required for denitrification, strain 5N harbours numerous other genes of environmental and climatic importance. These include those required for DMSP metabolism and production, CO oxidation, nitrification and aromatic compound degradation (Chapter 3).

The introduction of marker genes, such as the fluorescent marker *gfp* demonstrated in this study, facilitates the detection of host. Fluorescent markers can be used to study cell motility, biofilm formation or to identify and localise the organism within its environment (Chudakov and Lukyanov, 2003; Ma and Bryers, 2010; Gomes and Mergulhão, 2018). Fluorescent tools such as this are widely utilised to study interactions between bacteria and their host organisms. For example, *gfp*-labelled bacteria have been successfully used for investigating how soil bacteria, such as *Klebsiella pneumoniae* and *Pseudomonas* spp., interact with the roots of their plant hosts (Normander *et al.*, 1999; Marasco *et al.*, 2012). In marine systems, the extent of bacterial colonisation of marine cyanobacteria has been visualised using *gfp*-tagged bacteria in the case of *Nodularia* spp. (Eigemann *et al.*, 2019) and fluorescence assays in the case of *Trichodesmium* (As *et al.*, 2011). When re-introduced to the consortia it is known to associate with, strain 5N's relative abundance and interactions within these communities could be studied. pBBR1MCS-5-*gfp* could be used experimentally to visualise the localisation of *Labrenzia* sp. strain 5N within *Trichodesmium* colonies and the surrounding medium. This would provide a better understanding of the distribution of related denitrifiers within the colonies and whether these cells are evenly distributed through the colony interior and periphery. As strain 5N is a metabolically versatile, facultative anaerobe its distribution may not be restricted to the central suboxic microenvironments within the colonies, even if the denitrification process itself may be limited to such zones.

Furthermore, the use of fluorescent markers as reporter genes would enable *in vivo* gene expression assays in strain 5N. Promoter probe vectors are widely used to study gene expression patterns in bacteria (Karunakaran *et al.* 2018). This technique involves the construction of a vector containing a fusion between the promoter sequence for the gene of interest and a readily detected reporter, such as *gfp*. The expression of a gene can be detected and visualised *in vivo* by detecting and quantifying the fluorescent signal emitted by the downstream reporter. This approach would be invaluable in future studies on the denitrifying *Labrenzia*-like associates of the cyanobacterium, *Trichodesmium*. It would be particularly interesting to use the protocol developed in this study to investigate the expression of denitrification genes within colonies of *Trichodesmium*. For example, a promoter probe vector could be generated by creating a fusion gene between a fluorescent reporter (*gfp* or an anaerobic fluorescent reporter) and the promoter for nitrous oxide reductase (*nosZ*), using the pBBR1MCS-5 vector backbone. This vector could then be introduced into *Labrenzia* sp. strain 5N cells via the conjugation methods developed here.

The product of *nosZ* catalyses the terminal step in the denitrification pathway, reducing the greenhouse gas nitrous oxide to dinitrogen gas. Using a promoter probe vector in strain 5N, it would be possible to visualise the level of *nosZ* expression in the cells quantifiably using fluorescence microscopy. When grown in association with the host, *Trichodesmium*, this would provide a greater understanding of the physical localisation of denitrification within the colonies themselves. A combination of fluorescent reporters could be used to compare the overall distribution of *Labrenzia* sp. strain 5N-like cells within the colonies with that of the actively denitrifying cells. Additionally, the regulation of the denitrification genes under varying conditions, such as day-night cycles or ambient oxygen concentrations, could also be investigated. This system could be used to help unravel the dynamics of *Trichodesmium* associated denitrification. Indeed, the impetus for developing the genetic system for strain 5N was to facilitate just this type of investigation but work was curtailed by the restrictions imposed by the COVID-19 pandemic.

6.4.5 A starting point for the genetic manipulation of other *Labrenzia* spp.

The capacity to genetically manipulate *Labrenzia* sp. strain 5N has great potential for future work on the *Trichodesmium* holobiont and could also be adapted for studies involving other members of the *Labrenzia* genus. Over recent years, novel *Labrenzia* spp. from a wide variety of environments have been isolated (Pujalte *et al.*, 2005; Kim *et al.*, 2006; Weber and King, 2007; Bibi *et al.*, 2014). *Labrenzia* spp. contribute to marine nutrient cycling processes as well as to the climatically important production of dimethylsulfoniopropionate (DMSP) and nitrous oxide (Andrew R J Curson *et al.*, 2017b; Coates and Wyman, 2017). The potential to genetically manipulate these organisms should provide additional avenues to study members of this emerging group of globally important marine heterotrophs.

Despite the growing recent interest in *Labrenzia* spp., research to date has primarily focussed on descriptive studies of new isolates and/or genomic studies. At the time of writing, thirty-four draft genomes from members of the *Labrenzia* genus have been made publicly available. A third of these have been sequenced and published since 2019, demonstrating how research into this group of organisms is of accelerating interest and momentum. Whilst genome sequencing data can reveal a great deal of information about an organism, the study of functional pathways is limited by the availability of genetic manipulation tools adapted for members of the genus. *Labrenzia aggregata* LZB033 and *Labrenzia* sp. strain PHM005 are the only *Labrenzia* isolates to have undergone successful genetic manipulation to date (Curson *et al.*, 2017, Kačar *et al.*, 2019; Xu *et al.*, 2019). Suicide vectors harbouring genes of interest were introduced by triparental mating in both strains, to study the regulation of (i) labrenzin synthesis (a potential anti-cancer compound) and (ii) *rpoN*, respectively. Like strain 5N, both studies demonstrated that these *Labrenzia* spp. were capable of plasmid uptake, although the conjugation protocols used were not described in detail in either publication. Because suicide vectors were used to generate the gene knockouts described in these studies, it has yet to be demonstrated that they are able to maintain replicating plasmids stably. Potentially, however, the bi-partite conjugation protocol developed in the present study has wider promise for the genetic manipulation of other members of this genus.

Based on available genome sequencing data, plasmids have been identified in seventeen of the thirty-four sequenced *Labrenzia* spp. (Chapter 3). Additionally, bacterial *type IV secretion systems* (T4SSs) have also been detected across 22 *Labrenzia* species. The presence of these conjugal transfer systems strongly suggests the capacity for the conjugal transfer of plasmids is widespread among members of the genus. This natural ability to uptake foreign DNA through conjugation would suggest the protocol developed in this study could be easily adapted for use in a wide range of *Labrenzia* spp. Conjugation protocols may need to be optimised for different species, however, but the work reported here is a starting point for such optimisation. For example, it would be necessary to consider the mode of transconjugant selection. Like the present study, transconjugants of *Labrenzia* strains capable of partial or full denitrification could be selected by their ability to respire nitrate. The protocol could be modified also for non-denitrifying strains by using an auxotrophic donor strain, instead of *E. coli* MC1061, which would facilitate counterselection (Allard *et al.*, 2015). Syntenic plasmids are shared among a number of *Labrenzia* species, including strain 5N (Chapter 3). Given that these plasmids belong to the same incompatibility groups, it is likely that the pBBR1MCS derived plasmids used in this study would also be successfully maintained in a wider range of *Labrenzia* spp.

6.5 Conclusions

This study set out to develop a protocol to introduce a suitable plasmid vector into *Labrenzia* sp. strain 5N. To construct a plasmid vector capable of replicating in strain 5N and that would stably and quantifiably express the fluorescent marker gene *gfp*. Reliable method of Introducing this vector into the strain, following which it would be stably maintained. The ultimate aim is to implement this method for further studies investigating N cycling dynamics between *Trichodesmium* and *Labrenzia* denitrifiers.

This study assessed the capacity of *Labrenzia* sp. strain 5N to acquire and maintain plasmid DNA. It was determined that strain 5N was unreceptive to both chemical transformation and electroporation techniques under the experimental conditions tested. However, a successful protocol was developed for plasmid introduction through bi-partite conjugation using an *E. coli* donor strain. Plasmids belonging to the pBBR1MCS-5 vector series were shown to be stably maintained in strain 5N alongside

its two natural plasmids. Therefore, transformation via conjugation using this vector, or a derivative, is recommended for future studies with this strain. The widespread occurrence of syntenic natural plasmids and conjugation genes within the genus (Chapter 3), suggests conjugation-based procedures may be suitable for manipulating a wider range of *Labrenzia* strains also. There are many potential applications for gene specific studies using the procedures described. In particular, the use of promoter probes would be invaluable for transcriptional studies of the denitrification pathway in *Labrenzia* species forming associations with marine phytoplankton, including the colonial cyanobacterium, *Trichodesmium* (Coates and Wyman, 2017).

Chapter 7:

General Discussion

The research findings presented in chapters 2-6 report the results of an investigation into a model marine, denitrifying alphaproteobacterium from the suboxic waters of the Arabian Sea; a major Oxygen Minimum Zone (OMZ). Characterisation of the physical, biochemical and metabolic characteristics of this representative strain were complemented by genome-wide analyses into its genetic potential. How this bacterium adapts and modifies its proteome in response to anaerobiosis was investigated, as was its capacity for N transformations under different oxygen tensions. This chapter puts the work into context, by relating the laboratory findings presented in this thesis to the environmental challenges posed by the highly dynamic natural habitat of this organism. The potential significance of the results for understanding the constraints on denitrification by closely related organisms in OMZs and more specifically those within the *Trichodesmium* microbiome are explored. More generally, the implications for future rates of N₂O production by these denitrifiers are considered in the light of the projected expansion of low oxygen waters in response to climate change.

7.1 Denitrification in the Arabian Sea

7.1.1 Metabolic flexibility provides a competitive advantage to microbes inhabiting the Arabian Sea; a key OMZ.

Environmental conditions in the Arabian Sea, a major OMZ, are highly dynamic and not just in terms of the wide range of O₂ levels encountered. The region is subject to strong monsoonal winds from the south-west that drive seasonal upwelling between March and September. This brings nutrient-rich deep waters to the surface that fuel high primary productivity off the coasts of Africa and the Arabian peninsula (Olson *et al.*, 1993; Brock *et al.*, 1994; Morrison *et al.*, 1998; Ducklow *et al.*, 2001). The winds change in direction during the winter months (the North East Monsoon) reversing the prevailing circulating currents and leading to the development of highly oligotrophic conditions throughout much of the basin. It appears likely, therefore, that the organisms that make up the biota in this region need to be highly flexible in their response to the large gradients in both nutrient and oxygen availability.

This thesis focusses on the marine alphaproteobacterium, *Labrenzia* sp. strain 5N, isolated from suboxic waters of the Arabian Sea. Through experimental and genome analyses the metabolic potential of strain 5N was investigated. Results showed that in addition to this strain's capacity for denitrification, it is also highly metabolically versatile (Chapters 2 and 3). It was demonstrated experimentally that this strain can utilise a wide range of C and N substrates for growth (Chapter 2). Like many other *Labrenzia* spp. (Curson *et al.*, 2017a), genome analyses (Chapter 3) showed the strain also has the genetic potential to metabolise and synthesise DMSP, an important precursor of the climatically important gas, dimethylsulfide (DMS). Strain 5N has the enzymology required to oxidise carbon monoxide, as can all *Labrenzia* strains investigated experimentally to date (Weber and King, 2007).

Labrenzia sp. strain 5N harbours genes encoding a number of degradative pathways for aromatic compounds. Chapter 3 unveiled that strain 5N has the potential to degrade naturally occurring aromatic compounds as well as environmental pollutants such as polycyclic aromatic hydrocarbons (PAH's). The capacity of some bacteria to degrade PAH's is of considerable applied interest for bioremediation purposes (Pieper and Reineke, 2000). *Labrenzia* spp. have been detected in numerous oil contaminated areas including the well-known Deepwater Horizon (DWH) site as well as oil-contaminated sediments (Kostka *et al.*, 2011; Overholt *et al.*, 2013; Bacosa *et al.*, 2015; Militon *et al.*, 2015). Hydrocarbon-degrading bacteria are widespread in the oceans, even within unpolluted waters, and play an important role in the turnover of these compounds (Leahy and Colwell, 1990; Lea-Smith *et al.*, 2015). Perhaps more intriguingly recent work has demonstrated production of hydrocarbons by members of the two most abundant cyanobacteria, *Prochlorococcus* and *Synechococcus* (Lea-Smith *et al.*, 2015). The contribution of other abundant marine cyanobacteria to global hydrocarbon cycling has not yet been established. The genetic potential for hydrocarbon biosynthesis, however, has been identified in over 140 phylogenetically diverse strains of cyanobacteria, including *Trichodesmium* (Coates *et al.*, 2014; Klähn *et al.*, 2014). For isolate 5N, therefore, metabolic flexibility appears to be a key factor in accounting for its wide geographic distribution within waters of highly contrasting environmental conditions (Wyman *et al.*, 2013).

7.1.2 *Labrenzia*-type denitrifying bacteria in the Arabian Sea and potential associated N losses.

When dissolved oxygen (DO) levels fall far enough, nitrate becomes the primary respiratory substrate. The Arabian Sea, the largest OMZ in the world, is one of the three major regions of marine denitrification along with the eastern tropical North Pacific (ETNP) and the eastern tropical South Pacific (ETSP) (Codispoti and Richards, 1976; Codispoti, 2010; Packard, 1980; Naqvi, 1987; Ward *et al.*, 2009). The Arabian Sea OMZ accounts for ~50% of total N₂ production from all OMZs globally (Devol *et al.*, 2006). Of this total, it has been estimated that denitrification accounts for 87-99% of N losses in the OMZ of the central Arabian Sea, with the remainder attributed to annamox (Ward *et al.*, 2009). Over the Omani shelf, however, annamox in combination with DNRA may be much more significant, at least seasonally (Jensen *et al.*, 2011).

The expansion of hypoxic zones in the decades ahead, due to oceanic surface water warming, will lead to increased denitrification rates. The resulting impacts on the balance of global marine N budgets will have related knock-on effects on the ocean's biota. In the Bay of Bengal OMZ, for example, current O₂ levels are only marginally (by a few nmol.L⁻¹) above the threshold that would switch the system completely to nitrate respiration (Bristow *et al.*, 2017). The majority of net losses of fixed N from marine systems are from denitrification, with estimates generally in the region of 300 Tg N.yr⁻¹ (Codispoti *et al.*, 2001; Galloway *et al.*, 2004; Gruber, 2004), of which OMZs are responsible for ~50% (DeVries *et al.*, 2013). The considerably lower estimated inputs of newly fixed N (100-150 Tg N.yr⁻¹) suggest either a severe imbalance in global N cycling or substantial underestimation of N₂-fixation (Gruber and Sarmiento, 1997; Galloway *et al.*, 2004; Gruber, 2004; Deutsch *et al.*, 2007). Either way, higher rates of denitrification associated with the expansion of OMZs could further increase the oceanic sink for N. Although the implications of increased denitrification on global N budgets are hard to predict, it's worth taking into account that, in a warming climate with increased CO₂, it is plausible that N inputs from N₂-fixation might also increase (Deutsch *et al.*, 2007).

Overall estimates of heterotrophic denitrifying bacteria numbers within the secondary nitrite maximum (NO₂⁻ > 3 µM) of the Arabian Sea OMZ (<5 µM O₂) range from 1.5x10⁴ to 1x10⁸ cells.L⁻¹ (Ward *et al.*, 2009; Lam *et al.*, 2011). *Labrenzia*-type denitrifying bacteria, closely related to the model strain used in this study, have a widespread

distribution throughout the Arabian Sea and are not restricted to the OMZ (Wyman *et al.*, 2013). *Labrenzia* spp. *nosZ* genes were most abundant ($\sim 1 \times 10^4$ copies L⁻¹) at ~ 45 m depth, within well oxygenated waters, and at 200 m depth within the secondary nitrite maximum of the OMZ. In Chapter 3, it was demonstrated that *nosZ* is present as a single copy gene within the genome of strain 5N. Genome analyses in Chapter 3 revealed that *nosZ*, and other key denitrification genes, are located on a megaplasmid. This plasmid is present at a similar copy number to that of the chromosome and its replication is not upregulated under denitrifying conditions (Chapter 3). Based on total estimates of denitrifying bacteria in the secondary nitrite maximum of the Arabian Sea OMZ, these observations suggest that *Labrenzia* spp. could represent a considerable proportion (as much as two thirds, based on estimates by Lam *et al.*, 2011) of the overall community of denitrifiers at some depths.

7.1.3 Denitrification potential of *Labrenzia*-type denitrifiers under low oxygen conditions.

Labrenzia-type denitrifiers have been shown to express *nosZ* in suboxic waters of the Arabian Sea as well as in oxic surface waters (Wyman *et al.*, 2013). Chapter 4 demonstrates that the detection of *nosZ* transcripts from this strain, and likely from other closely related organisms, indicates the expression of a complete suite of denitrification reductases at not only the transcriptional level but, more importantly, at the protein level also. Strain 5N does not require complete anoxia for denitrification and can denitrify under suboxic/hypoxic conditions. It does not appear to denitrify under fully aerobic conditions, but denitrification occurs once oxygen levels decline to $\sim 50\%$ saturation (Chapter 5). Stable isotope analyses showed that strain 5N does not reduce nitrate immediately in newly inoculated aerobic batch cultures. However, once O₂ levels fall owing to aerobic respiration nitrate and O₂ are used simultaneously. *Labrenzia* sp. strain 5N, therefore, meets the generally accepted definition of an aerobic denitrifier (Robertson and Kuenen, 1984; Robertson *et al.*, 1995). The capacity of strain 5N for aerobic denitrification is consistent with recent studies identifying members of the *Labrenzia* (formerly *Stappia*) and closely related *Stappia* genera within mixed communities of aerobic denitrifiers (Du *et al.*, 2017; Yuan *et al.*, 2020).

Chapter 5 complements the work of Wyman *et al.* (2013); it confirms that the detection of *nosZ* transcripts in the suboxic waters of the Arabian Sea is indeed indicative of the

capacity for active denitrification. When comparing the end-products produced under aerobic and anaerobic conditions, however, it was shown that the activity of NosZ was impaired at higher oxygen levels. During aerobic denitrification, the reduction of nitrate leads to a high accumulation of N₂O, while N₂ was produced at comparatively low levels owing to the apparent O₂ sensitivity of NosZ. Considering that the expression of *nosZ* itself is also known to be negatively regulated by high O₂ levels (Coates and Wyman 2017), the use of *nosZ* mRNA transcript abundance as an indicator of the scale of denitrification in marine waters almost certainly underestimates the scale of nitrate removal by these organisms. The present work did not investigate the O₂ sensitivity of the denitrification apparatus (nor NosZ, in particular) in detail but future studies should look at how expression of the denitrification genes is regulated under varying O₂ concentrations. It would be possible then to determine at which O₂ concentration thresholds individual genes are activated and use this information to predict the potential extent of denitrification outside of OMZs. Based on the stable isotope work in Chapter 5 it would be anticipated that *nap*, *nir*, and *nor* genes should all be upregulated at relatively high oxygen concentrations whilst *nos* appears somewhat more sensitive.

This is the first detailed report of aerobic/anaerobic denitrification in *Labrenzia* spp. and, more widely, for related *Roseobacter* clade members that also harbour denitrification genes (Chapter 3). *Labrenzia* spp. have a widespread geographical range and inhabit a diversity of free living and particle-associated habitats (Wang *et al.*, 2016), where varying O₂ conditions are likely to be encountered. As previously discussed, the sensitivity of the denitrification pathway, and in particular that of NosZ, can differ even within a species. This variability between strains has been suggested to correlate with distinct genetic differences in the structure of key reductases themselves (Takaya *et al.*, 2003). It was shown in this study that a number of *Labrenzia* strains harbour highly related, syntenic “denitrification plasmids” and that the major denitrification genes in these strains share very high (>99%) sequence similarity (Chapter 3). In addition, *napA* which is harboured by all ten *Labrenzia* strains carrying the “denitrification plasmid” is considered to be a marker of an organism’s capacity for aerobic denitrification (Ji *et al.*, 2015). As such, it might be speculated that the facility for aerobic denitrification and N₂O production is not unique to strain 5N but is more widely spread among *Labrenzia* strains harbouring this plasmid. Given that six

Labrenzia strains also harbour syntenic conjugative plasmids (Chapter 3), it is possible that the method of plasmid transfer developed in Chapter 6 might be transferable to other members of the genus, allowing for wider genetic studies amongst these strains.

7.1.4 Marine hypoxia/suboxia as a source of nitrous oxide.

Nitrous oxide production from both nitrification and denitrification is stimulated under hypoxic and suboxic conditions (Suntharalingam *et al.*, 2000; Canfield *et al.*, 2010). Nitrous oxide is the most important greenhouse gas in terms of its warming potential, around 300 times that of CO₂ (per molecule over a 100 year period), and is the main natural source of emissions responsible for stratospheric ozone depletion (Ravishankara *et al.*, 2009). OMZs are the principal sources for the majority of oceanic N₂O emissions, with an estimated production of 4 Tg N.yr⁻¹ N₂O annually; a rate that is expected to increase with global warming and ocean deoxygenation (Nevison *et al.*, 2003; Naqvi *et al.*, 2010). As such, research into low oxygen regions, and the organisms and processes that dominate them is essential for estimating the likely scale of future oceanic N₂O emissions.

Generation of N₂O by *Labrenzia* sp. strain 5N as a by-product of nitrification was not detected in this study (Chapter 5). During both aerobic and anaerobic denitrification, however, N₂O accumulated in the headspace above the culture medium. In the presence of oxygen, N₂O concentrations reached a noteworthy ~10mmoles.L⁻¹ in the overlying gas phase. Although emissions were considerably lower under anaerobic conditions (~0.26mmoles.L⁻¹), the results demonstrate that N₂O is also a by-product of denitrification under complete anoxia. The ratio N₂O/(N₂O+N₂) varied greatly with oxygen availability and by the end of the experimental period (48hours) reached 75.5 ± 0.28% in the aerobic cultures and 0.13 ± 0.0016% under anoxia (Figure 7.1). Higher oxygen availability significantly increased the proportion of N₂O produced but did not exclude the complete reduction of some nitrate to N₂. Under both conditions, the concentration of N₂O produced by strain 5N was within the mM range. This is significant in that *in situ*, this gas would be released to the atmosphere (Robertson and Kuenen, 1984; Takaya *et al.*, 2003). The environments in which strain 5N and close relatives have been detected, both in the Arabian Sea and within the *Trichodesmium* microbiome (discussed below in section 7.3.1) feature a wide range of ambient O₂

concentrations. It seems likely, therefore, that the by-production of N₂O is commonplace among these organisms.

Laboratory measurements of N₂O production by strain 5N can be put into an environmental context using the results presented in Chapter 5 and the known abundance of *Labrenzia*-type denitrifiers in the natural environment (Wyman *et al.*, 2013; Coates and Wyman, 2017). With the working assumption that 1OD unit is equivalent to $\sim 1.93 \times 10^8$ cells/ml (Ichikawa *et al.*, 2015) and that the amount N₂O produced during anaerobic and aerobic denitrification represent the minimum and maximum levels of N₂O production possible by this strain, it is possible to scale the range of emissions that might occur within natural waters. The potential production of N₂O by these organisms in the secondary nitrite maximum of the Arabian sea OMZ could reach between ~ 0.003 - 0.126 nmoles N₂O.L⁻¹ seawater per day. At an average *Trichodesmium* colony volume of 0.002–0.01 ml (Sheridan *et al.*, 2002), N₂O production by *Labrenzia*-type organisms within the microbiome could reach ~ 0.17 - 1.28 μ moles N₂O.ml⁻¹ colony per day. These projected rates could only be realised, of course, if adequate nitrate were available to support denitrification. This would be the case for the Arabian Sea OMZ and the nutrient-rich waters overlying the Omani shelf. Except at depth, however, it is less likely they could be achieved in more oligotrophic regions unless nitrification rates were high enough to supply the nitrogen oxides necessary.

The Arabian Sea is an area of intense denitrification. The total (N₂O + N₂) flux to the atmosphere from the oxygen depleted waters of the Arabian Sea has been estimated at 11.9 Tg N yr⁻¹, approximately 10% of the global water column total (Fauzi *et al.*, 1993). Surface oxic and deeper upwelling waters are supersaturated with N₂O (up to 246%) while within the OMZ itself this increased up to 1264%. The annual contribution of denitrification to excess N₂O produced in the Arabian Sea amounts to 2.55 ± 1.3 Tg N₂O-N (Fauzi *et al.*, 1993).

N₂O will become increasingly less soluble as waters warm over the next century, making it likely that emissions to the atmosphere will be greater than present day (Freing *et al.*, 2012). If a nitrification or denitrification zone becomes warmer and more active, it is prone to export more N₂O to near surface waters unless it can be reduced further before being lost to the atmosphere. On the other hand, increased water

column stratification reduces mixing between major denitrification regions such as OMZs and surface waters. In certain regions this could potentially keep waters with higher N₂O contents from coming into contact with the air–sea interface, thereby lessening their gas release into the atmosphere. Denitrifiers like strain 5N, therefore, may have an important role in controlling future N₂O emissions. In oxic surface waters overlying the eastern tropical South Pacific OMZ, it is of note that high densities of active N₂O-reducers harbouring an atypical Nos have been reported in addition to conventional denitrifiers (Sun *et al.*, 2017).

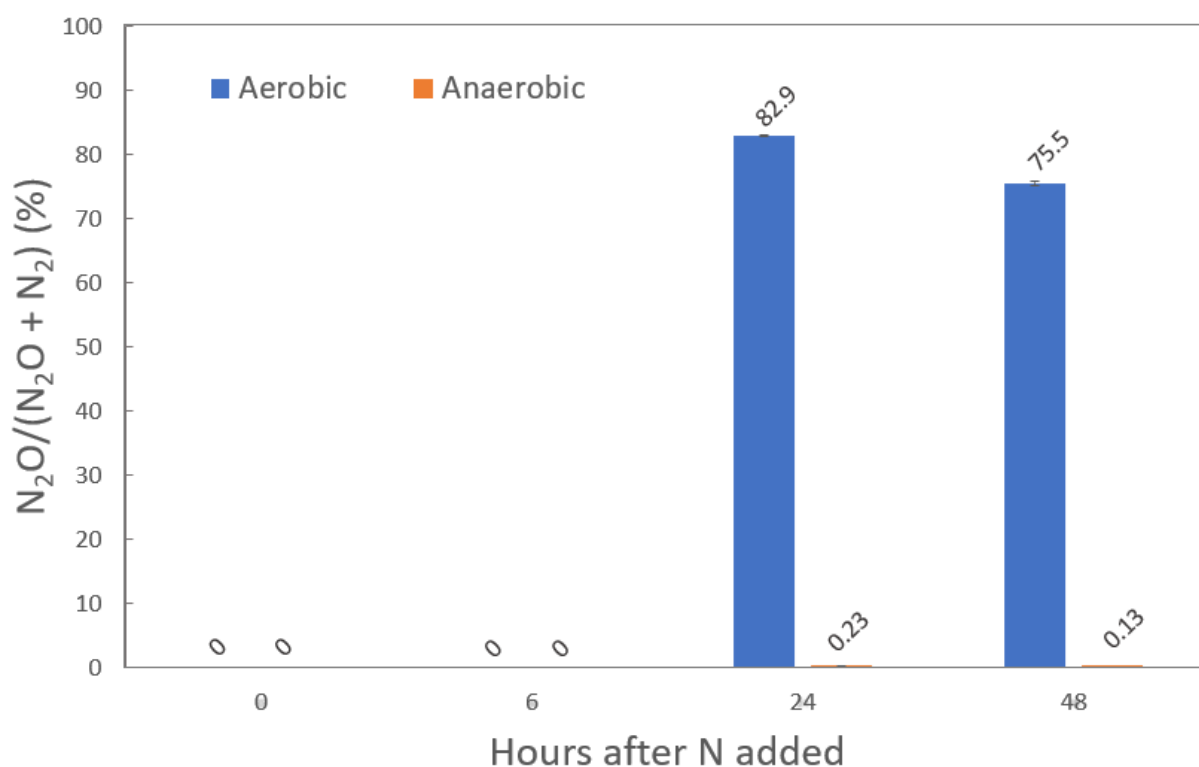


Figure 7.1. Relative N₂O production (expressed as the N₂O / (N₂O + N₂) molar ratio (n=3, ±sd)) by *Labrenzia* sp. strain 5N in the presence (blue bars) and absence (orange bars) of oxygen. Cultures were supplied with acetate as a C source and ammonium and nitrate as N sources.

7.2 Denitrification potential of organisms living in association with *Trichodesmium* colonies.

7.2.1 Small-scale N cycling within *Trichodesmium* colonies.

Trichodesmium colonies host an extensive array of associated organisms of which attached bacteria are the most abundant numerically (O'Neil JM and Roman, 1992; Selner KG, 1992; Nausch M., 1996; Sheridan C. C. *et al.*, 2002). Our current understanding of, and ability to predict, *Trichodesmium* bloom patterns and nitrogen fixation in oceans is incomplete. This might reflect the lack of knowledge of *Trichodesmium*'s epibionts, their metabolic contributions and how members of these complex communities interact. Metagenomic studies have shown that the epibiotic bacteria associated with *Trichodesmium* have the functional potential for a range of important biogeochemical processes including N cycling (Gradoville *et al.*, 2017). Surprisingly, >80% of the N₂-fixation related *nif* sequences retrieved originated from other cyanobacteria rather than the *Trichodesmium* host. Cyanobacterial nitrate assimilatory genes were abundant within the colonies and at higher concentrations than in the surrounding seawater. Gradoville *et al.* (2017) also found numerous dissimilatory nitrate reduction and denitrification genes within the colonies; a finding supporting earlier work by Wyman *et al.* (2013). Coates and Wyman (2017) demonstrated that at least one of these genes (*nosZ*) is actively expressed within the *Trichodesmium* colony environment raising the possibility that the colonies might be sites of denitrification in oxic waters (discussed in further detail in 7.3.2). Similar observations have been reported for the cultured laboratory isolate, *Trichodesmium* sp. IMS101. Transcripts for dissimilatory nitrate/nitrite reductases (*narG/nirB*) and *nosZ* were detected in cultures grown under both high and ambient CO₂ concentrations (Lee *et al.*, 2018). Most of these were related to genes from the phylum Bacteroidetes, suggesting that they may fill the same niche as the *Labrenzia*-like organisms found within natural populations by Coates and Wyman (2017).

Subsequent work by Klawonn *et al.* (2020) using stable isotopes found that *Trichodesmium* released ~10% newly fixed N as ammonium but that this did not appear to promote nitrification within the colony. Rates of complete denitrification (from nitrate to N₂) were insignificant and the authors suggested that O₂ levels within the colonies (minimum >60% air-saturation) were too high for this to occur. Unfortunately, neither nitrite or nitric oxide were analysed and so the study could not exclude partial,

incomplete denitrification. Klawonn *et al.* (2020) analysed small (0.5-1mm diameter) “puff” type colonies that are known to display higher internal oxygen concentrations than those that are larger (Eichner *et al.*, 2018). Gradoville *et al.* (2017) found that denitrification genes were significantly more abundant in more compact, higher biomass, ‘non-radial’ puffs than the smaller ‘radial’ puffs investigated by Klawonn *et al.* (2020). It has been suggested that exopolysaccharides, which are produced in abundance by many biofilm-forming bacteria, may be key to restricting oxygen diffusion within larger colonies (Lee *et al.*, 2017). Stable isotope experiments have detected the co-occurrence of aerobic and anaerobic N transformations within *anoxic microzones* of other cyanobacterial aggregates (*Nodularia spumigena*) that are of a comparable size to larger *Trichodesmium* colonies (Klawonn *et al.*, 2015).

As previously discussed in Chapter 1, oxygen gradients found within *Trichodesmium* colonies can be extreme. Concentrations ranging from completely anaerobic (Paerl and Bebout, 1992) to as high as 203% saturation have been reported (Eichner *et al.*, 2017). Oxygen concentrations fluctuate diurnally owing to photosynthetic O₂ evolution during daylight hours leading to supersaturation. However, *Trichodesmium* spp. have high rates of endogenous oxygen consumption (Kana, 1993) and so concentrations within the colony fall rapidly in dim light and at night (Paerl and Babout, 1992). Except in bright sunlight, therefore, colonies have the potential to support oxygen-sensitive processes like denitrification during some periods of the day. It was demonstrated in Chapter 4 that strain 5N is capable of denitrification when oxygen levels are below ~50% air saturation, well within the range of O₂ concentrations that have been found within *Trichodesmium* colonies.

A protocol for the introduction of plasmid DNA into *Labrenzia* sp. strain 5N cells was developed in this study (Chapter 6). It would be particularly interesting to study the expression of denitrification genes within colonies of *Trichodesmium* using this procedure. By introducing a fluorescent promoter probe vector into strain 5N, it would be possible to visualise the level of *nosZ* expression quantifiably using fluorescence microscopy. This would facilitate studies on denitrification in the *Trichodesmium* microbiome, including the localisation of the actively denitrifying cells within the colonies and the regulation of denitrification genes under varying O₂ conditions.

The detection of *nosZ* mRNAs might not be indicative of active denitrification, of course, if the gene is constitutively expressed. Coates and Wyman (2017) showed, however, that *nosZ* and *nirS* were highly expressed in denitrifying cultures but were not detectable under fully oxic, non-denitrifying conditions. Nonetheless, possible translational and post-translational regulation of gene expression means that the presence of transcripts alone does not confirm the synthesis of a functional protein (Cox and Mann, 2007; Aslam *et al.*, 2017). Chapter 4 complements the work of Coates and Wyman (2017) and compares the proteome of strain 5N under oxic and anoxic conditions. Denitrification proteins were absent under fully oxic conditions but a switch to anaerobic respiratory enzymes was seen under anoxia. Results showed a complete reorganisation of the electron transport chain during denitrification and suggest that the detection of *nosZ* mRNAs is indeed indicative of a fully functional denitrification pathway. Stable isotope analyses (Chapter 5) showed that strain 5N did not denitrify in aerobic conditions until O₂ levels fell (as a result of aerobic respiration) below the organism's threshold for active transcription/translation. Combined with the proteomics analyses, these results further suggest that under fully oxic conditions the strain is incapable of denitrification. These findings fully support the notion that the expression of *Labrenzia*-like *nosZ* transcripts in fully oxic surface waters must be confined to populations living within suboxic microzones including those that exist within *Trichodesmium* colonies (Coates and Wyman, 2017).

7.2.2 Is biofilm development promoted during anaerobic respiration?

Proteomics studies (Chapter 4) revealed that strain 5N cells exhibited a reduction in the expression of proteins essential for motility and an increased potential to form biofilms under denitrifying conditions. A connection between anoxia and biofilm formation has been observed previously in *Neisseria gonorrhoeae* and other organisms (Falsetta *et al.*, 2009; Phillips *et al.*, 2012). Under anaerobic conditions the Entner-Doudoroff pathway was downregulated in strain 5N, there was an increase in phenylacetic acid catabolism and the TDA pathway was upregulated. Each of these changes is known to be conducive to the establishment of biofilms and to promote host associations (Patra *et al.*, 2012; Sule and Belas, 2013; Timmermans *et al.*, 2017). Strain 5N also showed upregulation of *cbb*₃ oxidases during denitrification which are also implicated in biofilm formation via the control of NO concentrations (Justice *et al.*,

2008; Yoon *et al.*, 2011; Hamada *et al.*, 2014). The NO signalling pathway in the epibiont *Silicibacter* sp. strain TrichCH4B is activated by peptides secreted by *Trichodesmium* and induces biofilm formation and the establishment of the association with its cyanobacterial host (Rao *et al.*, 2015).

Combined, results suggest that strain 5N enters a more sedentary lifestyle in environmental conditions that promote denitrification and has a greater facility to attach to substrates and/or other organisms. This observation is of note in the context of the association of closely related *Labrenzia* spp. with the cyanobacterium *Trichodesmium*. It implies that denitrification is only possible, in the upper water column at least, when *Labrenzia* spp. are in close physical association with the trichomes.

7.2.3 Nitrification as a possible source of nitrite/nitrate for denitrification within *Trichodesmium* colonies.

Although the genetic potential for denitrification by *Trichodesmium* epibionts has been demonstrated (Wyman *et al.*, 2013; Gradoville *et al.*, 2017), and appears to be an active process within the colonies (Coates and Wyman, 2017), the origin of the source of nitrate/nitrite for denitrification in these surface waters remains unclear. *Trichodesmium* colonies undertake diurnal vertical migrations between surface waters and deeper waters enriched in nitrates and other nutrients like phosphate (Walsby, 1978). Migrations to these deeper waters occur primarily at night; the period of the day during which denitrification is most likely to occur within the colonies, as O₂ tensions will be at their lowest as photosynthesis ceases and respiration rates increase (Paerl and Bebout, 1988; Villareal and Carpenter, 2003). *Trichodesmium* also releases newly fixed N into the surrounding seawater, ~10% of this in the form of ammonium (Mulholland *et al.*, 2006; Klawonn *et al.*, 2020). Coates and Wyman (2017) hypothesized that the liberated ammonium could be oxidized to nitrite/nitrate by nitrifiers and then used by the denitrifying associates as a substrate for denitrification, as depicted in Figure 7.2. Studies to date have not detected nitrification within the *Trichodesmium* microbiome itself (Gradoville *et al.*, 2017; Klawonn *et al.*, 2020) though interestingly, ammonium released by *Trichodesmium* has been shown to support nitrification in the surrounding waters (Sutka *et al.*, 2004; Gandhi *et al.*, 2010). Nitrification in the Arabian Sea occurs primarily above the OMZ although lower rates

were also detected within the OMZ to depths of ~900m (Newell *et al.*, 2011). In addition, Gradoville *et al.* (2017) detected the significant expression of assimilatory nitrate reductases within the colonies, though not within the surrounding seawater. Klawonn *et al.* (2020) observed significant nitrate reduction, though the distinction between assimilatory or dissimilatory processes was not possible. These findings hint at the presence of nitrate within the colonies, potentially resulting from nitrification. Intriguingly, although Klawonn *et al.* (2020) did not detect significant rates of nitrification within *Trichodesmium* colonies, they did detect low levels of $^{15}\text{N}_2\text{O}$ that could only arise from the oxidation of the labelled ammonium supplied. The authors acknowledge that the high levels of nitrate consumption seen during their experiments may also have hidden any simultaneous nitrification. Combined with the habitual slow growth rates of nitrifiers, this finding suggests that some nitrification might have been taking place.

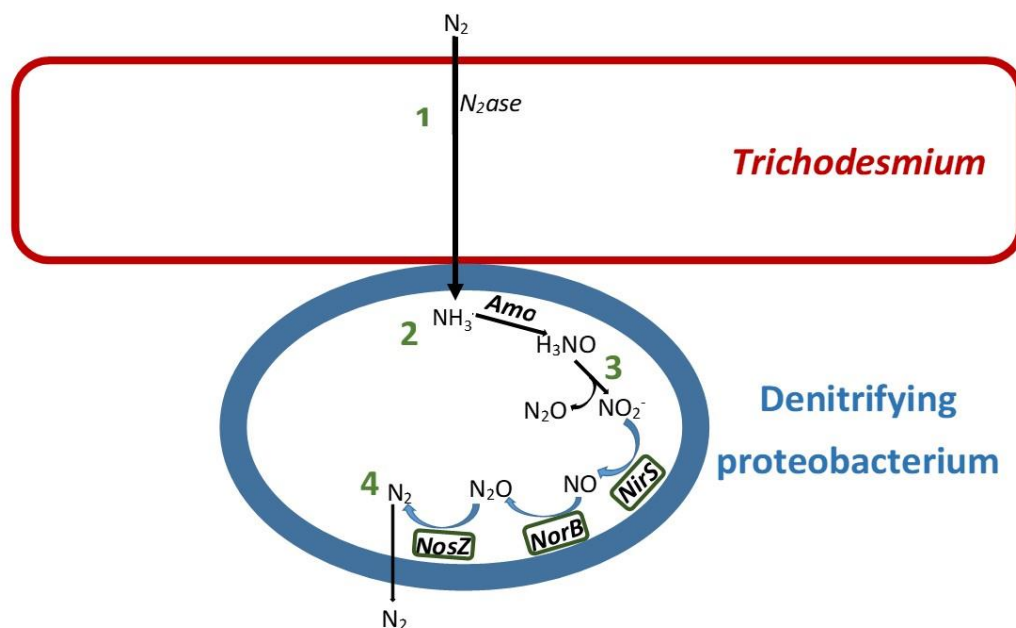


Figure 7.2. Hypothetical N-cycling within a *Trichodesmium*-denitrifier association. *Trichodesmium* cells are shown in red and the denitrifying epibiont in blue. 1, N₂-fixation; 2, ammonium oxidation; 3, hydroxylamine oxidation; 4, denitrification. N₂ase, nitrogenase; Amo, ammonia monooxygenase; NirS, nitrite reductase; NorB, nitric oxide reductase; NosZ, nitrous oxide reductase.

In Chapter 3 it was suggested through genome analyses that strain 5N has the genetic potential for nitrification. Results from Chapter 5 supported this; *Labrenzia* sp. strain 5N is capable of heterotrophic nitrification. When supplied with ammonium, in addition to an organic carbon source, strain 5N oxidised the ammonium to nitrite towards the end of exponential growth. When nitrate was supplied in addition to ammonium, the strain was capable of simultaneous heterotrophic nitrification and aerobic denitrification. In this case, nitrite derived from ammonium oxidation was denitrified to $\text{N}_2\text{O}/\text{N}_2$ though the added nitrate was used as the main source for denitrification because it was supplied in excess. Though observed levels of ammonium oxidation were low, likely due to the optimal conditions for nitrification not being met in the experimental study, these results demonstrate that this *Labrenzia* strain does indeed have the ability to nitrify. As such, we propose that nitrification of the ammonium released by *Trichodesmium* (Mulholland *et al.*, 2006; Klawonn *et al.*, 2020) is a plausible/viable route by which nitrite/nitrate might be supplied as a substrate for denitrification within the colony environment.

7.3 General Conclusions

Oxygen is essential for all aerobic life in the Oceans, from the smallest of bacteria to the largest of fish. Worryingly, the oceans are losing oxygen at an increasing pace due to the direct impact of anthropogenic activity. There are two main drivers of oxygen depletion; (i) eutrophication associated with nutrient inputs from agricultural runoff and waste disposal and (ii) the warming of the oceans driven by climate change (Keeling *et al.*, 2010). As air temperatures on Earth increase, the oceans are warming and warmer waters hold less oxygen (IPCC, 2014).

Whether we live by the sea, eat seafood or not, the consequences of ocean deoxygenation impact us all. Annually the ocean's primary producers generate at least half of the oxygen we need to breathe (Walker, 1980; Behrenfeld *et al.*, 2001). They also absorb anthropogenic CO_2 and excess heat from the atmosphere, helping to buffer the effects of climate change (Levitus *et al.*, 2009; Gruber *et al.*, 2019). The health of the oceans is directly linked to the health of the rest of the planet. Marine oxygen concentrations are declining markedly and the areal extent of low oxygen regions is growing in size. For the ~3 billion humans who rely on seafood as their

primary source of protein (primarily in developing countries), the implications of deoxygenation on fisheries are uncomfortably close to home (FAO, 2018). The socio-economic impacts of ocean deoxygenation are severe, leaving communities suffering poverty, malnutrition and hunger. Deoxygenation impacts directly on the fisheries and aquaculture industries especially due to habitat loss, (with the related economic impacts). Individual fish species differ in their tolerance of low oxygen tensions, with larger fish generally requiring higher concentrations for healthy growth and reproduction (Nilsson *et al.*, 2010; Vaquer-Sunyer and Duarte, 2010). Being mobile, fish and crustaceans actively avoid low oxygen areas, migrating vertically or horizontally to seek out more favourable conditions. The loss of animals of higher trophic status precipitates large scale changes in nutrient cycling and the structure of foodwebs, shifting predator-prey interactions significantly. As fish distributions change so will that of larger predatory marine mammals, with the associated losses of tourism-related activities.

The ocean oxygen budget needs our urgent attention. Marine phytoplankton and bacteria are key to controlling the biological processes that add/remove oxygen and recycle essential nutrients in the oceans, which in turn impacts on the global distribution of other species. We need to better understand the metabolic processes that sustain the organisms that live in low oxygen waters and, in particular, those that will become more abundant as low oxygen zones grow. The expansion of hypoxic zones in the decades ahead will promote increased rates of redox-sensitive metabolisms including denitrification (Voss *et al.*, 2013). The resulting impacts on the balance of global marine N budgets will have significant knock-on effects on the ocean's biota.

The Arabian Sea is an area of intense denitrification, accounting for approximately 10% of global marine N losses annually (Mantoura *et al.*, 1993). Total estimates of denitrifying bacteria in the secondary nitrite maximum of the Arabian Sea OMZ indicate that *Labrenzia* spp. represent a sizeable proportion (as much as two thirds, based on estimates by Lam *et al.*, 2011) of the overall community of denitrifiers at some depths (Lam *et al.*, 2011; Wyman *et al.*, 2013). My research has revealed that these organisms are capable of performing a variety of N transformations depending on prevailing oxygen conditions. The findings presented highlight that these biogeochemically significant processes may be more widespread in the oceans than

that assumed by current models and that the scale of marine N losses and rates of N₂O production may be underestimated considerably. It is critical, therefore, to assess how organisms such as these might respond to future oceanic conditions, to better understand the likely implications for global N cycling. A more in-depth understanding of these processes will help to guide the selection of more appropriate and realistic models for N cycling and N₂O budgets. Small-scale, organism specific studies such as those presented in this thesis, in combination with larger scale *in situ* experiments, will provide a more complete understanding of these processes and help identify those aspects requiring urgent attention.

These findings and their implications also extend to the *Trichodesmium* microbiome. The nature of the metabolic pathways occurring within the microbiome are yet to be understood but interactions between the microbial epibionts and the cyanobacterial host are likely to be complex (Lee *et al.*, 2017). Processes carried out by one species will almost certainly impact on the physiology and growth of another. The wider environmental impact of these associations might be unexpectedly significant but is largely underestimated, highlighting the importance of identifying key epibionts and determining their functional role (Frischkorn *et al.*, 2017). Associated bacteria have the potential to modulate *Trichodesmium* productivity directly impacting CO₂ and N₂ fixation capacity and, more widely, in regulating the flux of essential nutrients, influencing marine biogeochemical cycling at a larger scale (Frischkorn *et al.*, 2017; Lee *et al.*, 2017).

These bacterial associations almost certainly play an essential role in the ecophysiology of *Trichodesmium* and yet our knowledge of the functional role of the microbes involved is limited. Further studies on the diversity of the epibiotic bacteria *Trichodesmium* hosts are necessary and will broaden our understanding of these communities as a functional unit; the holobiont. The present study highlights that members of the epibiotic *Labrenzia* community have the metabolic potential for N-cycling within *Trichodesmium* colonies under environmentally relevant oxygen tensions. *Trichodesmium* colonies represent potentially important sites of fixed N losses from the ocean (as well as inputs) and a novel source of N₂O production in surface waters. Our current understanding of *Trichodesmium* bloom patterns and nitrogen fixation potential in the oceans is poor. This thesis has shown that this understanding might be improved by taking into account its numerous epibionts and

their metabolic contributions. Research into low oxygen regions, and the organisms that inhabit them, is highly relevant in a changing world and contributes to the development of strategies to predict and mitigate the ongoing challenges the oceans face.

Chapter 8:

Appendices

Appendices for Chapter 2

Table 8.1. PCR primers used in this study

Oligonucleotide	Target gene	Primer sequence 5'–3'	Annealing temperature (°C)	Product size (bp)	Reference
StanosF StanosR	<i>nosZ</i>	GCTGAAACCGGAGAATGAAC TGTTTCTTGGCATCGTCAAA	52	252	Wyman <i>et al.</i> , 2013
Form1C_For Form1C_Rev	<i>cbbL</i>	GAYGAYGARAACATYAACTC TGGTGCATYTGNCNGCRTG	53	561	This study
#5N_NarB_For #5N_NarB_R	<i>narB</i>	CGCCAGGATATCGCACTCTT CGACGACTGACTGGTTGACA	55	91	This study
M13F M13R	M13 multiple cloning site	GTAAACGACGGCCAGT CAGGAAACAGCTATGAC	55	variable	Universal
GenRFor GenRRev	Gentamycin resistance gene	ACCTACTCCCAACATCAGCC TAGATCTCACTACGCGGCTG	55	120	This study

Table 8.11. A5 Stock solution

Compound	Quantity (g.L-1)
H ₃ BO ₃	2.86
MnCl ₂	1.81
ZnSO ₄	0.222
Na ₂ MoO ₄	0.39
CuSO ₄	0.079

Table 8.12. f/2 Trace Metal Solution (Guillard & Ryther 1962, Guillard 1975)

To 1L dH₂O add:

Quantity	Compound	Stock solution	Molar concentration in final medium
3.15 g	FeCl ₃ · 6H ₂ O		1 x 10 ⁻⁵ M
4.36 g	Na ₂ EDTA · 2H ₂ O		1 x 10 ⁻⁵ M
1 mL	CuSO ₄ · 5H ₂ O	9.8 g/L dH ₂ O	4 x 10 ⁻⁸ M
1 mL	Na ₂ MoO ₄ · 2H ₂ O	6.3 g/L dH ₂ O	3 x 10 ⁻⁸ M
1 mL	ZnSO ₄ · 7H ₂ O	22.0 g/L dH ₂ O	8 x 10 ⁻⁸ M
1 mL	CoCl ₂ · 6H ₂ O	10.0 g/L dH ₂ O	5 x 10 ⁻⁸ M
1 mL	MnCl ₂ · 4H ₂ O	180.0 g/L dH ₂ O	9 x 10 ⁻⁷ M

Table 8.2. Average Nucleotide Identity (ANI) estimates between alphaproteobacterium strain 5N (GCA_012395805.1), *Labrenzia aggregata* RMAR6-6 (GCF_001999245.1), *Labrenzia* sp. CP4 (GCF_001579905.1), *Labrenzia aggregata* IAM12614 (GCF_000168975.1), *Labrenzia alexandrii* strain DFL-11T (GCF_000158095.1), *Labrenzia alba* CECT5095 (GCF_001404515.1), *Labrenzia marina* KCTC 12288T (GCF_002906165.1) and *Labrenzia suaedae* YC6927T (GCF_900142725.1) calculated using the online ANI calculator (Rodriguez-R and Konstantinidis, 2016). ANI estimates between the two genomes and standard deviation values are given. NCBI RefSeq Accession numbers are provided in brackets. An ANI value of 95% is used for species delineation.

	Alphaproteobacterium strain 5N	<i>Labrenzia aggregata</i> RMAR6-6	<i>Labrenzia</i> sp. CP4
<i>Labrenzia aggregata</i> RMAR6-6	Two-way ANI: 98.12% (SD: 1.40%)		
<i>Labrenzia</i> sp. CP4	Two-way ANI: 97.64% (SD: 1.36%)	Two-way ANI: 97.76% (SD: 1.38%)	
<i>Labrenzia aggregata</i> IAM12614	Two-way ANI: 87.30% (SD: 4.14%)	Two-way ANI: 86.98% (SD: 4.32%)	Two-way ANI: 87.07% (SD: 4.39%)
<i>Labrenzia alexandrii</i> strain DFL-11 ^T	Two-way ANI: 79.83% (SD: 4.79%)	Two-way ANI: 79.74% (SD: 6.01%)	Two-way ANI: 79.69% (SD: 6.01%)
<i>Labrenzia alba</i> CECT5095	Two-way ANI: 79.47% (SD: 4.24%)	Two-way ANI: 78.72% (SD: 4.75)	Two-way ANI: 78.72% (SD: 4.83%)
<i>Labrenzia marina</i> KCTC 12288 ^T	Two-way ANI: 80.92% (SD: 4.31%)	Two-way ANI: 79.94% (SD: 4.49%)	Two-way ANI: 80.02% (SD: 4.50%)
<i>Labrenzia suaedae</i> YC6927 ^T	Two-way ANI: 79.18% (SD: 4.56%)	Two-way ANI: 78.87% (SD: 4.94%)	Two-way ANI: 79.03% (SD: 4.93%)

Table 8.3. In silico DNA-DNA hybridisation estimates between alphaproteobacterium strain 5N (GCA_012395805.1), *Labrenzia aggregata* RMAR6-6 (GCF_001999245.1), *Labrenzia* sp. CP4 (GCF_001579905.1), *Labrenzia aggregata* IAM12614 (GCF_000168975.1), *Labrenzia alexandrii* strain DFL-11T (GCF_000158095.1), *Labrenzia alba* CECT5095 (GCF_001404515.1), *Labrenzia marina* KCTC 12288T (GCF_002906165.1) and *Labrenzia suaedae* YC6927T (GCF_900142725.1) were calculated using the GGDC web server (Auch et al., 2010; Meier-Kolthoff et al., 2013). Inter-genomic distances and DDH estimates [CI] obtained using formula 2 are given. NCBI RefSeq accession numbers for each genome are provided in brackets. A DDH estimate of 70% is used for species delineation.

	Alphaproteobacterium strain 5N	<i>Labrenzia aggregata</i> RMAR6-6	<i>Labrenzia</i> sp. CP4
<i>Labrenzia aggregata</i> RMAR6-6	Distance: 0.0159 DDH estimate: 86.60% [84 - 88.8%]		
<i>Labrenzia</i> sp. CP4	Distance: 0.0194 DDH estimate: 83.40% [80.6 - 85.9%]	Distance: 0.0208 DDH estimate: 82.20% [79.4 - 84.8%]	
<i>Labrenzia aggregata</i> IAM12614	Distance: 0.1045 DDH estimate: 38.50% [36.1 - 41%]	Distance: 0.1254 DDH estimate: 33.40% [30.9 - 35.9%]	Distance: 0.1248 DDH estimate: 33.50% [31.1 - 36%]
<i>Labrenzia alexandrii</i> strain DFL-11^T	Distance: 0.1675 DDH estimate: 25.90% [23.6 - 28.4%]	Distance: 0.2121 DDH estimate: 20.70% [18.5 - 23.1%]	Distance: 0.2164 DDH estimate: 20.30% [18.1 - 22.7%]
<i>Labrenzia alba</i> CECT5095	Distance: 0.1727 DDH estimate: 25.20% [22.9 - 27.7%]	Distance: 0.2178 DDH estimate: 20.20% [18 - 22.6%]	Distance: 0.2182 DDH estimate: 20.10% [17.9 - 22.5%]
<i>Labrenzia marina</i> KCTC 12288^T	Distance: 0.1637 DDH estimate: 26.50% [24.1 - 29%]	Distance: 0.2043 DDH estimate: 21.50% [19.2 - 23.9%]	Distance: 0.2037 DDH estimate: 21.50% [19.3 - 24%]
<i>Labrenzia suaedae</i> YC6927^T	Distance: 0.1866 DDH estimate: 23.40% [21.1 - 25.9%]	Distance: 0.2132 DDH estimate: 20.60% [18.4 - 23%]	Distance: 0.2166 DDH estimate: 20.30% [18.1 - 22.7%]

Appendices for Chapter 3

Table 8.4. **Average Relative Synonymous Codon Usage** values for the replicons of *Labrenzia* sp. strain 5N, *Labrenzia aggregata* RMAR6-6 and *Labrenzia* sp. CP4. Preferred codons are shown in bold.

Amino acid	Codon	<i>Labrenzia</i> sp. strain 5N			<i>Labrenzia aggregata</i> RMAR6-6			<i>Labrenzia</i> sp. CP4	
		Chromosome	Plasmid 1	Plasmid 2	Chromosome	Plasmid 1	Plasmid 2	Chromosome	pBCL-1
Phe	TTT	0.62	0.62	0.7	0.61	0.62	0.76	0.61	0.78
	TTC	1.34	1.35	1.13	1.36	1.35	1.18	1.37	1.18
Leu	TTA	0.05	0.04	0.15	0.05	0.04	0.17	0.04	0.18
	TTG	0.7	0.66	1.03	0.64	0.7	1.02	0.65	1.07
	CTT	1.16	1.13	1.2	1.11	1.13	1.17	1.09	1.14
	CTC	1.17	1.3	1.46	1.19	1.3	1.49	1.17	1.43
	CTA	0.11	0.09	0.27	0.1	0.07	0.32	0.09	0.37
	CTG	2.81	2.76	1.86	2.91	2.74	1.84	2.95	1.81
Ile	ATT	0.76	0.71	0.8	0.73	0.73	1	0.73	0.96
	ATC	2.04	2.05	1.84	2.09	2.04	1.71	2.09	1.68
	ATA	0.16	0.17	0.28	0.15	0.18	0.29	0.15	0.33
Val	GTT	0.94	0.88	0.9	0.89	0.9	1.1	0.89	1.09
	GTC	1.56	1.69	1.53	1.62	1.66	1.35	1.62	1.43
	GTA	0.25	0.2	0.31	0.22	0.2	0.36	0.22	0.32
	GTG	1.22	1.23	1.22	1.25	1.23	1.13	1.26	1.1
Ser	TCT	0.49	0.4	0.68	0.45	0.43	0.66	0.44	0.68
	TCC	1.83	1.74	1.47	1.85	1.75	1.49	1.87	1.52
	TCA	0.48	0.46	0.61	0.44	0.47	0.7	0.43	0.72
	TCG	1.37	1.49	1.42	1.4	1.5	1.06	1.39	1.04
	AGT	0.41	0.46	0.45	0.39	0.42	0.66	0.39	0.65
	AGC	1.39	1.45	1.34	1.46	1.42	1.29	1.46	1.3

Phe	CCT	0.55	0.5	0.67	0.51	0.51	0.62	0.5	0.63
	CCC	0.81	0.96	1.01	0.84	0.97	0.9	0.85	0.86
	CCA	0.42	0.4	0.62	0.37	0.39	0.68	0.36	0.74
	CCG	2.19	2.09	1.62	2.24	2.09	1.74	2.26	1.74
Thr	ACT	0.33	0.26	0.46	0.3	0.25	0.54	0.3	0.53
	ACC	1.82	1.8	1.46	1.87	1.78	1.27	1.88	1.33
	ACA	0.59	0.58	0.82	0.56	0.64	0.8	0.56	0.79
	ACG	1.22	1.33	1.2	1.23	1.31	1.33	1.23	1.35
Ala	GCT	0.53	0.44	0.66	0.49	0.43	0.58	0.48	0.6
	GCC	1.58	1.65	1.49	1.67	1.66	1.45	1.68	1.43
	GCA	0.8	0.78	0.75	0.74	0.78	0.74	0.74	0.75
	GCG	1.08	1.12	1.07	1.1	1.13	1.23	1.09	1.22
Tyr	TAT	0.93	0.95	0.87	0.92	0.92	0.94	0.91	0.96
	TAC	0.95	0.97	0.89	0.97	0.98	0.9	0.97	0.89
His	CAT	0.87	0.93	0.85	0.87	0.94	0.81	0.87	0.83
	CAC	0.99	0.98	0.91	1	0.98	1.01	0.99	1.01
Gln	CAA	0.42	0.44	0.69	0.39	0.43	0.73	0.38	0.72
	CAG	1.54	1.52	1.17	1.57	1.53	1.07	1.58	1.11
Asn	AAT	0.63	0.64	0.7	0.61	0.63	0.62	0.61	0.61
	AAC	1.3	1.3	1.12	1.33	1.31	1.14	1.33	1.1
Lys	AAA	0.64	0.64	0.86	0.62	0.65	0.97	0.61	0.99
	AAG	1.33	1.34	1.05	1.35	1.31	0.99	1.36	0.98
Asp	GAT	0.8	0.78	0.78	0.76	0.77	0.87	0.76	0.84
	GAC	1.18	1.2	1.15	1.21	1.2	1.08	1.22	1.1
Glu	GAA	1.25	1.22	1.07	1.23	1.21	1.07	1.24	1.07
	GAG	0.73	0.76	0.92	0.75	0.77	0.9	0.74	0.89
Cys	TGT	0.42	0.45	0.5	0.41	0.44	0.39	0.41	0.4
	TGC	1.17	1.25	0.99	1.2	1.31	1.07	1.19	1.05

Arg	CGT	1.02	0.83	0.78	0.94	0.82	0.84	0.95	0.88
	CGC	2.35	2.22	1.98	2.47	2.21	1.9	2.47	1.93
	CGA	0.37	0.46	0.68	0.34	0.42	0.7	0.33	0.71
	CGG	1.45	1.55	1.58	1.49	1.56	1.47	1.49	1.44
	AGA	0.3	0.31	0.43	0.28	0.36	0.53	0.28	0.47
	AGG	0.48	0.59	0.51	0.46	0.57	0.56	0.46	0.57
Gly	GGT	0.9	0.79	0.81	0.84	0.82	0.77	0.84	0.75
	GGC	2.02	1.98	1.77	2.1	1.95	1.72	2.11	1.7
	GGA	0.54	0.6	0.73	0.52	0.62	0.8	0.52	0.8
	GGG	0.53	0.61	0.66	0.53	0.6	0.64	0.53	0.72

Table 8.5. Distribution of plasmids and denitrification genes among *Labrenzia* species. RepAB, plasmid replication proteins; *napA*, dissimilatory nitrate reductase subunit A; *nirS*, dissimilatory nitrite reductase; *norBC*, nitric oxide reductase subunits B and C; *nosZ*, nitrous oxide reductase. ^a Type II Toxin-antitoxin plasmid maintenance system. ^b The strain harbours a copy of *nirK* rather than *nirS*.

Organism	Previously identified plasmids	Newly predicted plasmids	Contig/replicon	Size (bp)	RepA B	ccdAB Type II T/AT system ^a	<i>napA</i>	<i>nir</i> ^b	<i>norB C</i>	<i>nosZ</i>	Plasmid located denitrification genes	Conjugative transfer genes	GC content	NCBI Accession
<i>Labrenzia</i> sp. THAF82			chromosome	6395015			Y	Y	Y	Y		Y	55%	NZ_CP045354.1
<i>Labrenzia</i> sp. THAF35	3		pTHAF35_a	418081	Y	Y		Y	Y	Y	Y		58.80%	NZ_CP045381.1
			pTHAF35_b	152967	Y							Y	57.90%	NZ_CP045382.1
			pTHAF35_c	70393	Y							Y	55.80%	NZ_CP045383.1
			chromosome	6170751			Y					Y	59%	NZ_CP045380.1
<i>Labrenzia</i> sp. PHM005	1		p1BIR	19450	Y								48.50%	NZ_CP041190.1
			chromosome	6167349			Y	Y	Y	Y			55%	NZ_CP041191.1
<i>Labrenzia</i> sp. THAF191b	4		pTHAF191b_a	538462	Y	Y		Y	Y	Y	Y		58.60%	NZ_CP045329.1
			pTHAF191b_b	120832	Y	Y						Y	57.70%	NZ_CP045330.1
			pTHAF191b_c	93994	Y	Y						Y	56.60%	NZ_CP045331.1
			pTHAF191b_d	22652		Y						Y	63%	NZ_CP045332.1
			chromosome	6069958			Y						59.10%	NZ_CP045328.1
<i>Labrenzia</i> sp. THAF191a	4		pTHAF191a_b	539584	Y	Y		Y	Y	Y	Y		58.50%	NZ_CP045335.1
			chromosome	6069958			Y						59.10%	NZ_CP045333.1
			pTHAF191a_a	120832	Y	Y						Y	57.70%	NZ_CP045334.1
			pTHAF191a_c	93994	Y	Y						Y	56.60%	NZ_CP045336.1
			pTHAF191a_d	22652		Y						Y	63%	NZ_CP045337.1
<i>Labrenzia</i> sp. THAF187b	4		pTHAF187b_b	120832	Y							Y	57.70%	NZ_CP045346.1
			pTHAF187b_c	93994	Y							Y	56.60%	NZ_CP045347.1
			pTHAF187b_d	22652	Y							Y	63%	NZ_CP045348.1
			pTHAF187b_a	538462	Y	Y		Y	Y	Y	Y		58.60%	NZ_CP045345.1

			chromosome	6069958	Y		Y						59.10%	NZ_CP045344.1
Organism	Previousl y identified plasmids	Newly predicted plasmids	Contig/replicon	Size (bp)	RepA B	ccdAB Type II T/AT system ^a	nap A	nir ^b	norB C	nos Z	Plasmid located denitrificatio n genes	Conjugativ e transfer genes	GC content	NCBI Accession
<i>Labrenzia</i> sp. VG12			chromosome	5977548									59.90%	NZ_CP022529.1
<i>Labrenzia</i> sp. CP4	1		pBCL1	98619	Y							Y	55.90%	NZ_CP011928.1
			chromosome	5952682			Y						59%	NZ_CP011927.1
<i>Labrenzia</i> sp. CE80			chromosome	4737606									57.10%	NZ_WAJT00000000.1
<i>Labrenzia</i> sp. PO1		1	Scaffold5_1	407976	Y	Y		Y	Y	Y	Y		59%	NZ_JAAXCR010000005 .1
			whole genome	6218130									59%	NZ_JAAXCR000000000 .1
<i>Labrenzia</i> sp. Alg231-36			chromosome	7393951									56.30%	NZ_FREW00000000.1
<i>Labrenzia</i> sp. PT13C1		1	contig Ga0206394_109	80647	Y	Y								NZ_SOCB01000020.1
			whole genome	5206673									56.50%	NZ_SOCB00000000.1
<i>Labrenzia</i> sp. C1B10		2	contig 4	314973	Y	Y		Y	Y	Y	Y		59.20%	NZ_AXBY01000034.1
			contig 11	63047	Y	Y								NZ_AXBY01000003.1
			whole genome	6831407			Y					Y	58.80%	NZ_AXBY00000000.1
<i>Labrenzia</i> sp. C1B70		2	contig 11	314730	Y	Y		Y	Y	Y	Y		59.10%	NZ_AXCE01000003.1
			contig3	61646	Y	Y								NZ_AXCE01000023.1
			whole genome	6831462			Y					Y	58.50%	NZ_AXCE00000000.1
<i>Labrenzia</i> sp. 5N	2		plasmid 1	410093	Y	Y		Y	Y	Y	Y		58.90%	
			chromosome	5918759			Y						59%	
			plasmid 2	135383	Y	Y						Y	57%	
			whole genome	6464235										NZ_JAAZTM000000000 .1
<i>Labrenzia</i> sp. EL143			chromosome	7279421			Y	Y	Y	Y		Y	56.30%	NZ_OGUZ00000000.1
<i>Labrenzia</i> sp. OB1			chromosome	6002344									59.40%	NZ_JSEP00000000.1

Labrenzia sp. 011	1	NODE_19	90112	Y	Y	Y ^b							57.40%	QCYM01000018.1
		whole genome	5102962										61.60%	NZ_QCYM00000000.1
Organism	Previousl y identified plasmids	Newly predicted plasmids	Contig/replicon	Size (bp)	RepA B	ccdAB Type II T/AT system ^a	nap A	nir ^b	norB C	nos Z	Plasmid located denitrificatio n genes	Conjugativ e transfer genes	GC content	NCBI Accession
Labrenzia sp. DG1229			chromosome	7550056				Y	Y	Y		Y	56.30%	NZ_AYYG00000000.1
Labrenzia sp. UBA4493			chromosome										59%	DGNL00000000.1
Labrenzia sp. AG- 365-D23			chromosome										59.10%	CACDCE00000000.1
Labrenzia aggregata RMAR6-6	2		unnamed_1	407152	Y	Y		Y	Y	Y	Y		58.90%	NZ_CP019631.1
			unnamed_2	94445	Y							Y	55.80%	NZ_CP019632.1
			chromosome	6149397			Y						58.90%	NZ_CP019630.1
Labrenzia aggregata IAM 12614	1		scaffold 1101096003804	154164	Y	Y						Y	59.50%	NZ_AA UW01000020.1
			whole genome	6561391			Y	Y	Y	Y			59.40%	NZ_AA UW00000000.1
Labrenzia aggregata CECT480 1		1		scaffold 0004	380054	Y	Y		Y	Y	Y		58.70%	CXST01000004.1
				whole genome	6695,95			Y						58.90%
Labrenzia aggregata LZB033				6431067		Y	Y	Y	Y	Y		Y	59.10%	NZ_JXMT00000000.1
Labrenzia marina DSM 17023				chromosome	6057182			Y ^b	Y			Y	60.30%	NZ_PPCN00000000.1
Labrenzia suaedae DSM 2153				contig: Ga0131142_105	348869	Y		Y ^b	Y	Y				FRBW01000005.1
					5142828									
				chromosome									60.20%	NZ_FRBW00000000.1

<i>Labrenzia alba</i> CECT509 5	chromosome	6901468	Y	Y	Y	Y		56.40%	NZ_CXWE00000000.1
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Organism	Previously identified plasmids	Newly predicted plasmids	Contig/replicon	Size (bp)	RepA B	ccdAB Type II T/AT system ^a	<i>napA</i>	<i>nir^b</i>	<i>norB C</i>	<i>nos Z</i>	Plasmid located denitrification genes	Conjugative transfer genes	GC content	NCBI Accession
<i>Labrenzia alexandrii</i> DFL-11	2		pLADFL_1	93929	Y	Y						Y	56.70%	NZ_CM011003.1
			pLADFL_2	68647	Y	Y							56.30%	NZ_CM011004.1
			chromosome	5300354									56.40%	NZ_CM011002.1
<i>Labrenzia alexandrii</i> CECT 5112		1	predicted plasmid	79683	Y	Y							54.60%	NZ_CXWD01000021.1
			whole genome	5351857									56.30%	NZ_CXWD00000000.1

Table 8.6. Gene assignment to COG functional categories

Code	Value	Percentage of total genome	Description
B	4	0.07	Chromatin structure and dynamics
C	316	5.15	Energy production and conversion
D	49	0.80	Cell cycle control, cell division, chromosome partitioning
E	588	9.59	Amino acid transport and metabolism
F	100	1.63	Nucleotide transport and metabolism
G	478	7.79	Carbohydrate transport and metabolism
H	207	3.38	Coenzyme transport and metabolism
I	222	3.62	Lipid transport and metabolism
J	197	3.21	Translation, ribosomal structure and biogenesis
K	515	8.40	Transcription
L	145	2.36	Replication, recombination and repair
M	280	4.57	Cell wall/membrane/envelope biogenesis
N	109	1.78	Cell motility
O	184	3.00	Posttranslational modification, protein turnover, chaperones
P	289	4.71	Inorganic ion transport and metabolism
Q	179	2.92	Secondary metabolites biosynthesis, transport and catabolism
R	704	11.48	General function prediction only
S	521	8.50	Function unknown
T	360	5.87	Signal transduction mechanisms
U	98	1.60	Intracellular trafficking, secretion, and vesicular transport
V	65	1.06	Defence mechanisms
Z	1	0.02	Cytoskeleton
Uncategorised	521	8.50	Not assigned to COGs

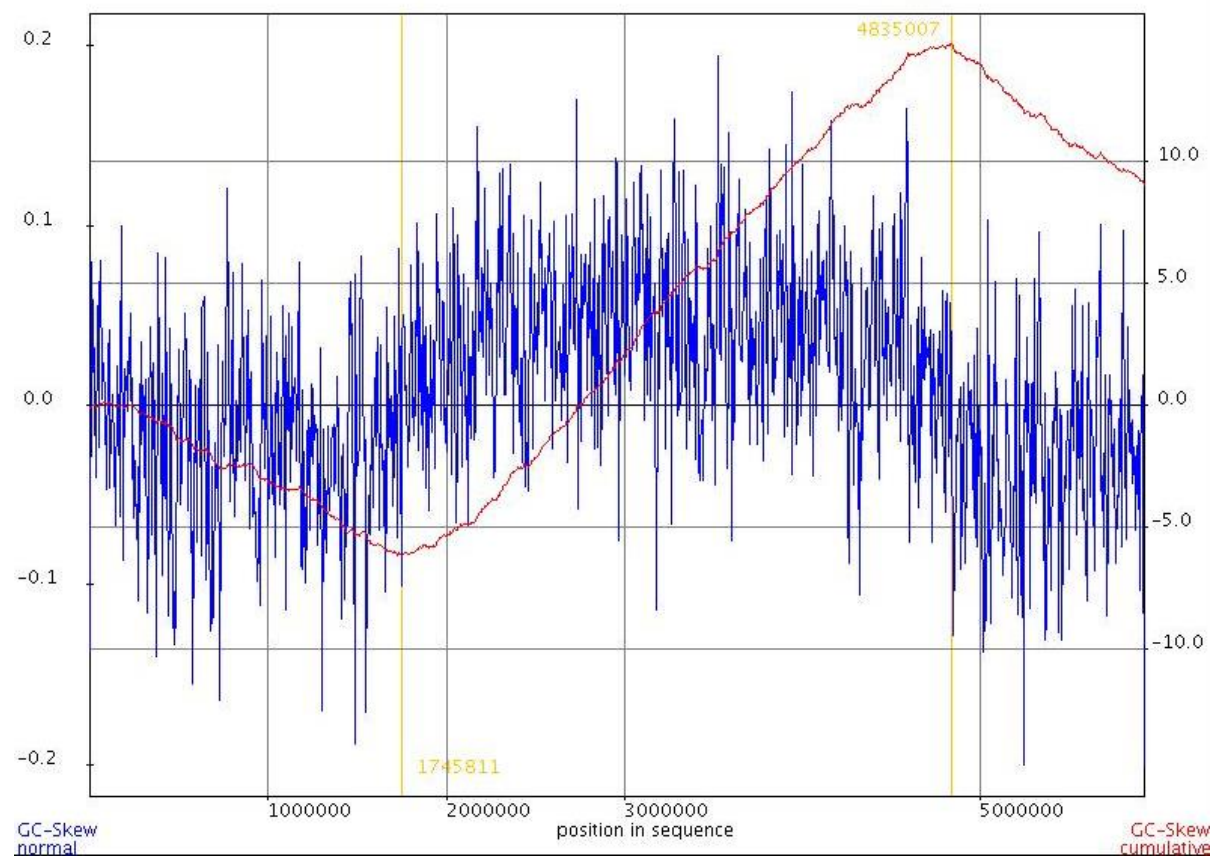


Figure 8.1. Cumulative GC Skew and coverage profile of *Labrenzia* sp. strain 5N chromosome using GenSkew (<http://genskew.csb.univie.ac.at/>). GC-skew is shown in blue, the cumulative GC-skew in red and the minimum (ori) and maximum (ter) are shown in yellow.

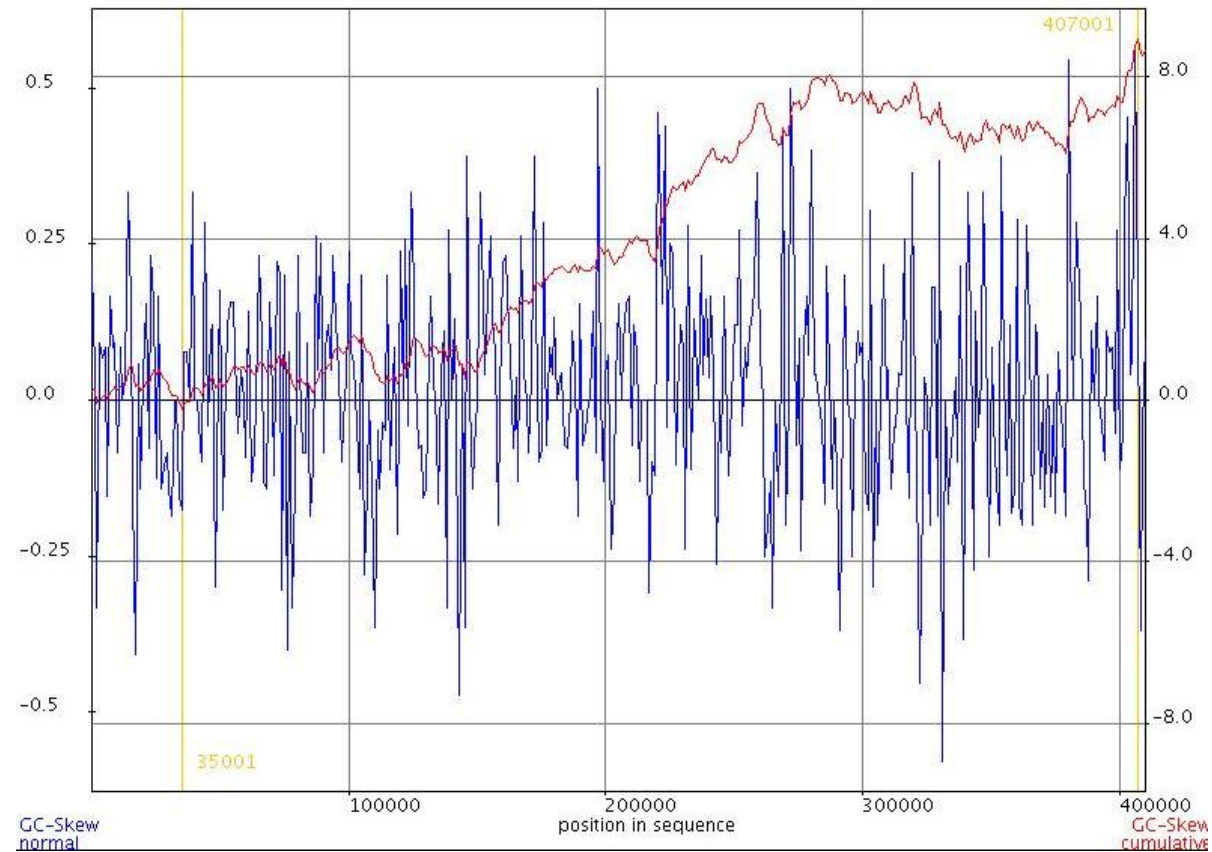


Figure 8.2. Cumulative GC Skew and coverage profile of *Labrenzia* sp. strain 5N plasmid 1 using GenSkew (<http://genskew.csb.univie.ac.at/>). GC-skew is shown in blue, the cumulative GC-skew in red and the minimum (ori) and maximum (ter) are shown in yellow.

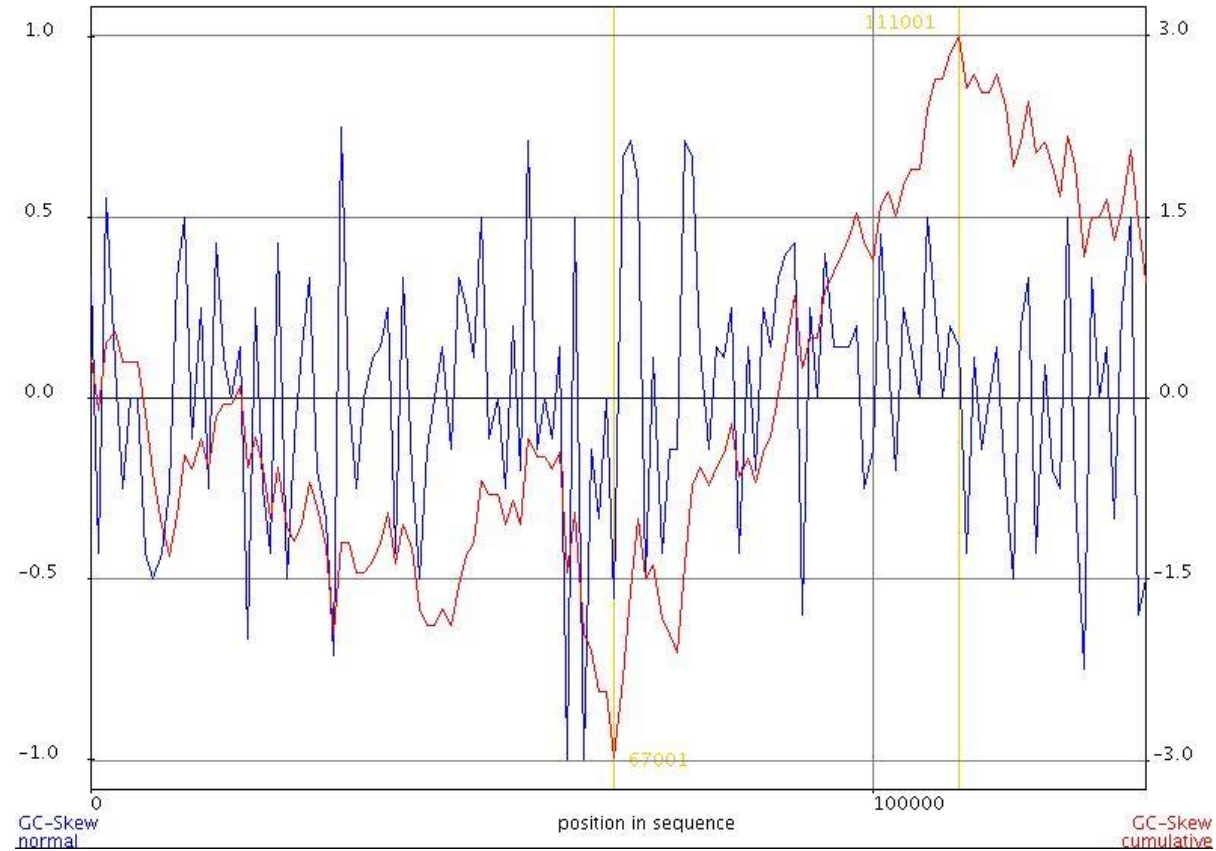


Figure 8.3. Cumulative GC Skew and coverage profile of *Labrenzia* sp. strain 5N plasmid 2 using GenSkew (<http://genskew.csb.univie.ac.at/>). GC-skew is shown in blue, the cumulative GC-skew in red and the minimum (ori) and maximum (ter) are shown in yellow.

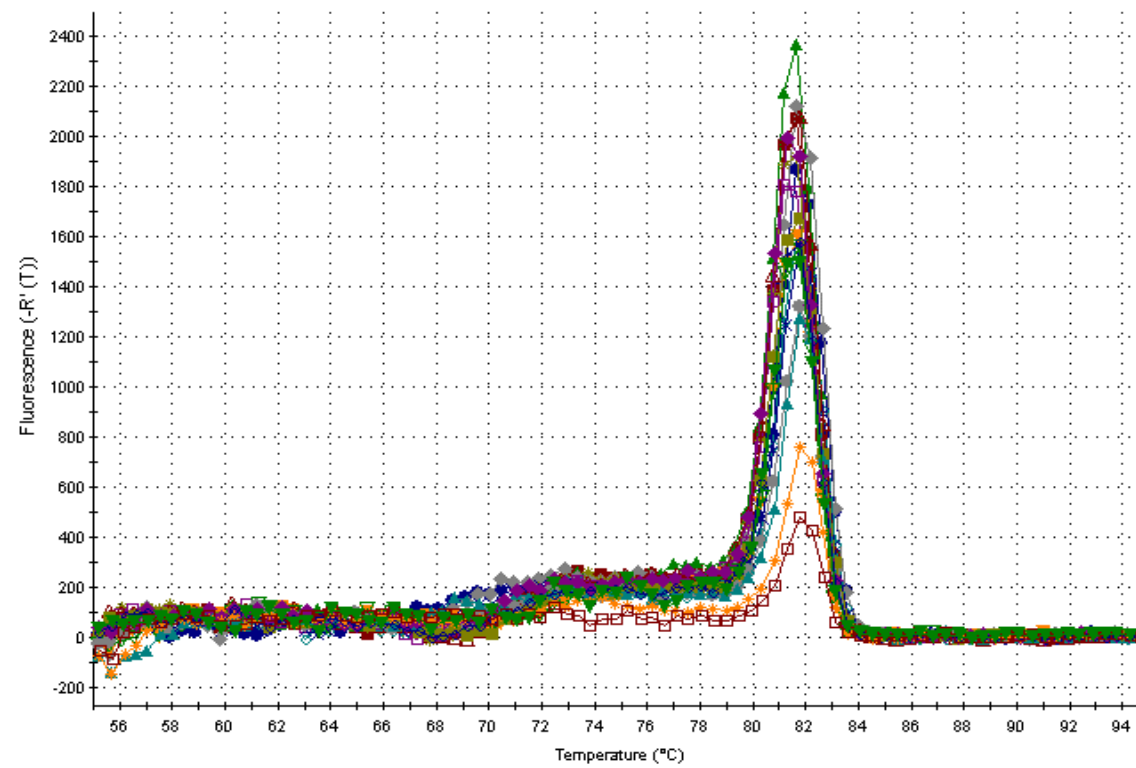


Figure 8.4. Dissociation curve of narB qPCR standards and samples for the determination of *Labrenzia* sp. strain 5N plasmid 1 copy number.

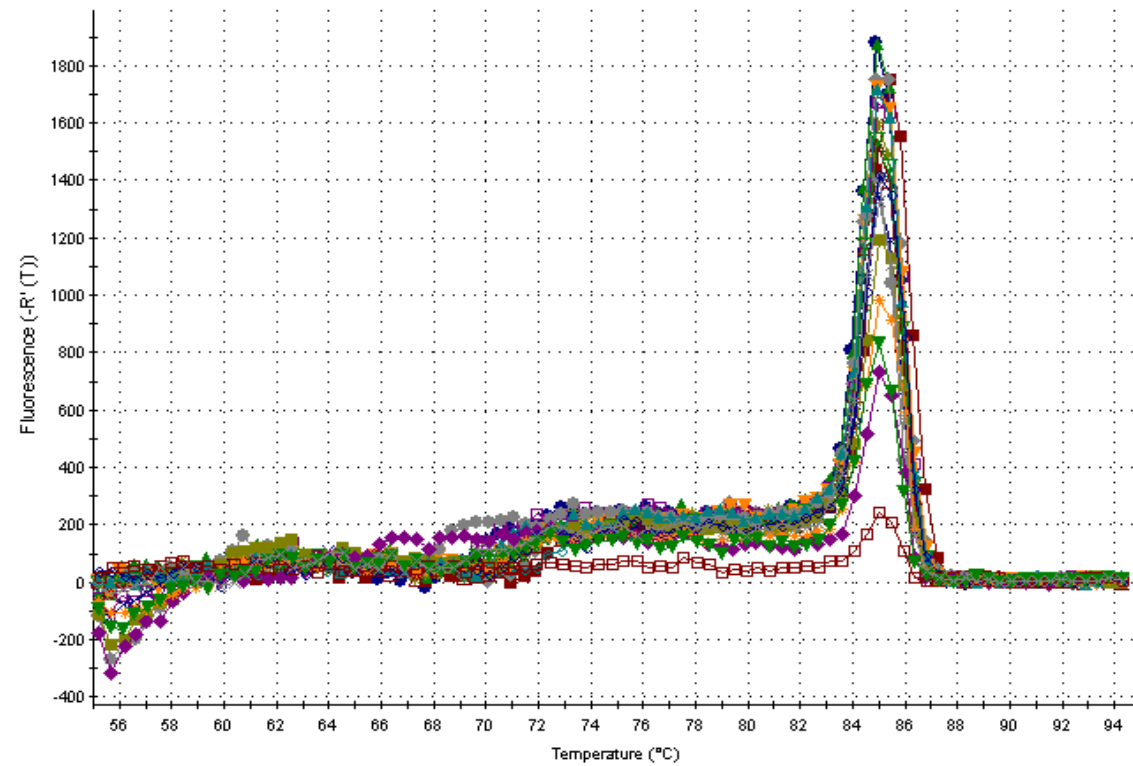


Figure 8.5. Dissociation curve of *nosZ* qPCR standards and samples for the determination of *Labrenzia* sp. strain 5N plasmid 1 copy number.

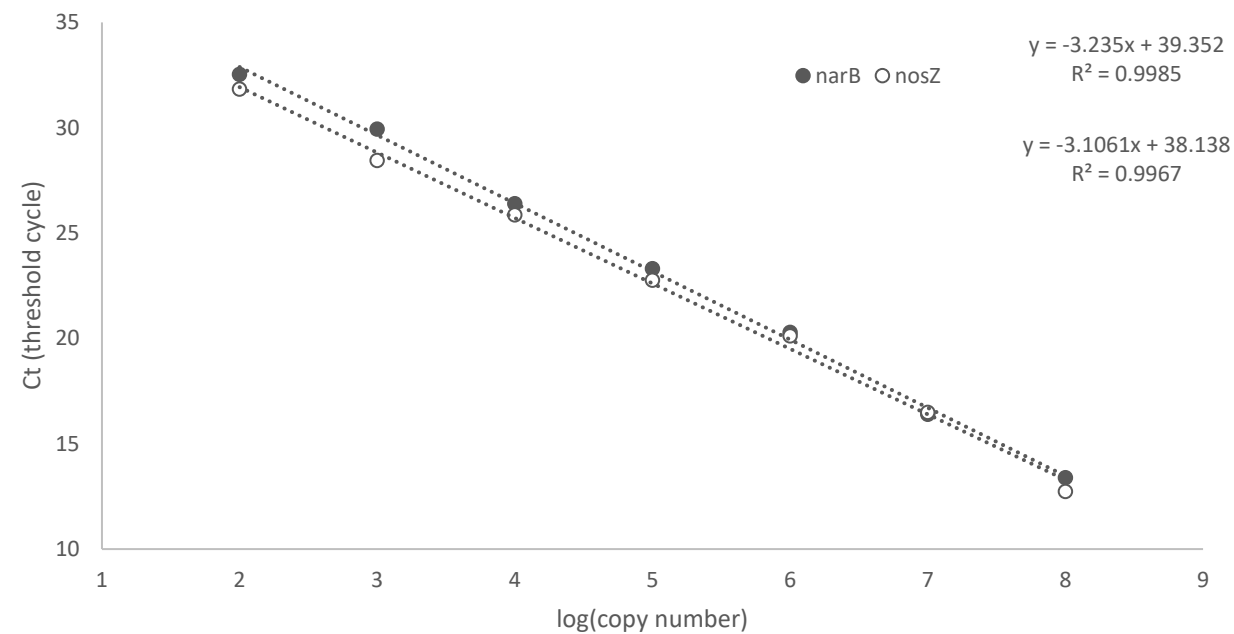


Figure 8.6. Standard curves for quantification by RT-qPCR of the *narB* and *nosZ* copy numbers in *Labrenzia* sp. strain 5N. Equations are shown.

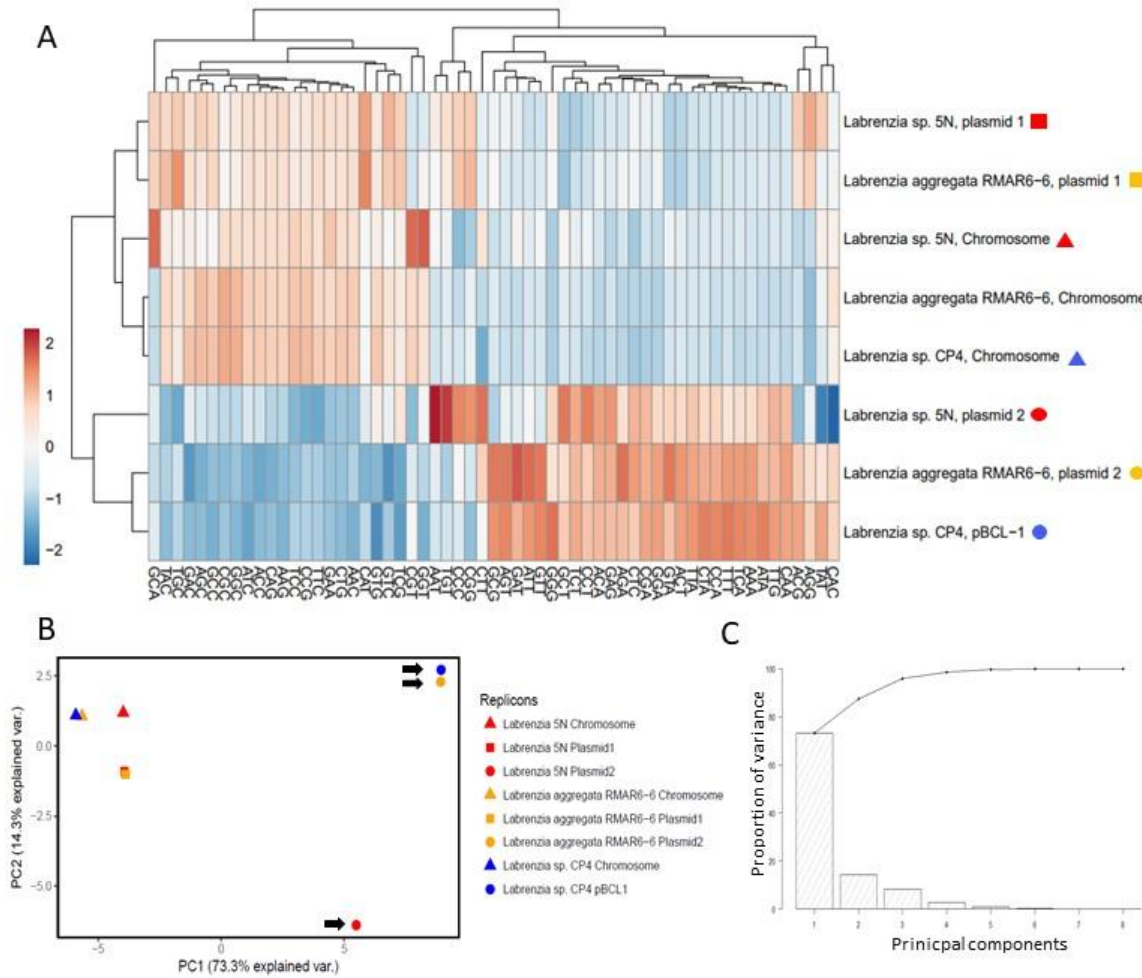


Figure 8.7. Heat map and PCA analysis of RSCU of synonymous codons from the 8 replicons of *Labrenzia* sp. strain 5N, *Labrenzia aggregata* RMAR6-6 and *Labrenzia* sp. CP4. (A) Heatmap of RSCU of 59 codons from the 8 replicons of *Labrenzia* sp. strain 5N, *Labrenzia aggregata* RMAR6-6 and *Labrenzia* sp. CP4 using Euclidean distance and average linkage clustering module. Heatmap generated using the ClustVis webtool (Metsalu et al. 2015). Rare codons are shown in blue and frequent codons are shown in red for each replicon. (B) The PCA of RSCU values of 59 codons from the *Labrenzia* sp. strain 5N (red), *Labrenzia aggregata* RMAR6-6 (orange) and *Labrenzia* sp. CP4 (blue) replicons (n=8), on the primary and secondary axes (accounting for 73.3% and 14.3% of variance, respectively). Black arrows indicate the positioning of the three conjugative plasmids. (C) Variance contribution (bars) and cumulative variance contribution (circles) of each of the 8 principal components (x-axis) from the PCA analysis of RSCU of 59 codons from the 8 replicons of *Labrenzia* sp. strain 5N, *Labrenzia aggregata* RMAR6-6 and *Labrenzia* sp. CP4.

Appendices for Chapter 4

Table 8.7. List of proteins implicated in the main pathways of interest as depicted in Figure 4.2.

Process		Protein ID	Description	Fold change
Central metabolism	C4 uptake	HEP89_13540	C4-dicarboxylate ABC transporter [Labrenzia aggregata]	5.090
		HEP89_03490	TRAP transporter substrate-binding protein DctP [Labrenzia sp. 5N]	3.784
		HEP89_25890	TRAP transporter permease [Labrenzia sp. 5N]	2.798
		HEP89_15385	MULTISPECIES: C4-dicarboxylate ABC transporter substrate-binding protein [Labrenzia]	1.764
	CO uptake	HEP89_21400	carbon monoxide dehydrogenase [Labrenzia aggregata]	2.927
		HEP89_21395	carbon monoxide dehydrogenase [Labrenzia aggregata]	2.200
		HEP89_21405	carbon monoxide dehydrogenase [Labrenzia aggregata]	2.191
	EDP	HEP89_17120	phosphogluconate dehydratase [Labrenzia sp. 5N]	0.276
		HEP89_17115	keto-deoxy-phosphogluconate aldolase [Labrenzia aggregata]	0.473
	DNA metabolism	HEP89_24295	type I DNA topoisomerase [Labrenzia sp. 5N]	0.613
		HEP89_25650	DNA topoisomerase IV subunit A [Labrenzia sp. 5N]	0.399
		HEP89_06420	DNA topoisomerase (ATP-hydrolyzing) subunit B [Labrenzia sp. 5N]	0.623
		HEP89_22020	DNA gyrase subunit A [Labrenzia sp. 5N]	0.597
		HEP89_24765	MULTISPECIES: DNA helicase [Labrenzia]	0.431
		HEP89_06410	DNA polymerase III subunit beta [Labrenzia sp. 5N]	0.632
		HEP89_06405	chromosomal replication initiator protein DnaA [Labrenzia sp. 5N]	0.204

		HEP89_2462 5	nucleoside-diphosphate kinase [Labrenzia sp. 5N]	0.502
		HEP89_2065 5	vitamin B12-dependent ribonucleotide reductase [Labrenzia sp. 5N]	0.388
		HEP89_2144 5	MULTISPECIES: CTP synthetase [Labrenzia]	0.610
		HEP89_1654 0	DNA repair protein RecN [Labrenzia sp. 5N]	0.661
		HEP89_1652 5	cell division protein FtsZ [Labrenzia sp. 5N]	0.539
		HEP89_2856 5	DNA translocase FtsK [Labrenzia sp. 5N]	0.312
		HEP89_1993 0	DNA-directed RNA polymerase subunit beta [Labrenzia sp. 5N]	0.434
		HEP89_2009 0	MULTISPECIES: DNA-directed RNA polymerase subunit alpha [Labrenzia]	0.431
		HEP89_2559 0	MULTISPECIES: DNA-directed RNA polymerase subunit omega [Labrenzia]	0.403
		HEP89_1993 5	DNA-directed RNA polymerase subunit beta' [Labrenzia sp. 5N]	0.384
		HEP89_1990 5	MULTISPECIES: transcription termination/antitermination protein NusG [Labrenzia]	0.487
		HEP89_0623 5	transcription termination/antitermination protein NusA [Labrenzia sp. 5N]	0.470
		HEP89_0819 0	MULTISPECIES: transcription termination factor Rho [Labrenzia]	0.397
Transcriptional regulation	Regulators/ repressors	HEP89_1465 5	TetR/AcrR family transcriptional regulator [Labrenzia sp. 5N]	19.341
		HEP89_2803 5	autoinducer 2 ABC transporter substrate-binding protein [Labrenzia sp. 5N]	2.421
		HEP89_1850 0	MULTISPECIES: AsnC family transcriptional regulator [Labrenzia]	1.995
		HEP89_2261 0	GntR family transcriptional regulator [Labrenzia sp. 5N]	1.731
		HEP89_1885 5	helix-turn-helix transcriptional regulator [Labrenzia sp. 5N]	1.690
		HEP89_2550 5	MULTISPECIES: NrdR family transcriptional regulator [Labrenzia]	1.590

Protein synthesis	Transcription termination factors	HEP89_2391 0	Lrp/AsnC family transcriptional regulator [Labrenzia sp. 5N]	0.635
		HEP89_2665 5	MULTISPECIES: ArsR family transcriptional regulator [Labrenzia]	0.594
		HEP89_0174 0	MULTISPECIES: transcriptional regulator [Labrenzia]	0.584
		HEP89_0900 0	MULTISPECIES: CarD family transcriptional regulator [Labrenzia]	0.444
		HEP89_1207 5	YebC/PmpR family DNA-binding transcriptional regulator [Labrenzia sp. 5N]	0.399
		HEP89_0625 5	helix-turn-helix transcriptional regulator [Labrenzia sp. 5N]	0.347
		HEP89_1155 0	LysR family transcriptional regulator [Labrenzia sp. 5N]	0.283
		HEP89_1522 5	MerR family transcriptional regulator [Labrenzia sp. 5N]	0.191
		HEP89_1990 5	MULTISPECIES: transcription termination/antitermination factor NusG [Labrenzia]	0.487
		HEP89_0623 5	transcription termination/antitermination protein NusA [Labrenzia sp. 5N]	0.470
		HEP89_2548 5	MULTISPECIES: transcription antitermination protein NusB [Labrenzia]	0.453
		HEP89_0819 0	MULTISPECIES: transcription termination factor Rho [Labrenzia]	0.397
		HEP89_1698 5	acetolactate synthase 3 large subunit [Labrenzia sp. 5N]	0.273
		HEP89_0832 0	3-isopropylmalate dehydratase large subunit [Labrenzia sp. 5N]	0.575
		HEP89_2095 0	proline--tRNA ligase [Labrenzia sp. 5N]	0.654
		HEP89_0316 5	isoleucine--tRNA ligase [Labrenzia sp. 5N]	0.586
		HEP89_2496 5	cysteine--tRNA ligase [Labrenzia sp. 5N]	0.559
		HEP89_0259 5	glycine--tRNA ligase subunit alpha [Labrenzia sp. 5N]	0.559
		HEP89_1951 5	threonine--tRNA ligase [Labrenzia sp. 5N]	0.532

	HEP89_1803		
	5	alanine--tRNA ligase [Labrenzia sp. 5N]	0.524
	HEP89_0331		
	5	histidine--tRNA ligase [Labrenzia sp. 5N]	0.503
	HEP89_0487		
	5	phenylalanine--tRNA ligase subunit alpha [Labrenzia sp. 5N]	0.497
	HEP89_2500		
	5	glutamate--tRNA ligase [Labrenzia sp. 5N]	0.476
	HEP89_0488		
	5	phenylalanine--tRNA ligase subunit beta [Labrenzia sp. 5N]	0.473
	HEP89_0823		
	5	leucine--tRNA ligase [Labrenzia sp. 5N]	0.452
	HEP89_2584		
	5	methionine--tRNA ligase [Labrenzia sp. 5N]	0.448
	HEP89_2648		
	5	Asp-tRNA(Asn)/Glu-tRNA(Gln) amidotransferase subunit GatB [Labrenzia sp. 5N]	0.448
	HEP89_1687		
	0	glutamyl-tRNA amidotransferase [Labrenzia aggregata]	0.447
	HEP89_2110		
	5	glutamate--tRNA ligase [Labrenzia sp. 5N]	0.440
	HEP89_0447		
	0	methionyl-tRNA formyltransferase [Labrenzia sp. 5N]	0.427
	HEP89_2199		
	5	tRNA preQ1(34) S-adenosylmethionine ribosyltransferase-isomerase QueA [Labrenzia sp. 5N]	0.408
	HEP89_0182		
	5	lysine--tRNA ligase [Labrenzia sp. 5N]	0.378
	HEP89_2250		
	5	valine--tRNA ligase [Labrenzia sp. 5N]	0.343
	HEP89_0282		
	5	tyrosine--tRNA ligase [Labrenzia sp. 5N]	0.137
50S large ribosomal subunit proteins (L1-31)	HEP89_2432		
	0	MULTISPECIES: 50S ribosomal protein L33 [Labrenzia]	0.491
	HEP89_1992		
	0	MULTISPECIES: 50S ribosomal protein L10 [Labrenzia]	0.487
	HEP89_1992		
	5	MULTISPECIES: 50S ribosomal protein L7/L12 [Labrenzia]	0.467
	HEP89_2002		
	0	MULTISPECIES: 50S ribosomal protein L24 [Labrenzia]	0.465

HEP89_2006 5	MULTISPECIES: 50S ribosomal protein L15 [Labrenzia]	0.453
HEP89_1997 5	MULTISPECIES: 50S ribosomal protein L23 [Labrenzia]	0.444
HEP89_2006 0	MULTISPECIES: 50S ribosomal protein L30 [Labrenzia]	0.438
HEP89_0129 5	MULTISPECIES: 50S ribosomal protein L21 [Labrenzia]	0.433
HEP89_1991 0	MULTISPECIES: 50S ribosomal protein L11 [Labrenzia]	0.414
HEP89_2476 0	MULTISPECIES: 50S ribosomal protein L9 [Labrenzia]	0.409
HEP89_0893 0	MULTISPECIES: 50S ribosomal protein L20 [Labrenzia]	0.409
HEP89_1193 5	MULTISPECIES: 50S ribosomal protein L31 [Labrenzia]	0.408
HEP89_0129 0	MULTISPECIES: 50S ribosomal protein L27 [Labrenzia]	0.404
HEP89_2009 5	50S ribosomal protein L17 [Labrenzia aggregata]	0.401
HEP89_2002 5	MULTISPECIES: 50S ribosomal protein L5 [Labrenzia]	0.399
HEP89_0989 5	MULTISPECIES: 50S ribosomal protein L28 [Labrenzia]	0.382
HEP89_1997 5	50S ribosomal protein L4 [Labrenzia aggregata]	0.377
HEP89_0831 5	50S ribosomal protein L19 [Labrenzia aggregata]	0.376
HEP89_2004 5	MULTISPECIES: 50S ribosomal protein L6 [Labrenzia]	0.364
HEP89_1991 5	50S ribosomal protein L1 [Labrenzia aggregata]	0.356
HEP89_1999 5	MULTISPECIES: 50S ribosomal protein L22 [Labrenzia]	0.355
HEP89_2002 0	MULTISPECIES: 50S ribosomal protein L14 [Labrenzia]	0.351
HEP89_1455 5	MULTISPECIES: 50S ribosomal protein L25 [Labrenzia]	0.350

HEP89_1997 0	MULTISPECIES: 50S ribosomal protein L3 [Labrenzia]	0.343
HEP89_2005 0	MULTISPECIES: 50S ribosomal protein L18 [Labrenzia]	0.339
HEP89_2000 5	MULTISPECIES: 50S ribosomal protein L16 [Labrenzia]	0.322
HEP89_2359 5	MULTISPECIES: 50S ribosomal protein L13 [Labrenzia]	0.248
HEP89_2001 0	MULTISPECIES: 50S ribosomal protein L29 [Labrenzia]	0.190
HEP89_2432 0	MULTISPECIES: 50S ribosomal protein L33 [Labrenzia]	0.491
HEP89_1992 0	MULTISPECIES: 50S ribosomal protein L10 [Labrenzia]	0.487
HEP89_1992 5	MULTISPECIES: 50S ribosomal protein L7/L12 [Labrenzia]	0.467
HEP89_2002 0	MULTISPECIES: 50S ribosomal protein L24 [Labrenzia]	0.465
HEP89_2006 5	MULTISPECIES: 50S ribosomal protein L15 [Labrenzia]	0.453
HEP89_1997 5	MULTISPECIES: 50S ribosomal protein L23 [Labrenzia]	0.444
HEP89_2006 0	MULTISPECIES: 50S ribosomal protein L30 [Labrenzia]	0.438
HEP89_0129 5	MULTISPECIES: 50S ribosomal protein L21 [Labrenzia]	0.433
HEP89_1991 0	MULTISPECIES: 50S ribosomal protein L11 [Labrenzia]	0.414
HEP89_2476 0	MULTISPECIES: 50S ribosomal protein L9 [Labrenzia]	0.409
HEP89_0893 0	MULTISPECIES: 50S ribosomal protein L20 [Labrenzia]	0.409
HEP89_1193 5	MULTISPECIES: 50S ribosomal protein L31 [Labrenzia]	0.408
HEP89_0129 0	MULTISPECIES: 50S ribosomal protein L27 [Labrenzia]	0.404
HEP89_2002 5	MULTISPECIES: 50S ribosomal protein L5 [Labrenzia]	0.399

	HEP89_0989		
	5	MULTISPECIES: 50S ribosomal protein L28 [Labrenzia]	0.382
	HEP89_1997		
	5	50S ribosomal protein L4 [Labrenzia aggregata]	0.377
	HEP89_0831		
	5	50S ribosomal protein L19 [Labrenzia aggregata]	0.376
	HEP89_2004		
	5	MULTISPECIES: 50S ribosomal protein L6 [Labrenzia]	0.364
	HEP89_1991		
	5	50S ribosomal protein L1 [Labrenzia aggregata]	0.356
	HEP89_1999		
	5	MULTISPECIES: 50S ribosomal protein L22 [Labrenzia]	0.355
	HEP89_2002		
	0	MULTISPECIES: 50S ribosomal protein L14 [Labrenzia]	0.351
	HEP89_1455		
	5	MULTISPECIES: 50S ribosomal protein L25 [Labrenzia]	0.350
	HEP89_1997		
	0	MULTISPECIES: 50S ribosomal protein L3 [Labrenzia]	0.343
	HEP89_2005		
	0	MULTISPECIES: 50S ribosomal protein L18 [Labrenzia]	0.339
	HEP89_1998		
	5	50S ribosomal protein L2 [Labrenzia aggregata]	0.339
	HEP89_2000		
	5	MULTISPECIES: 50S ribosomal protein L16 [Labrenzia]	0.322
	HEP89_2359		
	5	MULTISPECIES: 50S ribosomal protein L13 [Labrenzia]	0.248
	HEP89_2001		
	0	MULTISPECIES: 50S ribosomal protein L29 [Labrenzia]	0.190
30S small ribosomal subunit proteins (S1-21)	HEP89_2008		
	5	MULTISPECIES: 30S ribosomal protein S11 [Labrenzia]	0.475
	HEP89_0829		
	5	30S ribosomal protein S16 [Labrenzia sp. 5N]	0.466
	HEP89_2005		
	5	MULTISPECIES: 30S ribosomal protein S5 [Labrenzia]	0.459
	HEP89_1996		
	5	MULTISPECIES: 30S ribosomal protein S10 [Rhodobacteraceae]	0.451
	HEP89_0639		
	5	MULTISPECIES: 30S ribosomal protein S20 [Labrenzia]	0.450

	HEP89_1817		
	5	MULTISPECIES: 30S ribosomal protein S4 [Labrenzia]	0.450
	HEP89_2475		
	5	MULTISPECIES: 30S ribosomal protein S18 [Labrenzia]	0.450
	HEP89_2004		
	0	MULTISPECIES: 30S ribosomal protein S8 [Labrenzia]	0.446
	HEP89_2475		
	0	MULTISPECIES: 30S ribosomal protein S6 [Labrenzia]	0.444
	HEP89_1188		
	0	MULTISPECIES: 30S ribosomal protein S21 [Labrenzia]	0.442
	HEP89_0620		
	5	30S ribosomal protein S15 [Labrenzia sp. 5N]	0.435
	HEP89_0543		
	5	30S ribosomal protein S1 [Labrenzia sp. 5N]	0.427
	HEP89_1995		
	0	MULTISPECIES: 30S ribosomal protein S7 [Labrenzia]	0.427
	HEP89_2001		
	5	MULTISPECIES: 30S ribosomal protein S17 [Labrenzia]	0.420
	HEP89_2008		
	0	MULTISPECIES: 30S ribosomal protein S13 [Labrenzia]	0.407
	HEP89_2359		
	0	MULTISPECIES: 30S ribosomal protein S9 [Labrenzia]	0.406
	HEP89_1999		
	0	MULTISPECIES: 30S ribosomal protein S19 [Labrenzia]	0.402
	HEP89_2101		
	5	MULTISPECIES: 30S ribosomal protein S2 [Labrenzia]	0.356
	HEP89_1994		
	5	MULTISPECIES: 30S ribosomal protein S12 [Labrenzia]	0.334
	HEP89_2000		
	0	MULTISPECIES: 30S ribosomal protein S3 [Labrenzia]	0.318
translational elongation factors	HEP89_1989		
	0	elongation factor Tu [Labrenzia aggregata]	0.568
	HEP89_0484		
	0	MULTISPECIES: elongation factor 4 [Labrenzia]	0.558
	HEP89_0026		
	0	elongation factor 3 [Labrenzia aggregata]	0.547
	HEP89_1521		
	0	MULTISPECIES: transcription elongation factor GreA [Labrenzia]	0.524

Nitrogen metabolism		HEP89_11995	MULTISPECIES: elongation factor P [Labrenzia]	0.516
		HEP89_21020	elongation factor Ts [Labrenzia sp. 5N]	0.496
		HEP89_19955	elongation factor G [Labrenzia sp. 5N]	0.394
	Translation initiation factors	HEP89_00640	MULTISPECIES: translation initiation factor IF-1 [Rhodobacteraceae]	0.393
		HEP89_06225	MULTISPECIES: translation initiation factor IF-2 [Labrenzia]	0.389
		HEP89_10605	translation initiation factor IF-3 [Labrenzia sp. 5N]	0.211
	Peptide chain release factors	HEP89_10800	peptide chain release factor 1 [Labrenzia sp. 5N]	0.522
		HEP89_00465	peptide chain release factor 3 [Labrenzia sp. 5N]	0.236
	Dissimilatory nitrate reduction	HEP89_19450	periplasmic nitrate reductase subunit alpha [Labrenzia sp. 5N]	10.035
		HEP89_19455	chaperone NapD [Labrenzia sp. 5N]	22.192
		HEP89_19445	nitrate reductase cytochrome c-type subunit [Labrenzia sp. 5N]	6.219
		HEP89_27870	nitrite reductase NirS[Labrenzia aggregata]	695.167
		HEP89_27885	protein nirF [Labrenzia sp. 5N]	21.768
		HEP89_27895	protein nirG [Labrenzia aggregata]	18.684
		HEP89_27900	protein nirH [Labrenzia aggregata]	16.798
		HEP89_27905	heme d1 biosynthesis radical SAM protein NirJ [Labrenzia sp. 5N]	7.551
		HEP89_27910	NirN	35.762
		HEP89_03290	transcriptional regulator NsrR [Labrenzia aggregata]	10.185
		HEP89_27865	Nitric oxide reductase cytochrome c subunit [Labrenzia aggregata]	73.528

	HEP89_2785		
	5	NorQ	12.643
	HEP89_2795		117.36
	5	nitrous-oxide reductase [Labrenzia aggregata]	6
	HEP89_2795		
	0	nitrous oxide reductase family maturation protein NosD [Labrenzia sp. 5N]	19.984
	HEP89_2794		
	5	ABC transporter ATP-binding protein nosF [Labrenzia aggregata]	6.394
	HEP89_2796		
	0	regulatory protein NosR [Labrenzia sp. 5N]	4.989
N assimilation	HEP89_1792	MULTISPECIES: quinoxemoprotein amine dehydrogenase Gamma subunit [Labrenzia]	69.507
	5		
	HEP89_1793		
	0	quinoxemoprotein amine dehydrogenase subunit beta [Labrenzia sp. 5N]	35.605
	HEP89_1791		
	5	quinoxemoprotein amine dehydrogenase subunit alpha [Labrenzia sp. 5N]	12.486
	HEP89_1792		
	0	quinoxemoprotein amine dehydrogenase maturation protein [Labrenzia sp. 5N]	31.187
Urea cycle	HEP89_1521		
	5	carbamoyl-phosphate synthase large subunit [Labrenzia sp. 5N]	0.484
	HEP89_1523	glutamine-hydrolyzing carbamoyl-phosphate synthase small subunit [Labrenzia sp. 5N]	0.382
	0		
	HEP89_0851		
	5	argininosuccinate lyase [Labrenzia sp. 5N]	0.631
	HEP89_0191		
	0	ornithine carbamoyltransferase [Labrenzia sp. 5N]	0.516
Urease activity	HEP89_0838		
	0	urease subunit alpha [Labrenzia sp. 5N]	1.882
	HEP89_0840		
	0	MULTISPECIES: urease subunit gamma [Labrenzia]	2.683
	HEP89_0837		
	0	urease accessory protein UreE [Labrenzia sp. 5N]	1.907
	HEP89_0836		
	0	urease accessory protein UreG [Labrenzia sp. 5N]	1.762
	HEP89_0839		
	0	MULTISPECIES: urease subunit beta [Labrenzia]	0.443
Electron transport	HEP89_2676		
	5	cytochrome c-type biogenesis protein CcmH [Labrenzia sp. 5N]	8.721

HEP89_2677		
5	cytochrome c maturation protein CcmE [Labrenzia sp. 5N]	7.589
HEP89_2678		
0	c-type cytochrome biogenesis protein CcmI [Labrenzia sp. 5N]	5.841
HEP89_2791		
0	cytochrome C oxidase Cbb3 [Labrenzia sp. 5N]	35.762
HEP89_0156		
5	cytochrome-c oxidase, cbb3-type subunit III [Labrenzia sp. 5N]	12.975
HEP89_0157		
5	cytochrome-c oxidase, cbb3-type subunit II [Labrenzia sp. 5N]	5.943
HEP89_0156		
0	cytochrome c oxidase accessory protein CcoG [Labrenzia sp. 5N]	25.254
HEP89_0155		
5	FixH family protein [Labrenzia sp. 5N]	10.675
HEP89_1517		
5	cytochrome c2 oxidoreductase [Labrenzia aggregata]	22.963
HEP89_1893		
5	MULTISPECIES: cytochrome C2 signal peptide protein [Labrenzia]	14.838
HEP89_2405		
0	cytochrome bd-I ubiquinol oxidase subunit CydA [Labrenzia sp. 5N]	2.103
HEP89_0219		
0	ubiquinone biosynthesis protein [Labrenzia aggregata]	26.348
HEP89_2799		
5	UbiD family decarboxylase [Labrenzia sp. 5N]	21.942
HEP89_2800		
0	UbiX family flavin prenyltransferase [Labrenzia sp. 5N]	19.511
HEP89_1186		
0	AarF/ABC1/UbiB kinase family protein [Labrenzia sp. 5N]	7.528
HEP89_2191		
0	UbiH/UbiF family hydroxylase [Labrenzia sp. 5N]	3.188
HEP89_0638		
0	MULTISPECIES: ubiquinone/menaquinone biosynthesis methyltransferase [Labrenzia]	1.549
HEP89_2866		
0	ferredoxin [Labrenzia aggregata]	4.508
HEP89_1885		
0	ferredoxin [Labrenzia aggregata]	2.142
HEP89_2555		
0	porphobilinogen synthase [Labrenzia sp. 5N]	2.476

	HEP89_10370	porphobilinogen deaminase [Labrenzia aggregata]	3.386
	HEP89_08180	uroporphyrinogen decarboxylase [Labrenzia sp. 5N]	3.859
	HEP89_14400	oxygen-dependent coproporphyrinogen oxidase [Labrenzia sp. 5N]	3.645
	HEP89_10385	heme biosynthesis protein HemY [Labrenzia sp. 5N]	2.987
	HEP89_11675	ferrochelatase [Labrenzia sp. 5N]	2.131
	HEP89_22450	Fe-S cluster assembly scaffold SufA [Labrenzia sp. 5N]	6.762
	HEP89_22475	Fe-S cluster assembly protein SufB [Labrenzia sp. 5N]	2.365
	HEP89_22470	Fe-S cluster assembly ATPase SufC [Labrenzia sp. 5N]	2.534
	HEP89_22465	Fe-S cluster assembly protein SufD [Labrenzia sp. 5N]	2.221
	HEP89_05975	MULTISPECIES: cysteine desulfurase [Labrenzia]	1.860
Motility and biofilm formation	HEP89_14285	MULTISPECIES: flagellar motor switch protein FliG [Labrenzia]	0.590
	HEP89_14295	MULTISPECIES: flagellar motor switch protein FliN [Labrenzia]	0.350
	HEP89_09865	BolA family transcriptional regulator [Labrenzia sp. 5N]	1.584
	HEP89_18855	helix-turn-helix transcriptional regulator [Labrenzia sp. 5N]	1.690
	HEP89_12075	YebC/PmpR family DNA-binding transcriptional regulator [Labrenzia sp. 5N]	0.399
	HEP89_14085	MULTISPECIES: transcriptional regulator [Labrenzia]	2.969
	HEP89_22245	1,2-phenylacetyl-CoA epoxidase subunit A [Labrenzia sp. 5N]	8.665
	HEP89_22205	hydroxyphenylacetyl-CoA thioesterase Paal [Labrenzia sp. 5N]	10.010
	HEP89_22215	phenylacetic acid degradation bifunctional protein PaaZ [Labrenzia sp. 5N]	8.376

HEP89_2221		
0	phenylacetate--CoA ligase [Labrenzia sp. 5N]	6.346
HEP89_2222		
0	phenylacetate-CoA oxygenase/reductase subunit PaaK [Labrenzia sp. 5N]	4.452
HEP89_2486		
0	3-oxoadipyl-CoA thiolase [Labrenzia sp. 5N]	4.352
HEP89_0174		
5	acyl-CoA dehydrogenase [Labrenzia sp. 5N]	4.534

Appendices for Chapter 5

Table 8.8. **Carbon and Oxygen budget modelling for an aerobic *Labrenzia* sp. strain 5N culture supplied with acetate as the C source and ammonium as the N source.** ^a based on 1OD unit = 1.93×10^{-8} cells/ml. ^b assumes a biomass of 100 fgC/cell. ^c RQ, Respiratory Quotient = 0.35 for growth on acetate. ^d amount of C remaining (in g/culture volume) in the medium at each time interval, corrected for the loss of medium acetate at each sampling point. ^e the equimolar equivalent loss due to respiration at RQ=0.35 with a starting concentration in the medium of 0.000934 g/150 ml (at 33°C and 27 psu).

Time (h)	Model OD	^a Cells/ml	Cells/culture	^b Cell C/Culture	^c C demand RQ=0.35	^d Residual C	^e Residual O ₂	O ₂ uptake rate
0	0.039	7.55E+06	1.13E+09	0.00011	0.00017	0.00720	0.00094	
1	0.053	1.03E+07	1.54E+09	0.00015	0.00024	0.00714	0.00087	0.00006
2	0.072	1.39E+07	2.09E+09	0.00021	0.00032	0.00705	0.00079	0.00009
3	0.098	1.90E+07	2.84E+09	0.00028	0.00044	0.00694	0.00067	0.00012
4	0.134	2.58E+07	3.87E+09	0.00039	0.00059	0.00678	0.00052	0.00016
5	0.182	3.50E+07	5.26E+09	0.00053	0.00081	0.00657	0.00030	0.00021
6	0.247	4.76E+07	7.14E+09	0.00071	0.00110	0.00628	0.00001	0.00029
7	0.335	6.47E+07	7.38E+09	0.00074	0.00114	0.00624	-0.00003	0.00004
8	0.456	8.80E+07	1.00E+10	0.00100	0.00154	0.00583	-0.00043	0.00041
9	0.620	1.20E+08	1.36E+10	0.00136	0.00210	0.00528	-0.00099	0.00055
10	0.843	1.63E+08	1.85E+10	0.00185	0.00285	0.00452	-0.00174	0.00075
11	1.146	2.21E+08	2.52E+10	0.00252	0.00388	0.00350	-0.00277	0.00103
12	1.558	3.01E+08	3.43E+10	0.00343	0.00527	0.00210	-0.00416	0.00139
13	2.117	4.09E+08	4.66E+10	0.00466	0.00717	0.00021	-0.00606	0.00190

Table 8.9. **Carbon and Oxygen budget modelling for an aerobic *Labrenzia* sp. strain 5N culture supplied with acetate as the C source and ammonium and nitrate as the N sources.** ^a based on 1OD unit = 1.93×10^{-8} cells/ml. ^b assumes a biomass of 100 fgC/cell. ^c RQ, Respiratory Quotient = 0.35 for growth on acetate. ^d amount of C remaining (in g/culture volume) in the medium at each time interval, corrected for the loss of medium acetate at each sampling point. ^e the equimolar equivalent loss due to respiration at RQ=0.35 with a starting concentration in the medium of 0.000934 g/150 ml (at 33°C and 27 psu).

Time (h)	Model OD	^a Cells/ml	Cells/culture	^b Cell C/Culture	^c C demand RQ=0.35	^d Residual C	^e Residual O ₂	O ₂ uptake rate
0	0.039	7.55E+06	1.13E+09	0.00011	0.00017	0.00720	0.00094	
1	0.053	1.03E+07	1.54E+09	0.00015	0.00024	0.00714	0.00087	0.00006
2	0.072	1.39E+07	2.09E+09	0.00021	0.00032	0.00705	0.00079	0.00009
3	0.098	1.90E+07	2.84E+09	0.00028	0.00044	0.00694	0.00067	0.00012
4	0.134	2.58E+07	3.84E+09	0.00038	0.00059	0.00678	0.00052	0.00015
5	0.182	3.50E+07	5.22E+09	0.00052	0.00080	0.00657	0.00031	0.00021
6	0.247	4.76E+07	7.10E+09	0.00071	0.00109	0.00628	0.00002	0.00029
7	0.335	6.47E+07	7.38E+09	0.00074	0.00114	0.00624	0.00003	0.00004
8	0.456	8.80E+07	1.00E+10	0.00100	0.00154	0.00583	0.00043	0.00041
9	0.620	1.20E+08	1.36E+10	0.00136	0.00210	0.00528	0.00099	0.00055
10	0.843	1.63E+08	1.85E+10	0.00185	0.00285	0.00452	0.00174	0.00075
11	1.146	2.21E+08	2.52E+10	0.00252	0.00388	0.00350	0.00277	0.00103
12	1.558	3.01E+08	3.43E+10	0.00343	0.00527	0.00210	0.00416	0.00139
13	2.117	4.09E+08	4.66E+10	0.00466	0.00717	0.00021	0.00606	0.00190

Appendices for Chapter 6

Table 8.10. Bacterial strains and plasmids used in this study.

Strain/Plasmid	Relevant characteristics	Reference or source
Bacterium		
<i>Labrenzia</i> sp. strain 5N	Wild-type	[1]
	pBBR1MCS-5-gfp	This study
<i>Escherichia coli</i> MC1061	Host for pRK24, pRL542 and pBBR1MCS-5 derived constructs; used as a donor in conjugations	[2]
Plasmids		
pGFP	<i>gfp</i> , <i>Plac</i> , Amp ^r	
pBBR1MCS-5	<i>Plac</i> , <i>lacZα</i> , Gm ^r , pBBR1 Rep, pBBR1 oriV	[5]
pBBR1MCS-5-gfp	<i>gfp</i> under control of <i>Plac</i> , <i>lacZα</i> , Gm ^r , pBBR1 Rep, pBBR1 oriV, 5536 bp	This study
pRK24	Tc ^r , Amp ^r ; conjugal plasmid	[3]
pRL542	Cm ^r ; helper plasmid, carries <i>mob</i> genes	[4]
pCR2.1-TOPO	Kan ^r , Amp ^r ; PCR product cloning vector	Invitrogen

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