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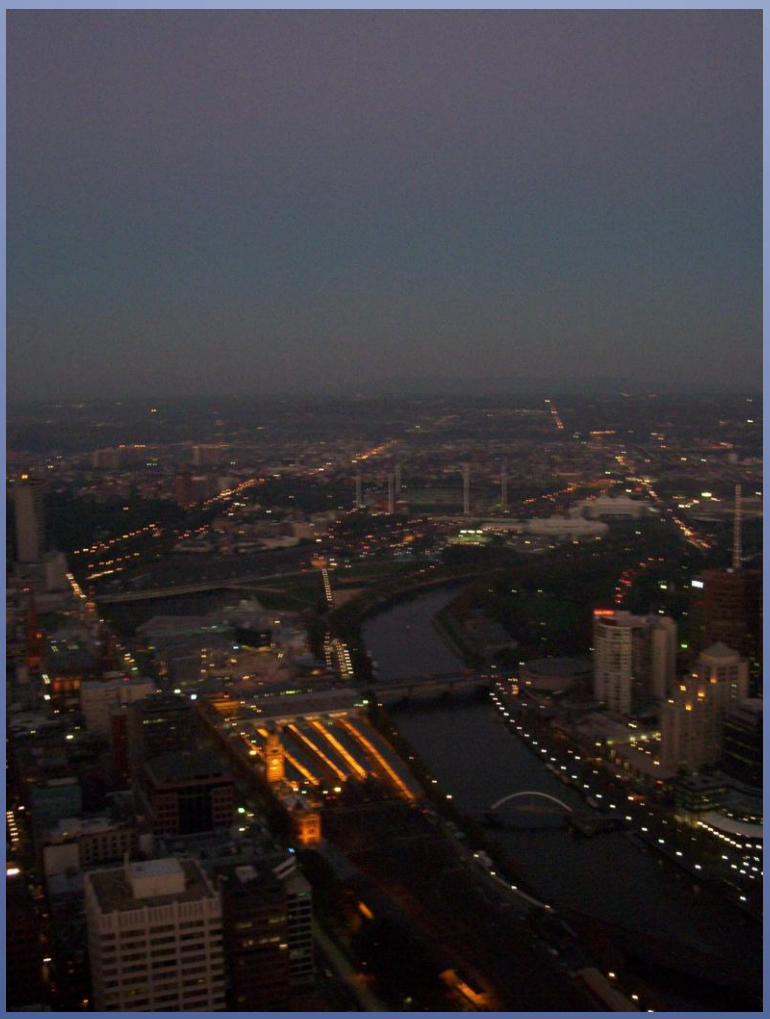

MICROBIAL COMMUNITY STRUCTURE & FUNCTION

The response of denitrifiers on an environmental gradient – stream urbanization.

Shane Elliott Perryman

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Cover Image: View of the Melbourne CBD and Yarra River looking east to the Dandenong Ranges at sunset. All sites used in this thesis were located between these two end points. The picture was taken from the Rialto Towers by the author in 2003.

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The response of denitrifiers on an environmental gradient – stream urbanization.

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WATER STUDIES CENTRE (WSC) AND SCHOOL OF CHEMISTRY

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A thesis submitted according to the requirements for the Degree of Doctor of Philosophy at Monash University, Victoria, Australia.

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Notice 1

ACKNOWLEDGMENTS

To my parents and family, who never tired of asking when this thing would be finished, but whose faith and support has been a great comfort.

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To Dr. Chris Walsh for access to the background data on the sites used in this research and for his advice on the advantages of ordination methods for examining community change.

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To my wife Sulfikar for her faith and patience – now hurry up and finish your thesis!

To prednisone and the sulfa class of medicines.

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PART A: GENERAL DECLARATION

Monash University
Monash Research Graduate School

Declaration for thesis based or partially based on conjointly published or unpublished work

General Declaration

In accordance with Monash University Doctorate Regulation 17/ Doctor of Philosophy and Master of Philosophy (MPhil) regulations the following declarations are made:

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes **2** original papers published in peer reviewed journals and **0** unpublished publications. The core theme of the thesis is the ***structure and function of denitrifying bacteria in relation to environmental variables.*** The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the candidate, working within the **School of Chemistry** under the supervision of:

Dr Mike Grace,

Prof Barry Hart,

Dr Gavin Rees.

In the case of **Chapter 2** my contribution to the work involved the following:

Thesis chapter	Publication title	Publication status	Nature and extent of candidate's contribution
2	**Ordination and significance testing of microbial community composition derived from terminal restriction fragment length polymorphisms: application of multivariate statistics.** *Antonie van Leeuwenhoek* 86: 339–347, 2004. Gavin N. Rees, Darren S. Baldwin, Garth O. Watson, Shane Perryman and Daryl L. Nielsen	**published**	Selection and discussion of the statistical method and data analysis. **20%**
2	**Analysis of denitrifying communities in streams from an urban and non-urban catchment.** *Aquatic Ecology*. Vol. 42, no. 1, pp. 95-101. Mar. 2008. Shane E. Perryman, Gavin N. Rees, Christopher J. Walsh	**published**	Primary author of the above article. Sample collection and analysis; data analysis. **80%**

I have renumbered sections of submitted or published papers in order to generate a consistent presentation within the thesis.

Signed: [redacted]

Date: 28/11/09

ABSTRACT.

Denitrification is an important step in the global nitrogen cycle, removing fixed bioavailable nitrogen from aquatic systems and returning it to the atmosphere. Understanding the factors that influence the rate of denitrification is an important management goal in streams receiving high inputs of nitrogen: especially those that discharge to sensitive receiving waters. While many studies have examined the role of physical and chemical parameters on rates of denitrification, fewer studies have explored the effects that these factors have on the distribution of the denitrifying community responsible.

Variation in the community structure of denitrifying bacteria was explored at sites influenced by the effects of urbanization. Variables were at two scales; those describing the sediment and stream, and those describing the catchment geography. Using multivariate analyses, significant correlations were found between micro habitat and catchment scale variables, suggesting that catchment scale processes modified by urban development play a part in structuring the dentrifying community.

'Texture' was an important variable at the micro scale that described the community structure. However, texture was correlated with other factors at multiple scales and these correlations are discussed in the context of the gradient of urban influences. The amount of community variance explained by this set of correlated variables was less than 20% indicating that other factors were also important.

The effect of sediment carbon composition was explored using multivariate decomposition of spectra recorded using Fourier Transform Infra Red (FT-IR) and Excitation Emission Matrices (EEM). Principal Components Analysis reduced the number of variables from these spectra to a few factors. Using the scores from the reduced factors demonstrated that carbon composition of the sediments also had a significant structuring effect on the denitrifying community.

Links between the denitrifying community structure and *function* were explored in a multiway ANOVA manipulation of sediments collected from streams heavily impacted

and lightly impacted by urbanization. Sediments were dosed with carbon substrates thought to mimic either an urban or non urban source, and changes in the response of CO_2, CH_4 and N_2 monitored for one month. Additionally, sediments were treated with the heavy metal zinc, found in higher concentrations in the urban sediments. The result of these manipulations suggested that community function was, depending on the treatment, significantly altered but that stream community structure was not: function was not limited by lack of diversity – the community was functionally adaptable.

This research contributes to a growing body of knowledge on the factors affecting the community structure of microorganisms and suggests several variables that should be consistently included in examinations of 'wild' bacterial communities. If futures studies consistently incorporated an agreed set of basic or core variables, the field of microbial ecology would benefit by making cross comparison and meta-analysis of related studies more practical.

ADDENDUM.

This is an electronic version of an original thesis with the same title and author housed within the Monash University Archive.

In the original thesis 5 pages were left to accomodate errors and changes in the text during the review process. These changes have been incorporated into this document and no longer appear as addenda. This has altered the pagination slightly.

The Table of Contents has been updated to incorporate these changes and does not match the original printed document.

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CHAPTER 1

NITROGEN, THE NITROGEN CYCLE AND THE ROLE OF BACTERIA.

OVERVIEW.

This introduction begins with a very broad historical account of nitrogen, its biological significance and since its discovery in the 18th century, the important role it has played in the evolution of human affairs. The purpose of this quick historical sojourn is to highlight the level of humanities dependence on nitrogen to maintain food supplies. This is followed by a discussion of the substantial human changes to the nitrogen cycle before a discussion of the role of bacteria in nitrogen cycling in aquatic environments. The diversity of the enzymes required to process nitrogen is discussed and the possible relationships between genes, enzymes, ecological function and habitat is briefly explored in the context of species competition and resource gradients. Project aims and objectives are then described and the thesis structure is provided at the end of the chapter.

1.1 INTRODUCTION: NITROGEN AND HUMANITY.

Nitrogen: a short historical 'big picture'.

Nitrogen is one of a core suite of elements that are absolutely essential to life. It plays a pivotal role as a building of block of the nucleotides of DNA and RNA, amino acids and thus ultimately proteins (Williams 1997, Wackett et al. 2004, Williams 2007, Falkowski and Godfrey 2008). Until a micro-organism evolved a mechanism to make atmospheric nitrogen biologically available, nitrogen supply was limited to abiological processes, principally lightning (Kasting and Siefert 2001, Navarro-Gonzalez et al. 2001). While the exact timing of this epochal event is still debated (Mancinelli and McKay 1988, Leigh 2000, Kasting and Siefert 2001, Navarro-Gonzalez et al. 2001, Anbar and Knoll 2002, Fennel et al. 2005, Glass et al. 2009) current evidence suggests that it is a biochemical process that has evolved only once (Mancinelli and McKay 1988, Leigh 2000, Anbar and Knoll 2002, Berman-Frank et al. 2003, Glass et al. 2009) Until the development of the Haber-Bosch synthesis of ammonia , biological fixation was the dominant nitrogen fixing

pathway (Smil 1997, Frink et al. 1999, Smil 2002, Galloway et al. 2003, Schlesinger 2009) .

Compounds containing nitrogen had been used by Alchemists for some time, and Saltpeter[1] was long an established commodity product, when nitrogen itself was discovered around 1770 by Daniel Rutherford (1749 – 1819)[2] (Muir 1913, Wisniak 2000, Dennis 2003). This led to Lavoisier's (1743 – 1794) 'oxygen theory of combustion' and the end of the 'phlogiston theory of combustion'[3] and ushered in the 'modern age' of chemistry (Muir 1913, Lamprecht 2003, McClellan and Dorn 2006). Due perhaps to the nature of some of the experiments involved in its discovery (i.e. suffocating animals) one of the original names for nitrogen, Azote, means lifeless (Smil 2004). It was not until the agricultural research of Liebig[4] (1803 – 1873) some half a century later that it was realized nitrogen was a key limiting nutrient for plant growth; the others being potassium and phosphorus (Martin 1991, van der Ploeg et al. 1999, Pollock 2007). The success of Liebigs 'mineral nutrition' ideas has led in many agricultural sectors to the gradual abandonment of the practice of fallowing and the recycling of organic matter. This has lead to greater dependence on the application of mineral fertilizers (Crews and Peoples 2004, Manlay et al. 2007). Organic materials had previously been recycled from towns back to nearby farms (Manlay et al. 2007, Pollock 2007).

As stated above, nitrogen is an essential component of living systems. Human nutrition requires the consumption of about 1 g protein per kg body weight to make up for excretion (as urea) and losses from hair and skin (Smil 2002). This amounts to approximately 2 kg N yr^{-1} as protein per person (Galloway and Cowling 2002). By the late 19th century concern was raised that the expanding populations of Europe, and to a lesser extent North America, were rapidly running out of fertile land to continue to feed themselves (Bryce 1918, Smil 2002, Foster and Clark 2004, Webb 2008). Realizing that the 'Chilean'[5] nitrate supplies would last no more than 30 years, and in the absence of

[1] Essential for the production of gunpowder. The name means "salt of/from rock": Peter means rock.
[2] But also Carl Wilhelm Scheele, Henry Cavendish, and Joseph Priestley at about the same time.
[3] That combustion of matter was due to the release of the substance phlogiston, thought to be contained within all substances.
[4] Though credit must also go to Carl Sprengel (1787–1859) (van der Ploeg et al. 1999)
[5] Rivalries between Bolivia, Peru (her ally) and Chile led to the 'War of the Pacific' (1879–1883) over the nitrate deposits of the Atacama Desert (Barnum 2003, Foster and Clark 2004, Mc Cray 2005, Bucheli 2007). Backed by British capital, Chile seized control of the Pacific coastal province of Bolivia.

'new lands'on which to expand the production of wheat, Sir William Crookes (1832 – 1919) argued that the only means of increasing production and avoiding this Malthusian scenario[1] was by finding a way of fixing nitrogen synthetically else "*[a]ll England and all civilized nations stand in deadly peril of not having enough to eat*" (Bryce 1918, Galloway and Cowling 2002, Rees et al. 2005).

It is thought that biological nitrogen fixation arose in response to a 'crisis' or shortage in the supply of available nitrogen (Navarro-Gonzalez et al. 2001, Fennel et al. 2005) – especially given the kinetic requirements and the complexity of the reaction (i.e. breaking the triply bonded N_2 and attaching three atoms of hydrogen to each N). In an event somewhat analogous to the evolution of biochemical nitrogen fixation, a small team of chemists led by Fritz Haber (1868 – 1934) and Carl Bosch (1874 – 1940) managed to create a practical means of fixing atmospheric nitrogen in 1909 (Smil 1999, Rees et al. 2005). Interestingly the metal iron features in catalysts used by both biological fixation and industrial fixation (Maxwell 2005, Rees et al. 2005)[2]. However, where biological fixation occurs at room temperatures and atmospheric pressures, the method of Haber and Bosch requires ~200 atm and 300 - 550°C (Maxwell 2005). Despite these technical barriers, humanity has managed to double the rate at which fixed nitrogen is entering the terrestrial environment in just 100 years. This has been achieved through the consumption of natural gas (or other fossil fuel), which is used to provide some of the energy for the process, and the source of the necessary hydrogen (Maxwell 2005, Bhattacharjee 2006).

The Haber-Bosch process has had far reaching consequences. In the short term it allowed Germany to prosecute WWI without recourse to foreign (and blockaded) sources of (chiefly 'Chilean') nitrate – required for the production of explosives (Pollock 2007). In the medium term it bankrupted the Chilean Government (already heavily indebted to her British creditors) by collapsing the price for nitrate (Barnum 2003, Foster and Clark 2004). But perhaps most significantly, the doubling of the worlds available nitrogen supply is arguably the prime reason global population has been able to expand

[1] "…that population, when unchecked, increased in a geometrical ratio, and subsistence for man in an arithmetical ratio." (Malthus 1798)
[2] Biological fixation also uses Molybdenum and Vanadium (Zehr et al. 2003, Rees et al. 2005).

exponentially for the last 100 years from ~1.5 billion at the turn of the century to over 6 billion now (Smil 1997, Vitousek et al. 1997a, Smil 1999, Galloway and Cowling 2002).

Anthropogenic changes to the nitrogen cycle.

Many recent reviews, papers and books have amply described the central role that nitrogen plays in the biosphere with particular emphasis on recent human induced perturbations (Smil 1997, Vitousek et al. 1997a, Vitousek et al. 1997b, Bouwman and Booij 1998, Frink et al. 1999, Socolow 1999, Asner et al. 2001, Rabouille et al. 2001, Galloway and Cowling 2002, Smil 2002, Vitousek et al. 2002b, Galloway et al. 2003, Townsend et al. 2003, Smil 2004, Schlesinger 2009). Table 1.1 summarizes the findings of many of these reviews – the list of effects and authors is not exhaustive. Figure 1.1 (below) shows the relative sizes of nitrogen reservoirs in present Terrestrial, Aquatic and Atmospheric environments. The single largest reservoir is atmospheric N_2: the stable triple bond of this molecule making it relatively un-reactive (Galloway and Cowling 2002, Berman-Frank et al. 2003, Maxwell 2005). In the terrestrial environment the largest pool of nitrogen is dead organic matter, or the humus, upon which a large proportion of living plants draw their nutrition (Wolf and Snyder 2003, Manlay et al. 2007). As a single species, people represent a small but significant pool of nitrogen. But coupled with the fact that nearly half of the animal biomass and half of planetary primary production have been appropriated for humanity – this small pool has had a major influence on rates of nitrogen fluxes in the terrestrial sphere (Vitousek et al. 1986).

The effect that industrial nitrogen fixation has had on terrestrial processes is shown in Figure 1.2. The single largest change in nitrogen flux is the ~85 – 130 Tg N yr^{-1} due to industrial fixation that did not exist prior to 1880. This has increased the rate of denitrification from ~110 to 200 Tg N yr^{-1} and along with changes in agricultural practice altered the way that nitrogen flows through the terrestrial ecosystem (Socolow 1999, Galloway et al. 2003). The centrality of nitrogen to life's processes, the scale of the perturbations and the multiple pathways that nitrogen can flow through the environment have led to these changes being dubbed "*The Nitrogen Cascade*" (Galloway et al. 2003).

Table 1.1 Some environmental effects of increased anthropogenic nitrogen loads.

Consequence of excess nitrogen	Review
Nitrogen-based compounds can contribute to the acidity of arable soils.	Smil 1997 Vitousek et al. 1997a, 1997b, Frink et al. 1999, Socolow 1999, Asner et al. 2001, Galloway and Cowling 2002.
Acidification can lead to increased loss of trace nutrients and cations.	Smil 1997,Vitousek et al. 1997a, Socolow 1999, Asner et al. 2001, Galloway and Cowling 2002.
High nitrate levels in groundwater. WHO guideline for safe drinking water =10 ppm. This standard is often exceeded. Can lead to methemoglobinemia and other diseases.	Smil 1997, Frink et al. 1999, Socolow 1999, Galloway and Cowling 2002, Townsend et al. 2003.
Fertilizer nitrogen escaping to ponds, lakes or ocean bays causes eutrophication, and hypoxia.	Smil 1997 Vitousek et al. 1997b, Frink et al. 1999, Socolow 1999, Rabouille et al. 2001, Galloway and Cowling 2002, Galloway et al. 2003, Townsend et al. 2003.
Changes in the composition and functioning of aquatic ecosystems.	Vitousek et al. 1997a, 1997b, Galloway and Cowling 2002.
Accelerated losses of biological diversity. Especially plants adapted to efficient use of nitrogen. Losses of animals and microorganisms that depend on these plants.	Vitousek et al. 1997a, Frink et al. 1999, Galloway and Cowling 2002, Galloway et al. 2003.
Nitrous oxide production. Destruction of ozone in the stratosphere. Atmospheric Lifetime > 100 years. Absorbs roughly 200 times more radiation than carbon dioxide per molecule.	Smil 1997, Vitousek et al. 1997a, 1997b, Frink et al. 1999, Socolow 1999, Galloway and Cowling 2002, Galloway et al. 2003.
Increased NOx leads to production of tropospheric smog, ozone and aerosols that induce serious respiratory illness, cancer, and cardiac disease in humans.	Socolow 1999, Galloway et al. 2003, Townsend et al. 2003.
"Human reliance on nitrogen fertilizer must further increase in order to feed the additional billions of people yet to be born before the global population finally levels off."	Smil 1997.

The causes and effects of these changes are perhaps best summarized by Socolow (1999).

> *The food system dominates anthropogenic disruption of the nitrogen cycle by generating excess fixed nitrogen. Excess fixed nitrogen, in various guises, augments the greenhouse effect, diminishes stratospheric ozone, promotes smog, contaminates drinking water, acidifies rain, eutrophies bays and estuaries, and stresses ecosystems. Yet, to date, regulatory efforts to limit these disruptions largely ignore the food system.*

There are many parallels between food and energy. Food is to nitrogen as energy is to carbon. Nitrogen fertilizer is analogous to fossil fuel. Organic agriculture and agricultural biotechnology play roles analogous to renewable energy and nuclear power in political discourse. Nutrition research resembles energy end-use analysis. Meat is the electricity of food.

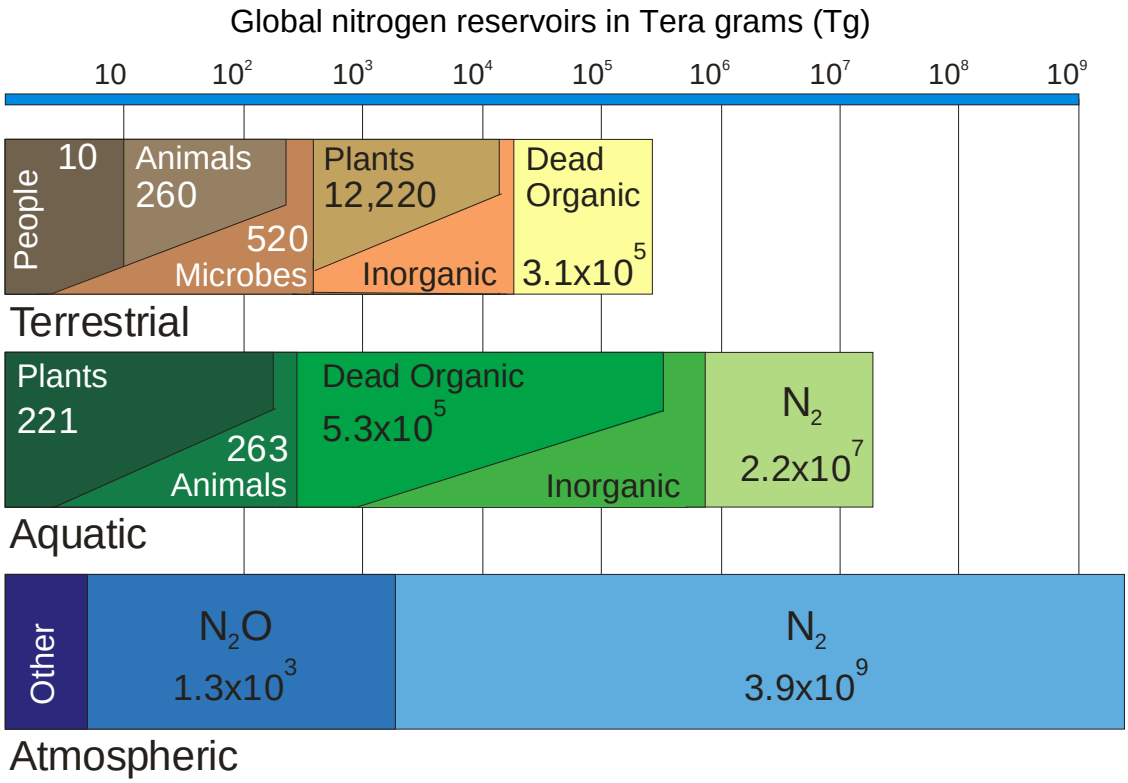

Figure 1.1 The relative size of nitrogen reservoirs in the terrestrial, aquatic and atmospheric environments, excluding the crust and mantle, in Tera grams N. Note log scale.[1]

From fixation to denitrification, the biological nitrogen cycle is tightly coupled to the carbon cycle (Vitousek et al. 1997a, Bernhardt and Likens 2002, Strauss and Lamberti 2002, Sobczak et al. 2003, Dodds et al. 2004, Lerman et al. 2004, Reich et al. 2006, Gruber and Galloway 2008, Thornton et al. 2009). The anthropogenic perturbation of the nitrogen cycle is no different, being tightly coupled to the consumption of fossil fuels – it resembles nothing less perhaps than heterotrophic fixation by bacteria[2]. In a similar vein, denitrification from agriculture is linked to the production of food (carbon). However, leakage from the human agricultural system has led in other ecosystems to localized

[1] Adapted from (Jaffe 2004)
[2] It would not be unreasonable to compare it to nitrogen fixation by methanotrophs (e.g. Murrell and Dalton 1983, Auman et al. 2001).

'decoupling' of the carbon and nitrogen cycles (Asner et al. 1997). Excess nitrogen can reduce the rate of natural nitrogen fixation, and nitrogen saturation leads to export, potentially causing eutrophication problems in receiving waters (see Table 1.1).

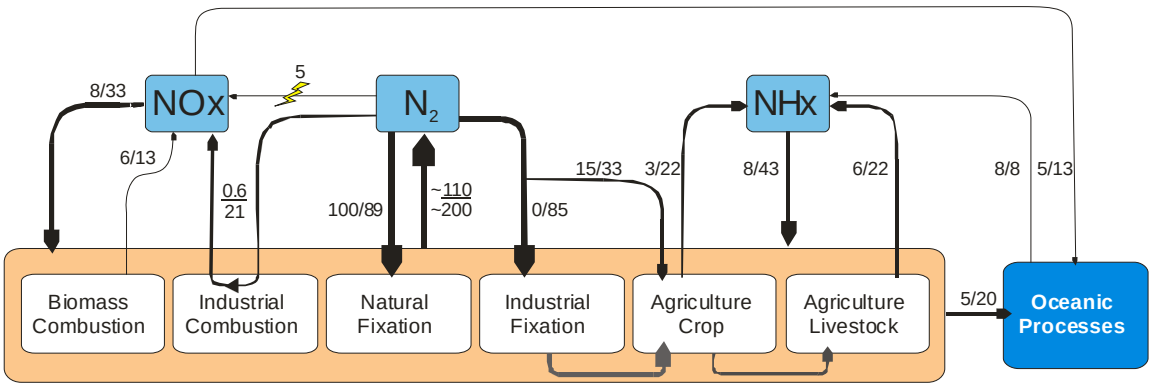

Figure 1.2 Changes in terrestrial nitrogen fluxes since the introduction of industrial fixation. Numbers for each flux are 'pre' (circa 1880) and 'post' (circa 1990) estimates of nitrogen fluxes in T g N yr^{-1}. Figures are from several sources and should be viewed as order of magnitude estimates only. Not shown are oceanic fixation and denitrification or atmospheric processes/gases other then NOx production from lightening.[1]

This link with carbon is particularly important in the context of global climate change. Population pressure (inset data in Figure 1.3) led to the increased consumption of nitrogen fertilizer (the 'Green Revolution') derived from fossil fuel. The increased population in turn has led to both an increase in energy consumption and further demand for nitrogen. This feedback loop has amplified the accumulation rate of the main greenhouse gas CO_2 (underway prior to the Haber-Bosch process) and led to the increase of the third[2] most significant greenhouse gas N_2O, a by-product of denitrification and nitrification (Hedin et al. 1998, Stein and Yung 2003).

The measured increase in atmospheric N_2O since 1900 (Figure 1.3) is one of the most obvious indicators of anthropogenic change to the nitrogen cycle (Stein and Yung 2003). This increase is primarily due to increased application of nitrogenous fertilizer to farmlands, but land use changes, increased use of biological nitrogen fixation (i.e. legumes) and combustion all play a part (Mosier et al. 1998, Stein and Yung 2003, Schlesinger 2009). Figure 1.3 A shows a 1000 year record of atmospheric N_2O concentrations together with the recorded global production of ammonia. Prior to the

[1] Adapted from Figure 3 Galloway and Cowling (2002) and Table 12-2 from Jaffe (2004)

[2] Not counting water.

introduction of the Haber-Bosch synthesis, N_2O concentrations did not exceed 290-300 parts per billion by volume (ppbv); longer ice core records suggest that these concentrations have been the natural upper limit for the last ~600,000 years (Sowers et al. 2003, Wolff and Spahni 2007). Since the 'Green Revolution', the N_2O concentration has risen by 12%[1], in line with fertilizer consumption (Figure 1.3 B) and is approaching 330 ppbv at an annual growth rate of ~0.6 ppbv y^{-1} (Khalil et al. 2002).

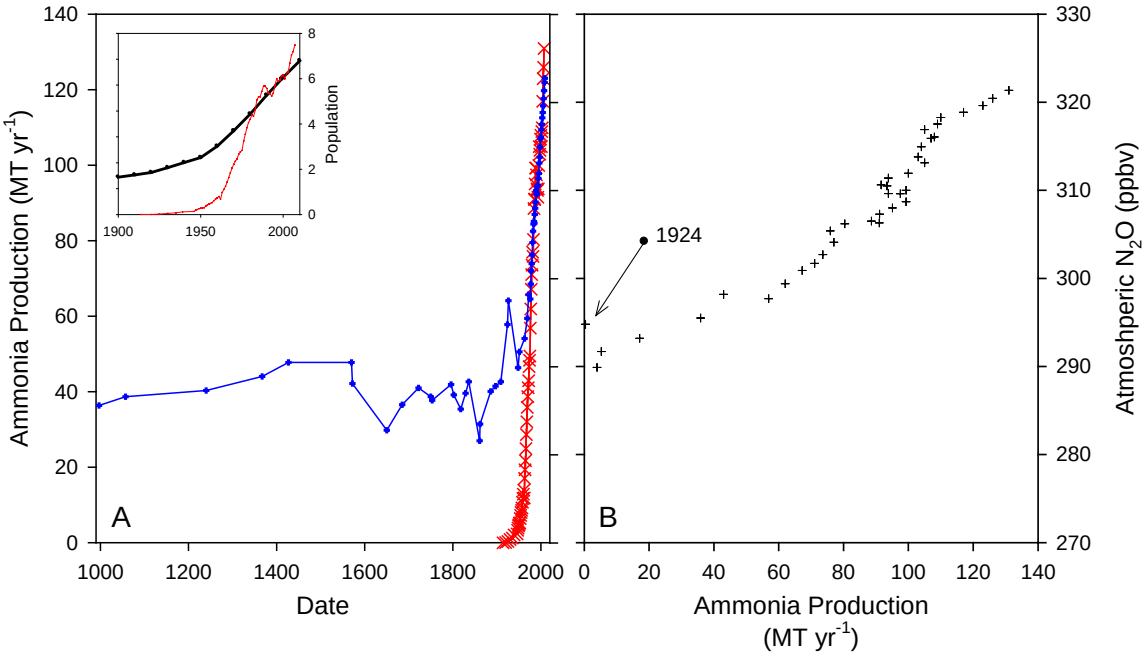

Figure 1.3 Changes in atmospheric nitrous oxide concentration (RHS), ammonia production (LHS) and population (inset). Panel **A)** shows global ammonia production (■) and atmospheric N_2O (■). Inset in **A)** shows ammonia production and global population (■) over the period 1900 – 2010). Panel **B)** indicates the relationship between ammonia production and global atmospheric N_2O concentrations since 1924.[2]

These changes are also a concern in Australia. Angus (2001) reported that nitrogen fertilizer use has grown by 14% annually in the 10 years up to 2000. Coupled with the finding that in many catchments the greatest input of nutrients to waterways is from diffuse sources associated with land use changes (Donnelly et al. 1998, Harris 2001), the potential exists for increased eutrophication of streams as a result of both hard to isolate sources and a direct increase in the load of nitrogen to the environment. A Land and Water Resources Research and Development Corporation (LWRRDC) sponsored

[1] To put this into perspective an increase of 12% represents ~160 Tg as nitrogen.
[2] N_2O data from Khalil (2002) and The World Meterological Organisation/World Data Centre for Greenhouse Gases (http://gaw.kishou.go.jp/wdcgg/ - Mauna Lao). Global ammonia production data from USGS (http://minerals.usgs.gov/ds/2005/140/) and Honti (1976). Population data from UN Population Division (http://www.un.org/esa/population/publications/sixbillion/sixbilpart1.pdf).

workshop on "*Sources, Transformations, Effects and Management of Nitrogen in Freshwater Ecosystems*" (Hart and Grace 2001) highlighted the difficulty that natural resource managers & researchers have in predicting the ecological effects of excess nitrogen in different waterways.

1.2 THE 'LITTLE PICTURE': NUTRIENT DYNAMICS, MICROBES AND GENES.

Nitrogen dynamics.

Studies into nitrogen dynamics and microbiological diversity are relatively advanced in the soil sciences (Angus 2001, Thies et al. 2001, Grandy et al. 2008), mainly in response to demand for a better understanding of processes in agricultural land and ultimately, improved nitrogen delivery to crops (Bøckman and Olfs 1998, Matson et al. 1998, Hayman and Alston 1999, Angus 2001, Brennan and Evans 2001, Wolfe 2001, Werner and Newton 2005). Studies in the aquatic realm, particularly flowing freshwaters, are still progressing (Howarth et al. 1988b, Hart and Grace 2001, Perakis and Hedin 2002, Rabalais 2002, van Breemen 2002, Lewis et al. 2007). Recent reports have highlighted the urgent need for a better understanding of both the 'source' of nutrients in waterways and the effect that this has on ecosystem function (Vitousek et al. 1997a, Carpenter et al. 1998, Donnelly et al. 1998, White and Kookana 1998, Boyer et al. 2002, Perakis and Hedin 2002, Vitousek et al. 2002b, Zehr and Ward 2002, Jarvie et al. 2008).

A simple conceptual model of the nitrogen cycle in the aquatic environment is presented in Figure 1.4. In this diagram, the major nitrogen processing pathways, directions and oxidation states of nitrogen in the various species are shown. The large pool of atmospheric nitrogen (Figure1.1), enters the biological cycle when it is 'fixed' by the action of bacteria as ammonium, which in turn is rapidly incorporated or assimilated into biomass. Under certain conditions ammonium is oxidized by bacteria via the process of nitrification[1]. Nitrate, the end product of this metabolic pathway, moves freely through the aquatic environment where it is either incorporated into biomass or reduced to nitrite and ultimately N_2. Because of its solubility, nitrogen in the form of nitrate has the potential to be rapidly removed from terrestrial aquatic environments potentially causing nitrogen limitation in headwater streams (Martin et al. 2001) or eutrophication in receiving waters (Smith et al. 1999, Alexander et al. 2000, Alexander et al. 2007).

[1] N_2O is a byproduct of this conversion.

Under reducing conditions (i.e. in the absence of oxygen) nitrate is reduced first to nitrite then nitrous oxide and ultimately back to nitrogen (Seitzinger 1988, Rysgaard et al. 1994). There are three major gaseous forms of nitrogen, namely nitrous oxide, ammonia and di-nitrogen. These chemical species form the major pathway by which nitrogen is redistributed globally.

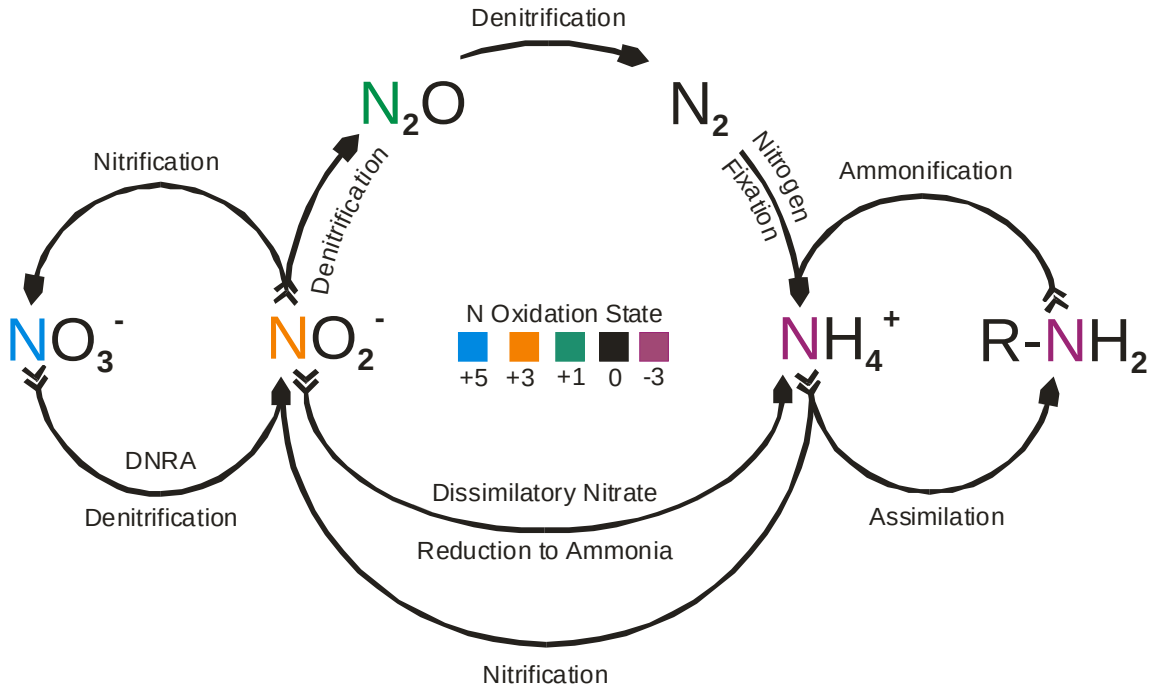

Figure 1.4 Simplified nitrogen cycle showing the major transformations and oxidation states[1]. R-NH_2 represents organic nitrogen; DNRA = Dissimilatory Nitrate Reduction to Ammonia.

The effect that the trophic status of an environment has on the nitrogen cycle is depicted in Figure 1.5. The size of each arrow is indicative of the relative rate of each process. The right hand diagram represents a hypothetical oligotrophic or low nutrient environment. Here, the main source of nitrogen is through the fixation of atmospheric nitrogen, which then becomes available to other organisms as it is processed through the various metabolic pathways. As the nutrient load within an environment increases it is said to become eutrophic. The process of nitrogen fixation by bacteria is energetically expensive. If available nitrogen species (e.g. NH_4^+ & NO_3^-) are present, bacteria preferentially utilize these species and nitrogen fixation stops (Merrick and Edwards 1995, Kessler et al. 2001). The scenario depicted in Figure 1.5 shows the probable effect of changes in nitrogen supply (in this case primarily as NH_4^+) on the nitrogen cycle which also depends intimately on the availability of carbon.

[1] Adapted from Zumft (1997), Richardson and Watmough (1999), Stolz and Basu (2002), Cabello (2004) and Jaffe (2004).

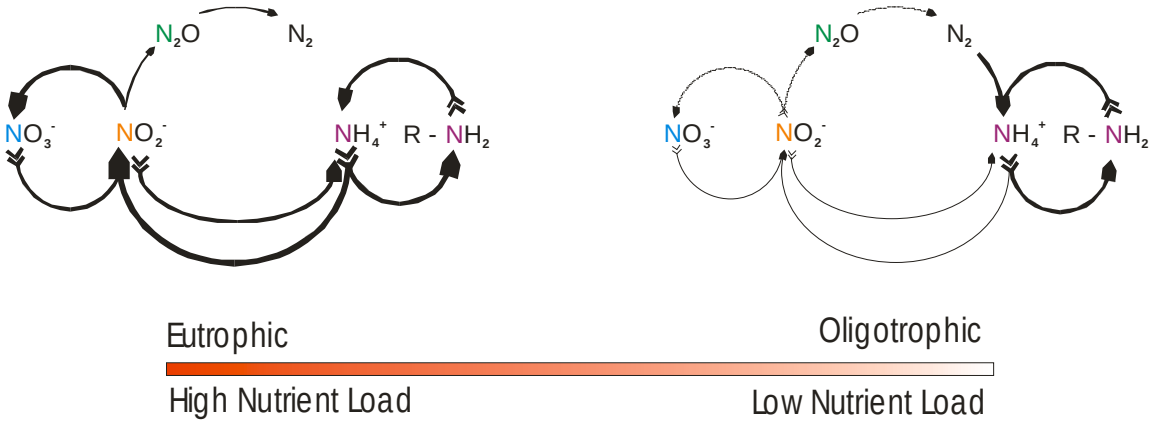

Figure 1.5 Changes in nitrogen cycle processes in response to changes in nitrogen loading. On the left an increase in NH_4^+ stops the process of fixation and potentially increases the rate of nitrification and denitrification. On the right, given sufficient energy, low NH_4^+ levels induce the process of fixation.

The nutrient status (i.e. eutrophic – oligotrophic) can be the result of a gradual *in situ* accumulation of nutrients, or as a result of increased loading to the environment as a result of anthropogenic activity. Individual steps (e.g. denitrification,) in the cycling of nitrogen are performed by a distinct flora/suite of micro organisms. Knowledge of the effect that different substrates (i.e. sources of nutrients; for example tree litter, food waste or fecal material) have on the different nitrogen pathways in the nitrogen cycle is lacking. Also, because different aquatic environments have different habitats relevant questions include; are the bacterial communities from different habitats the same or different, and do these habitats process the same substrate at the same rate?

<u>Diversity of nitrogen processing enzymes.</u>

Recent advances in our ability to extract DNA from soils and sediments (Steffan et al. 1988, Zhou et al. 1996, Yeates et al. 1997, Yeates et al. 1998) have increased the potential of genetic methods to assess environmental health by quickly and affordably measuring the community composition at the bacterial level. Genetic screening methods are preferred indicators of bacterial 'diversity' as it has been estimated that up to ~95% of soil and aquatic bacteria are un-culturable (Theron and Cloete 2000, Gray and Head 2001, Rappe and Giovannoni 2003, Riesenfeld et al. 2004). This is important as soils and the aquatic equivalent, sediments, are the site for a large proportion of the metabolic activity

of biodegradation and nutrient cycling (Nealson 1997, Boulton et al. 1998). These environments are complex and offer a wealth of microenvironments for different bacterial species to exploit (Torsvik and Øvreås 2002).

The transformations shown in Figure 1.4 are each catalyzed by a unique group of enzymes, in turn coded for by many and varied genes. The relationships between nitrogen processes and nitrogen genes for the simple nitrogen cycle of Figure 1.4 are shown in Table 1.2 and Figure 1.6, not including the many promoter, accessory and regulatory genes associated with each function (Merrick and Edwards 1995, Zumft 1997b). All microorganisms do not possess the complete set of genes shown in Table 1.2 and some organisms perform the same transformation using different sets of genes (e.g. some microbes use *nas* for nitrate assimilation whereas others use *nap*A and *nap*G : Table 1.2). Figure 1.6 shows the processes of denitrification and nitrogen fixation as a system of 'communicating' genes. Communicating is used here in the sense that the product of each enzyme acts as a signal for other genes (potentially located in different organisms) to be expressed or repressed.

Given that enzymes are not random strings of amino acids it follows that the genes encoding them are not random, although due to the degenerate nature of the genetic code, there is more variation in the gene sequence[1]. For each distinct enzymatic process, it also follows that there are critical operational regions in the enzyme that are nearly invariant. These regions appear in DNA as conserved (i.e. similar across species) segments of DNA. The ability to extract DNA from environmental samples coupled with primers targeting the conserved regions of genes coding for specific enzymatic pathways, enables the analysis of the diversity of specific functional groups (e.g. denitrifiers, methanogens etc) within the microbial community.

[1] E.g. in the universal genetic alphabet for microbes UCU, UCC, UCA and UCG all code for the amino acid Serine. The triplet base code is consistent but not 1:1. In this case it is 4 fold degenerate as there are four patterns that code for the same amino acid. Thus, where Serine is present in the protein of an enzyme, the corresponding DNA sequence could have been any one of these four codes.

Table 1.2 Key nitrogen transformations and some of the enzymes and associated genes responsible (not including promoter and regulatory genes)[1]. The gene group column shows the genes commonly studied and for which primers are available*.

Transformation		Enzyme	Gene Group
Nitrogen Fixation	$N_2 \rightarrow NH_3$	Nitrogenase	*nifH* (*nifHDK*)
Organic Nitrogen metabolism	$(NH_2)_2CO \rightarrow NH_3 + CO_2$	Eg: Urease. (but also proteases: peptidases, amidases etc)	*ure* (*ureABC*)
Nitrification	$NH_3 \rightarrow NH_2OH$	Ammonia mono-oxygenase	*amoA* (*amoCAB*)
	$NH_2OH \rightarrow NO_2$-	Hydroxylamine oxidoreductase	*hao*
	$NO_2^- \rightarrow NO_3^-$	Nitrite oxidoreductase	*nxr* (*nxrAB*)
Nitrate Assimilation	$NO_3^- \rightarrow NO_2^- \rightarrow NH_3$	Nitrate reductases	*nas* (*nasAB*) or *napA* (*napABC*) & *napG*
Dissimilatory Nitrate Reduction to Ammonia	$NO_3^- \rightarrow NO_2^- \rightarrow NH_3$	Nitrate reductases	*narG* (*narGHI*) & *nirB* or *napA* and *nrfA*
Nitrate and Nitrite respiration leading to Denitrification	$NO_3^- \rightarrow NO_2^-$	Nitrate reductase	*narG* or *napA*
	$NO_2^- \rightarrow NO_{(g)}$	Nitrite reductase	*nirS* or *nirK*
	$NO_{(g)} \rightarrow N_2O_{(g)}$	Nitric oxide reductase	*norB* (*norBC*) & *norZ*
	$N_2O_{(g)} \rightarrow N_{2(g)}$	Nitrous oxide reductase	*nosZ*

* Letters in brackets show gene clusters where the enzyme consists of several subunits. Urease is used as representative of the family of genes responsible for metabolizing organic nitrogen compounds.

Molecular screening of natural environments advanced when 'universal' primers for genes encoding ribosomal 16s rRNA[2] were applied to assessing microbial diversity (Woese and Fox 1977, Weisburg et al. 1991, Theron and Cloete 2000, Donachie et al. 2007a). Later, attempts were made to find specific primers for the 16s rRNA gene that targeted sub-groups of microorganisms (Prosser 2002). However, as the genetic lineage of 16s rRNA genes and other functional genes do not necessarily coincide, and it is not always possible to construct 16s rRNA gene primers that target specific functional groups[3], the last two decades have seen increasing use of primers targeting functional genes (Smith and Tiedje 1992, Ward 1996, Delorme et al. 2003, Wellington et al. 2003,

[1] Derived from Romero Lopez (1993), Merrick & Edwards (1995) Zumft (1997a), Sayavedra-Soto (1998), Richardson and Watmough (1999), Marusina (2001)Philippot (2002), Stolz and Basu (2002) and Butler and Richardson (2005).

[2] 16s rRNA is transcribed from DNA and folds to form the small sub unit of the Ribosome which synthesizes protein from RNA. It is present in all prokaryotes.

[3] This is true for the process of denitrification see Wellington et al. (2003)

Rosch et al. 2006). For a phylogenetic tree based on the 16s rRNA gene sequence and any other gene to be concordant, both genes must have a common line of descent. Horizontal gene transfer[1] of functional genes between 'species' can produce seemingly discordant trees – thus two organisms that appear phylogenetically similar using a metabolic gene sequence may appear phylogentically distant using the 16s rRNA gene sequence (and vice versa) (Jaspers and Overmann 2004, Beiko et al. 2005, Galtier 2007, Prosser et al. 2007).

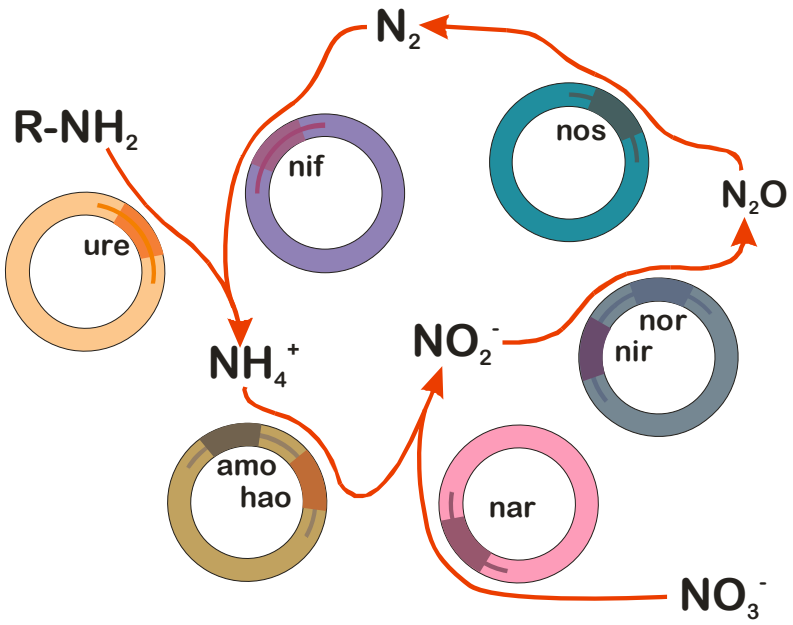

Figure 1.6 An alternative simplified representation of the nitrogen cycle as a system of interacting genes 'communicating' with each other as nitrogen species are transformed. This figure highlights the processes of denitrification and nitrogen fixation – nitrification is not shown. The separate circles represent hypothetical bacterial 'species'. In reality, microbial species posses more genes than are shown in this simple diagram (see Table 1.2).

Given that genes consist of conserved and variable segments and that different microbes possess different versions of the 'same' functional genes various methods have been devised to track changes in the diversity and structure of communities responsible for particular environmental functions (Liu et al. 1997, Hill et al. 2000, Ranjard et al. 2000, Theron and Cloete 2000). Due to the disturbances in the nitrogen cycle outlined above, two processes have received particular attention, nitrogen fixation and denitrification. Increases in anthropogenic nitrogen should (it is thought) lead to a decrease in biological fixation (e.g. Figure 1.2), and changes in the relative species diversity in aquatic environments by conferring an advantage to those species previously dependant on

[1] Any process by which genetic material is acquired from a non parent organism.

nitrogen fixing microorganisms (Howarth et al. 1988a, Vitousek et al. 2002a and references in Table 1.1). Denitrification has become a focus as researchers attempt to understand and perhaps optimize the mechanism of nitrate removal from aquatic environments – preventing eutrophication problems in downstream and receiving waters and to minimize the release of the greenhouse gas N_2O (Seitzinger 1988, Ward 1996, Zumft 1997a, Braker et al. 1998, Heggie et al. 1999, Inwood et al. 2005, Groffman et al. 2009 and many others throught this thesis).

Molecular methods for assessing the microbial diversity of environmental samples has led to the rapid accumulation of sequence fragments derived from environmental clones[1] (Rodríguez-Valera 2002, Rappe and Giovannoni 2003). The large majority of these sequences have come from the 16s rRNA gene and many researchers finish their analysis with a sequence based phylogenetic tree that places their "*unknown environmental clone*"[2] in clusters. Often, the only environmental context provided with these trees is the location or name of the soil or river, or maybe a brief description (e.g. 'sandy soil') and the inclusion of sequences from cultivated or 'known' species: the presumption being that organisms that cluster with these organisms must share some physiological characteristics. While these collections expand our knowledge of the 'diversity' of species and the potential evolutionary relationships – less insight is gained into the ecological function of species or the environmental pressures selecting for species.

1.3 Resource Competition, Resource Gradients and The Niche: The Ecological 'Big Picture'.

So far this chapter has provided a brief historical outline of the influence nitrogen, and its pursuit, has had on human affairs and the effects that anthropogenic influences have had on the natural nitrogen cycle – the "*why is nitrogen important?*" question. A brief outline of the aquatic nitrogen cycle and the importance of bacteria in driving the individual transformations was used to introduce the concept of the genetic diversity behind the enzymes that catalyze these processes – the "w*hy are bacteria important?*" question.

[1] The molecular process of cloning is used to extract and amplify DNA sequences from environmental samples. However, virtually all physiological information about the source organism is lost. This is inferred by comparing to known (i.e. cultured) phylogenetically related organisms.
[2] This is the frequently used phrase in the literature.

The combination of these themes leads to the question:

> *What are the environmental factors that select for the different denitrifying 'species' in the nitrogen cycle?*

The term 'species' is in quotes for two reasons;

1. the concept of the 'species' is still debated, especially when it comes to microbiota (Brower et al. 1996, Bengtsson 1998, Hey 2001, Cohan 2002, Prosser et al. 2007).
2. many molecular methods used to determine the 'diversity' or number of 'species' in a sample, such as the method employed in this research (Terminal Restriction Fragment Length Polymorphism: T-RFLP), do not really measure 'species' as such but rather some proxy indicator of the possible diversity, or present a fingerprint of changes in community structure (Marsh 1999, Kitts 2001, Forney et al. 2004, but see Donachie et al. 2007b for an interesting commentary).

For about a century the phrase "*Everything is everywhere—the environment selects*"[1] has been a maxim of microbial ecology (Fenchel 2005, Fierer and Jackson 2006). However this observation was based on the isolation of functional types of microorganisms based purely on selective media (Fenchel 2005). Modern molecular methods allow not only for expanding our appreciation of the genetic diversity of microbes, but to begin to probe the factors that determine the biogeographic distribution of microbes. This pursuit combines several of the important concepts at the heart of stream ecosystem theory namely, the biogeochemical cycling of elements (outlined briefly above), the 'niche' as the ecological 'space' of an organism, competition for resources, and the stability-diversity debate (Hutchinson 1957, Cummins 1974, Minshall 1988, Chapin et al. 1997, Tilman et al. 1997, Bengtsson 1998, Tilman 1999, McCann 2000, Begon et al. 2006). These ideas can be outlined with reference to Figure 1.7. The two axes represent resources required by the community of species. So long as the minimum resource requirement for all species is met (represented by the horizontal and vertical lines), at low (poor) 'concentrations' (**A** in Figure 1.7) it is thought that many species can coexist. Small variations or disturbances in the availability of resources

[1] This version of the concept was coined by Baas Becking in 1934.

maintain biotic diversity. This is represented by the overlapping semicircles near the origin – i.e. no single species dominates. The situation near the origin represents the case of a short environmental gradient, a point that becomes significant when considering the choice of statistical model to explore the effect of environmental variables on community structure (see Chapter 3).

At higher resource availability (**B**, Figure 1.7), theory suggests that the species that can utilize a potentially limiting resource the fastest should dominate. This is represented by the lower degree of overlap for the semicircles far from the origin: semicircle size represents abundance. At the extremes of the gradient a few species dominate – this is represented by the single semi circle at the ends of the gradient in Figure 1.7. In the extreme case (**C**), regions exist where single species dominate. This is best illustrated by the 'yellow species' in Figure 1.7. So long as the resource gradient remains stable, at this point in the resource gradient the yellow species is dominant as it can simultaneously use two resources more efficiently than either its neighbors, or the species that dominate the ends of the gradient. A key point here is that the gradient must have some degree of stability for the species distributions described by this theory to occur.

The simple two axis example is a simplification of the niche theory of Hutchinson (1957). The total niche space of an organism is made of all the resources (not just consumptive) required to describe the distribution of a 'species'. In a simple system (Figure 1.7) the habitat is not complex and the total number of niches is limited. As the habitat becomes more complex, the number of available niches increases, the niche space becomes hyper dimensional, and the potential for greater 'species' diversity increases. The potential also exists for multiple (partial) correlations between the axes representing the niche space potentially confounding interpretation of which variables are 'significant' drivers of community structure.

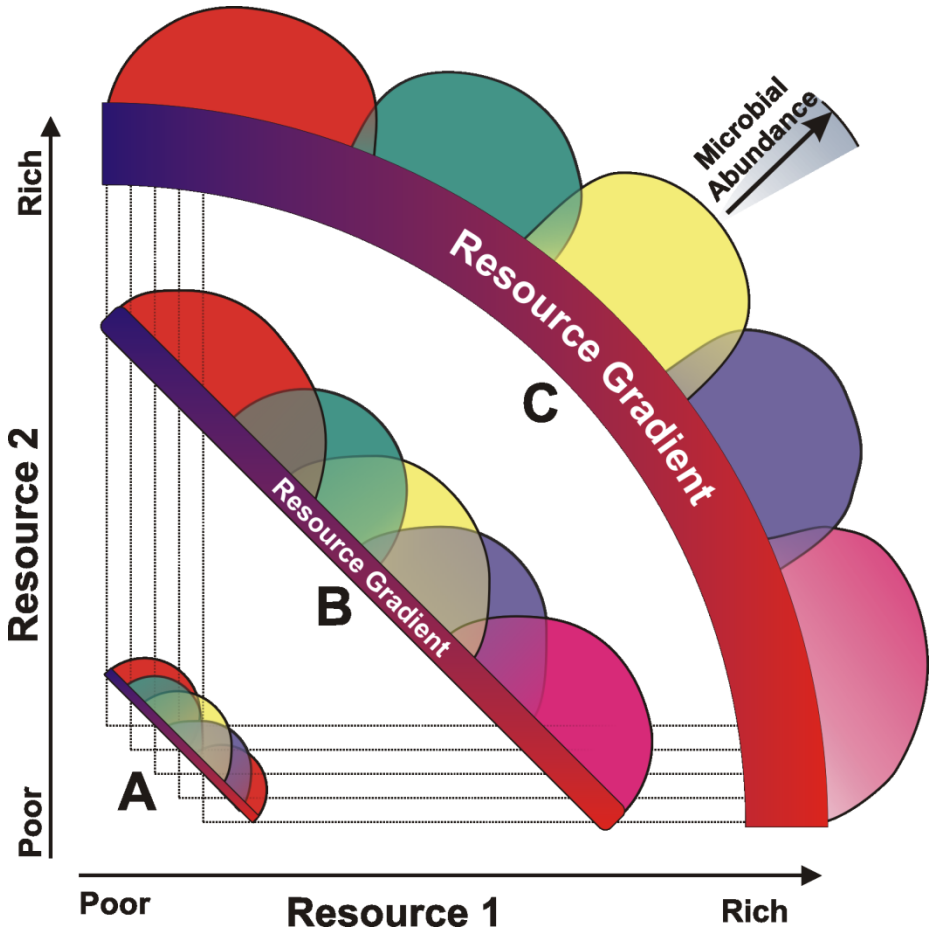

Figure 1.7 A resource gradient represented by two resources necessary for the growth of hypothetical microbial species (semicircles). At low resource availability no species is dominant and diversity is high – represented by the overlapping semicircles (**A**). At intermediate resource levels single species become dominant at the end members (**B**). In the extreme case single species dominate at intervals along the resource gradient (**C**).[1]

1.4 Research Rationale.

In light of the significant human alteration of the nitrogen cycle, nitrogen processing has become an important area of research, particularly in the urban setting where natural streams have been degraded due to the process of urbanization. In these streams understanding the factors that influence both the distribution and functioning of the denitrifying community are important if downstream systems are to be protected from enhanced nitrogen loading. Examining streams that have been progressively altered by the process of urbanization should help identify those factors that managers can control to enhance denitrification.

[1] Adapted from p425 Schulze (1994)

This thesis is an exploration of the methods and factors that explain the distribution or changes in the community structure of denitrifiers along an ecological gradient – the progressive urbanization of streams from a natural forested condition in the Australian city of Melbourne. The topics covered are multidisciplinary and I suspect that few readers have more than a general understanding in some areas. For this reason, the introductions to each chapter cover the material presented in some detail. This may be laborious to those familiar with the topic of that chapter: I apologize. To compensate each chapter has a very brief outline. The following summaries introduce the research questions explored and the links between each chapter.

1.5 THESIS STRUCTURE.

Chapter 1.

This chapter outlined the biotic importance of nitrogen in the environment and to humanity in general. A very short introduction to some of the geochemical (nutrient cycling), biochemical (nitrogen enzymes), bioinformatic (diversity of genes) and ecological (resource gradients) concepts behind the study of microbial communities followed. Hopefully this has set the scene for the more detailed exploration in subsequent chapters.

Chapter 2.

The hypotheses tested in this chapter are:

a. that the bacterial community structure of denitrifiers between two sites representing extremes in catchment urbanization is different, and
b. that the community structure between stream sediments and adjacent riparian soil is different.

Two methods used extensively in the thesis are introduced - one molecular and the other statistical. The main molecular method used throughout this thesis, Terminal Restriction Fragment Length Polymorphism (T-RFLP) is described before the manuscript "*Ordination and significance testing of microbial community composition derived from terminal restriction fragment length polymorphisms: application of multivariate statistics*" (Rees et al. 2004) is presented. This manuscript introduces microbial ecologists to robust multivariate tests (e.g. ANOSIM) commonly used by ecologists. This is followed by a second manuscript "*Analysis of denitrifying communities in streams from*

an urban and non-urban catchment" (Perryman et al. 2008) which reports the results of the two site comparison outlined above. Following these two manuscripts an initial attempt to use other multivariate techniques to relate the community structure to observed environmental variables is made.

Chapter 3.

The study in Chapter 2 showed denitrifying communities at two extremes of an urban gradient were different and that differences existed between riparian and in-stream sediments. The question then remaining was how denitrifiers would respond to an urban gradient. Chapter 3 begins with a description of the ecological changes that occur in streams due to land use changes resulting from urbanization. Across a range of sites differentially affected, these changes potentially form a gradient of significant ecological effects – and are the subject of much recent research. A review of the 'urban stream syndrome' literature is provided, and the rationale for using these sites for the research reported in this thesis.

This chapter resumes the exploration of the multivariate methods employed in Chapter 2 which provided suggestive but inconclusive results. A different technique is adopted to overcome the difficulties encountered in the multivariate analysis of Chapter 2. The constrained ordination method known as Canonical Correspondence Analysis (CCA) is introduced and later compared to Non Metric Multidimensional Scaling (NMDS) and Principle Components Analysis (PCA). CCA has only recently been applied to relate molecular data from environmentally isolated and amplified DNA samples with observed environmental variables. The chapter discusses the strengths and weaknesses of these three multivariate techniques and on the basis of the CCA results suggests possible variables responsible for structuring the microbial community. The study showed that there was a reasonable relationship between community structure and the urban gradient and that sediment composition, and possibly carbon, play important parts in structuring the community.

Chapter 4.

This chapter uses multivariate methods to qualitatively describe the type of carbon present in samples analyzed in Chapter 3 and to test for any relationship between the carbon 'type' and the microbial community structure. To do this two analytical

techniques were employed - Excitation Emission Matrices (EEM), a fluorescence method, and Fourier Transform Infra Red (FTIR) spectroscopy. Both techniques produce a lot of data that is susceptible to subjective interpretation. PCA was used to reduce the spectral data objectively to a set of factors that can be used to determine whether there are in fact qualitative differences in the type of carbon available to the sedimentary microbial communities along the gradient described in Chapter 3. CCA analysis indicated that community structure was significantly related to PCA Factors qualitatively describing the 'type' of carbon present along the gradient.

Chapter 5.

Preliminary observations from Chapter 2 and an examination of the literature indicated heavy metals can have a role in the ecology of urban streams. This is the first of two chapters that explore the effect of altering heavy metal concentration and carbon source on the sediment microbial community structure and function. The hypotheses for this research were:

a. that the bacterial community structures would converge when treated with a heavy metal, and
b. that when treated with a carbon substrate representative of an urban location, the non-urban bacterial community structure would shift to resemble an urban bacterial community and *vice versa*.

The chapter describes changes in the microbial communities from two sediment groups before and after a one month treatment with zinc, and carbon sources 'typical' of either an urban or non urban location. The community structure is examined using two genes: *nos*Z and the 16S rRNA gene. Comparisons between sediments for each treatment (carbon type, presence of zinc) for each gene are made using NMDS and ANOSIM.

Chapter 6.

The functional response of the microbial communities detailed in Chapter 5 is explored. The hypotheses for this research were:

a. that when exposed to a heavy metal typically encountered in urban sites, metabolic processes from non- urban site would be affected whereas those from the urban site would not, and

b. that when treated with a carbon substrate representative of an urban location, the metabolic processes of the bacterial community from the non-urban site would change to resemble an urban bacterial community and *vice versa*.

The results of headspace gas concentrations of nitrogen (N_2), methane (CH_4) and carbon dioxide (CO_2) are presented. Differences in the rates of gas production for each treatment were determined using ANOVA. Differences (or the lack thereof) are discussed and a synthesis of Chapters 5 and 6 is presented. The initial hypotheses for these chapters are reappraised in light of the results and the literature.

Chapter 7.

Final synthesis and conclusion.

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PART B: DECLARATION FOR THESIS CHAPTER

Monash University

Declaration for Thesis Chapter 2

Declaration by candidate

In the case of **Chapter 2**, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution
Ordination and significance testing of microbial community composition derived from terminal restriction fragment length polymorphisms: application of multivariate statistics. *Antonie van Leeuwenhoek* 86: 339–347, 2004. Gavin N. Rees, Darren S. Baldwin, Garth O. Watson, Shane Perryman and Daryl L. Nielsen Selection and discussion of the statistical method and data analysis.	**20%**
Analysis of denitrifying communities in streams from an urban and non-urban catchment. *Aquatic Ecology*. Vol. 42, no. 1, pp. 95-101. Mar. 2008. Shane E. Perryman, Gavin N. Rees, Christopher J. Walsh. Primary author of the above article. Sample collection and analysis, data analysis.	**60%**

The following co-authors contributed to the work. Co-authors who are students at Monash University must also indicate the extent of their contribution in percentage terms:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
Gavin N. Rees	Primary Author. Prepared Manuscript	**n/a**
Darren S. Baldwin	Co-worker, intellectual input, data analysis.	**n/a**
Garth O. Watson	Co-worker, intellectual input, data analysis.	**n/a**
Daryl L. Nielsen	Co-worker, intellectual input, data analysis.	**n/a**
Gavin N. Rees	Co-worker, intellectual input, data analysis.	**n/a**
Christopher J. Walsh	Co-worker, intellectual input, data analysis.	**n/a**

Candidate's Signature	[redacted]	**Date** **28/11/09**

Declaration by co-authors

The undersigned hereby certify that:

(1) the above declaration correctly reflects the nature and extent of the candidate's contribution to this work, and the nature of the contribution of each of the co-authors.
(2) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
(3) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
(4) there are no other authors of the publication according to these criteria;
(5) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and
(6) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

Location(s)	

[Please note that the location(s) must be institutional in nature, and should be indicated here as a department, centre or institute, with specific campus identification where relevant.]

Signature 1		**Date**
Signature 2		
Signature 3		
Signature 4		
Signature 5		

CHAPTER 2

COMMUNITY STRUCTURE VARIATION BETWEEN SITES WITH DIFFERENT HABITATS.

OVERVIEW.

A brief introduction to the Terminal Restriction Fragment Length Polymorphism (T-RFLP) method is provided followed by a demonstration of the technique in two papers. The first manuscript explores three ecologically different locations within the same stream. This paper details an ordination method commonly used in other ecological fields but less commonly used in microbiological ecology. The use of a Monte Carlo type significance test (ANOSIM) is also demonstrated. The second paper in this chapter expands on this and examines the community structure in two environments from sites representing either end of a disturbance gradient. The results from this study were used as an initial test of the methodology for examining the denitrifying communities as represented by the *nosZ* gene, which codes for a part of the enzyme responsible for converting nitrous oxide (N_2O) to di-nitrogen gas (N_2). The chapter closes with a preliminary investigation into methods linking community structure data to variables describing the environment that communities inhabited.

2.1 INTRODUCTION.

It has long been a goal of microbial ecologists to develop a more rigorous understanding of the nature and changes of the diverse microbial communities found in nature. Recent advances in culture methods aside (Joseph et al. 2003, Leadbetter 2003, Stevenson et al. 2004, Davis et al. 2005) it is recognized that culture based methods underestimate the total diversity of soil and sediment samples (Rappe and Giovannoni 2003, Riesenfeld et al. 2004, Rosch et al. 2006). A common remark in the literature is that 85-99% of the organisms from environmental samples are resistant to cultivation (Liu et al. 1997, Tiedje et al. 1999, Hill et al. 2000, Ranjard et al. 2000, Staley and Reysenbach 2002). More recently, the application of DNA based molecular methods to genomic material extracted from environmental samples has to some extent circumvented these problems by estimating the diversity of genes (Liu et al. 1997, Marsh 1999, Hill et al. 2000, Theron and Cloete 2000, Hughes et al. 2001, Norton et al. 2002, Prieme et al. 2002, Prosser 2002, Torsvik and Øvreås 2002, Zehr et al. 2003, Kirk et al. 2004, Case et al. 2007). Cloning of

templates and subsequent sequence analysis has been one of the most commonly used methods of estimating diversity (Purkhold et al. 2000, Hughes et al. 2001, Prieme et al. 2002, Rosch et al. 2002, Sekiguchi et al. 2002, Rappe and Giovannoni 2003). This approach relies on the construction of extensive clone libraries, from which the number of 'unique' sequences is determined and estimates of the diversity made on this basis. Obviously, this is a labour intensive approach.

Terminal Restriction Fragment Length Polymorphism (T-RFLP) is a DNA-based molecular technique (Avaniss-Aghajani et al. 1994, Liu et al. 1997, Marsh 1999, Osborn et al. 2000, Blackwood et al. 2003) that has found wide and increasing application to many environmental samples; activated sludge (Hiraishi et al. 2000), contaminated sediments (Flynn et al. 2000), the effect of transgenic crops on soils (Lukow et al. 2000) and components of different animal guts (Hongoh et al. 2006, Sait et al. 2003). In this method, genomic DNA extracted from a sample is used as a template for the polymerase chain reaction (PCR), in which at least one of the primers is labeled with a fluorescent dye. Primers are selected to target either particular microbial groups or physiological functions. Amplified products are cleaned and then digested with one or more restriction enzymes[1] and the sizes of the fluorescently labeled fragments determined. Traces, called electropherograms, typically exhibit a range of terminal fragment peak sizes of variable fluorescence intensity (Figure 2.1). To increase the number and distribution of peaks in the trace, restriction enzymes typically targeting a 4 base pair recognition site[2] are used as these sites are more likely to appear than more stringent (i.e. longer) recognition sites. The essential feature of T-RFLP is that it produces a unique but simplified pattern of fluorescent fragments that is a function of the sequence diversity, determined by the function and evolutionary history of the original template sequences. If the recognition site is located in a highly conserved region of the template sequences, or if a large number of the same organism were initially present in the sample, then a large number of fragments of the same length will result. In the T-RFLP trace this will appear as a peak with high fluorescence intensity. This means that information about the possible 'identity' of the organisms responsible for producing any particular peak is confounded, especially when using only one restriction enzyme. This seeming disadvantage, in the right context is actually the basis of the methods utility.

[1] Enzymes that cut DNA at specific target sites.
[2] A motif made up of a combination of the bases A,T,C or G

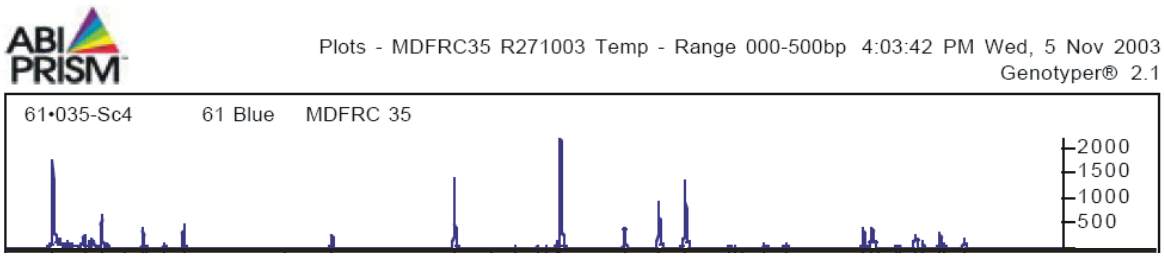

Figure 2.1 Example T-RFLP electropherogram or fingerprint for a sediment sample from Scotchmans Ck. The right axis is in arbitrary fluorescence units.

The T-RFLP method, by allowing a much more rapid estimate of sequence diversity in a single trace, lends itself to the examination of larger numbers of replicate environmental samples. Thus, although the phylogenetic potential of the information is lower, a measure of the overall bacterial community structure can still be made. By making repeated measurements of the community structure, either from different locations or by manipulating the environment, the factors that determine bacterial community structure can be investigated. As an assay of the general diversity of a particular habitat T-RFLP has been widely applied (see above) and in many studies has become the first step in determining whether or not particular environments show distinct patterns or changes in communities of organisms as defined by the targeting of particular genes. This initial screening may then be complemented by cloning and sequencing methods to provide more detailed phylogenetic information and to check that the material extracted and amplified is indeed homologous to the sequences from which the targeted primer set was constructed. This works as a check of the integrity of the method, especially for cases where the primer set may have been designed from relatively few sequences, and thus may not be targeting sufficiently conserved sites within the gene system of interest.

2.2 Manuscript 1 – Ordination and Significance Testing of Microbial Community Composition.

The following paper introduces some of the methods available for making valid statistical inference from data provided by T-RFLP using three spatially proximate but ecologically different sites where the community structure is represented by the 16s rRNA gene. This gene, which codes for a subunit of the ribosome (the structure responsible for

transcription in the cell) is present in all bacteria and archea and is widely used to depict

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Ordination and significance testing of microbial community composition derived from terminal restriction fragment length polymorphisms: application of multivariate statistics

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Key words: Microbial community analysis, Multivariate statistics, T-RFLP

Abstract

Terminal restriction fragment length polymorphism (T-RFLP) is increasingly being used to examine microbial community structure and accordingly, a range of approaches have been used to analyze data sets. A number of published reports have included data and results that were statistically flawed or lacked rigorous statistical testing. A range of simple, yet powerful techniques are available to examine community data, however their use is seldom, if ever, discussed in microbial literature. We describe an approach that overcomes some of the problems associated with analyzing community datasets and offer an approach that makes data interpretation simple and effective. The Bray-Curtis coefficient is suggested as an ideal coefficient to be used for the construction of similarity matrices. Its strengths include its ability to deal with data sets containing multiple blocks of zeros in a meaningful manner. Non-metric multi-dimensional scaling is described as a powerful, yet easily interpreted method to examine community patterns based on T-RFLP data. Importantly, we describe the use of significance testing of data sets to allow quantitative assessment of similarity, removing subjectivity in comparing complex data sets. Finally, we introduce a quantitative measure of sample dispersion and suggest its usefulness in describing site heterogeneity.

Abbreviations: T-RFLP – Terminal restriction fragment length polymorphism; *MDS* – non-metric multidimensional scaling; *ANOSIM* – analysis of similarity; *SIMPER* – similarity percentage

Introduction

Microbial ecologists have long strived to unravel the highly diverse microbial communities that exist in nature. Classical culture-based techniques to study microbiological communities have allowed microbiologists to identify many culturable organisms in given samples, but current application of culture techniques are recognized as having limited scope for studying microbial diversity in most environments. The application of DNA-based molecular tools has greatly enhanced understanding of microbial diversity as DNA techniques circumvent culturing problems by determining the sequence diversity in genes present within given samples. Terminal restriction fragment length polymorphism (T-RFLP) is a DNA-based molecular technique (Avaniss-Aghajani et al. 1994; Liu et al. 1997) that has been applied to activated sludge

the overall community structure. The page numbers have been changed.

(Hiraishi et al. 2000), anaerobic sediments (Lüdemann et al. 2000), contaminated sediments (Flynn et al. 2000), soils (Lucklow et al. 2000) and components of different animal guts (Gong et al. 2002; Sait et al. 2003). In this method, genomic DNA extracted from a sample is used as a template for the polymerase chain reaction (PCR), in which at least one of the primers is labeled with a fluorescent dye. Amplified products are digested with one or more restriction enzymes and the size of the terminal fragment, with the fluorescent label, is determined. Traces, termed electropherograms, typically exhibit a range of terminal fragments peak sizes of variable fluorescence intensity.

It is very important to chose an appropriate community analysis method for use with T-RFLP. The most simplistic approach has been to compare electropherograms of different samples and visually compare traces for the presence or absence of different peaks. Such an approach is valid, but lacks the benefits of a quantitative analysis. Many approaches have been used to analyze T-RFLP data sets quantitatively. Some of these include principal components analysis (PCA) (Clement et al. 1998), cluster analysis (Hiraishi et al. 2000; Urakawa et al. 2000; Sessitsch et al. 2002; Sait et al. 2003) and self organizing neural networks (Dollhopf et al. 2001). Unfortunately, approaches used for T-RFLP data analysis have not always made use of techniques that are ideally suited to this type of data (see below). It is apparent from our reading of the literature, the T-RFLP analysis requires a more standardized approach; in particular, one that has statistical rigor and that is easy to carry out.

Animal and plant ecologists examining community patterns have developed and applied a range of tools to analyze community structure (Lambshead 1986; Shiel 1990; Clarke 1993; – and references there in; Nielsen et al. 1997; Quinn and Keogh 2002). In particular, multivariate statistical methods have been extremely valuable in analyzing complex data sets. Multivariate techniques also have the advantage that datasets can comprise a variety of variables and need not be limited to species lists. In this paper we describe the rationale and application of several of multivariate statistical procedures to analyze T-RFLP data sets. To this end, we selected three sites along a 1km reach of an ephemeral stream and examined the bacterial communities in the sediment. The purpose in selecting this stream was that it provided us with a model system where we could sample three contrasting sediment types that occurred in close proximity. In our stream, differences in types of sediment, water depth and degree of impact from saline groundwater intrusions meant each of the three study sites differed in their physical and chemical characteristics. We describe the use of non-metric multidimensional scaling (MDS) as a simple method for preparing visual interpretations of sediment community data. Importantly, we also describe the use of the Analysis of Similarity (ANOSIM) procedure, which allows significance testing of our data groups. We used two exploratory tools to examine our data set. In the first instance, we used similarity percentage analysis (SIMPER) to explain aspects of the similarity between sites and to show that terminal fragments responsible for the differences between sites can be identified. In a further test, we calculated a dispersion index for our sites. Dispersion indices contrast the average rank of similarities between groups undergoing different treatments and until now, have only been used to describe perturbations on macroinvertebrate, coral and fish populations. In our study, we considered site heterogeneity, as indicated by physical features, as an indicator of perturbation to the community and examined within site data to see if heterogeneity was reflected in the degree of dispersion.

Materials and methods

Sample sites and sediment

The study site was a small ephemeral upland tributary of the Murrumbidgee River near Binalong, NSW, Australia (34° 40′ S, 148° 38′ E). Flow in the stream was highly variable and characterized by episodic rainfall events. As a consequence, the stream was a series of pools for much of the time. Although the pools were part of the same stream, the ephemeral nature of the stream, localized soil inputs from gully erosion and a groundwater salt intrusion meant the series of pools differed markedly from each other. Site 1 was approximately 15 m in length, up to 2 m wide and 50 cm deep and contained clear water. Sediments were a mixture of coarse sand and pebbles. Site 2 received fine sub soils from surface gully erosion. Sediments were fine ($<$ 1mm) and the water column contained a high suspended solid load with depth transparency less than 5cm. Water depth ranged from 0.5 to 0.75 m. Site 3 was a rocky stretch containing small riffles and pools up to 30cm deep. At

the time of sampling, flow through this site was predominately derived from ground water. Samples were taken from a range of bottom surfaces at site 3.

Ten sediment core samples were collected from each of three sites along the study stream. The top 2 cm of sediment of each of the cores were placed in sterile containers, placed immediately in freezers and stored frozen at −20 °C. In some instances, sediments at site 3 were less than 2 cm deep. In these cases, the maximum depth allowable was sampled. Analysis was carried out within 4 weeks of sampling.

Terminal restriction fragment length polymorphism

Immediately prior to analysis, the sediment cores were thawed and UltraClean Soil DNA Isolation Kits (Mo Bio Laboratories) were used to extract total community DNA from approximately 0.25 g wet sediment samples. Manufacturer's protocols were used throughout except cell disruption was by bead beating for 30 s at 2,500 rpm (Mini-beadbeater, Biospec Products). Genomic DNA quality and quantity was verified by agarose gel electrophoresis. The primer pair 27f (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492r (5'-ACGGCTACCTTGTTACGACTT-3' [Weisburg et al. 1991]) were used to amplify bacterial 16S rRNA genes, the forward primer being labeled with 5-carboxyfluorescein. The 50 μl PCR contained: PCR buffer, 2.5 U HotStar *Taq* polymerase (Qiagen), 0.8 μM of each primers, 200 μM of each dNTP, 1.5 mM $MgCl_2$ and template DNA. PCR was carried out in a Biorad thermal cycler with the following thermal profile: Hot Star*Taq* initialization at 95 ºC for 15 min, followed by 35 cycles involving denaturation at 94 ºC for 15 sec, primer annealing at 48 ºC for 15 sec and chain extension at 72 ºC for 1 min. Extension on the final cycle was for 6 min. Negative controls without template were always included in PCRs. Amplicon quality and quantity was determined in 1.0 % agarose electrophoresis gels stained with ethidium bromide. An UltraClean PCR Clean-up kit (Mo Bio Laboratories) was subsequently used to remove reactants from the PCR.

The purified PCR products were digested with *Hin*6 I (Geneworks, Australia) in 25μl reactions for 3 h at 37 ºC. The 25 μl digestion reactions contained between 80–100 ng of purified amplicon, 2.5 μl of incubation buffer (10 X), 3 U of endonuclease and sterile Milli Q water. Preliminary experiments showed 3 hours was sufficient to achieve optimal digestion. Post-digestion restriction fragments were precipitated with 2.5 μl of 5 M NaCl and 75 μl of 100 % ethanol, at −20 ºC overnight. Restriction digests were dried to remove ethanol. For final analysis, the dried pellets were resuspended in 10 μl water and 2.5 μl was added to 0.3 μl Genescan size standard (Applied Biosystems, Foster City, CA, USA), followed by 0.4 μl dextran Blue (50mg/mL in 25 mM Na-EDTA, pH=8), 1.8 μl formamide then denatured at 95 °C for 3 min. Final separation of restriction fragments was carried out with a 377 DNA sequencer (Applied Biosystems) at the Australian Genome Research Facility (Parkville, Victoria, Australia). The sizes and intensities of the terminal fragments (peak height and area) were calculated by Genotyper software (Applied Biosystems).

Data handling and statistical analysis

Raw data sets were generated that consisted of peaks that reflected the size of terminal fragments present, measured in base pairs, and the area and height of each peak, measured in fluorescence units. The Gene-scan software used in this study generally gave fragment sizes to 1/100th of a base pair. We developed a macro in Excel (Microsoft Corporation) that used a rounding function to convert peak sizes to the nearest integer value and then aligned all the peaks against the rounded sizes of the fragments. This 'automated' aligning macro had two purposes: (1) to check for subjective biases that might occur in the manual assignment of integer values for fragment length data when carrying out the aligning procedure manually and (2) to improve overall analysis time due to manual peak aligning. The macro also reorganized the data in a cross tabulated form to allow data importation into statistical analysis software. The crosstab macro was written by Dr C. Walsh and can be obtained from http://www.wsc.monash.edu.au/~cwalsh/treeflap.xls.

Data were standardized by calculating the area of each peak as a proportion of the total area. The data set was then manipulated by reassigning peaks with an area less than 1% of the total as zero and recalculating the proportion of each remaining peak. A second manipulation of the original dataset was then performed in a similar way that removed all peaks with an area less than 5%. A previous study has used a 5% threshold (Sait et al. 2003), who showed that a 5% threshold served to remove any bias caused by the amount of PCR product, while having a minor effect

on the overall area of the remaining T-RFs. The two resultant data sets were then imported into the multivariate statistical software package, Primer V5 (Primer-E Ltd, Plymouth, UK) and a similarity matrix was calculated using the Bray-Curtis coefficient as follows:

$$S_{jk} = 100\left\{1 - \frac{\sum_{i=1}^{n} |y_{ij} - y_{ik}|}{\sum_{i=1}^{n} (y_{ij} + y_{ik})}\right\}$$

where i is the peak function which is compared across j and k samples (Bray and Curtis 1957; Clarke 1993). For comparative purposes, some of our datasets were converted to presence/absence of peaks (binary data) and others used relative fluorescence (area) of peaks.

Non metric Multi Dimensional Scaling (MDS) was used to ordinate the similarity data. MDS uses an iterative algorithm that takes the multidimensional data of a similarity matrix and presents it in minimal dimensional space, typically two dimensions, although three dimensional plots can be employed to visualize group differences. In theory, the number of meaningful dimensions is only bound by the number of data points (n-1). The operator ultimately chooses the number of axes that they wish to produce. The result of MDS ordination is a map where the position of each sample is determined by its distance from all other points in the analysis. Since MDS ordination is an iterative algorithm that involves a 'goodness of fit' estimate, an important component of an MDS plot is a measure of the goodness of fit of the final plot. In the case of an MDS ordination, the latter is termed the 'stress' of the plot. A stress value greater than 0.2 indicates that the plot is close to random, stress less than 0.2 indicates a useful 2 dimensional picture and less than 0.1 corresponds to an ideal ordination with no real prospect of misinterpretation (Clarke 1993). For our analysis, stress was calculated as described by Kruskal (1964), within Primer. MDS ordinations are sensitive to the initial configurations chosen for each of the analyses. The ordinations in our analyses were all computed following 100 random restarts, with the best (final) configuration based on the number of times that a minimum stress was obtained over the course of the random restarts. An analysis of similarity (ANOSIM) routine was used to examine statistical significance between samples. ANOSIM tests the null hypothesis that the average rank similarity between objects within a group is the same as the average rank similarity between objects between groups. ANOSIM is based on the rank similarities between samples in the similarity matrix and produces a test statistic (R) which can range from -1 to 1. Objects that are more dissimilar between groups than within groups will be indicated by an R statistic greater than 0. An R value of 0 indicates the null hypothesis is true. A level of significance (p value) also is produced for the analysis. ANOSIM involves a test based on the permutations of all sites and replicate samples and is free from any assumptions of normality, but not from the assumption of equal within-group dispersion. For our T-RFLP data, we tested the null hypothesis that there was no difference in community composition between our three study sites. SIMPER analysis calculates the average contribution of individual terminal fragments to the average dissimilarity between samples. In our case, we examined the similarity of the 1% threshold data, comparing the presence/absence and relative abundance of fragments. Dispersion indices were also calculated in Primer to examine site heterogeneity. In essence, the dispersion index compares the average rank similarities across a given number of samples, with the average range derived from the underlying similarity matrix. The larger the value of the dispersion index indicates more within site heterogeneity (variability) compared to sites with smaller dispersion indices. The suite of multivariate analyses gave us rapid insight into the variability in microbial populations within and between different sediments.

Results

Data ordination

The traces in our data set contained between 16 and 44 terminal restriction fragments (T-RF) per sample. Manually and 'automatically' aligned data sets did not cause any clear shift in the resulting ordinations. Given the rapid nature of the automated process, compared with manually aligning data sets, we routinely used the 'automated' approach for all analyses and all results presented here are from 'automatically-aligned' data. In addition, we applied either 1% or 5% threshold to all our data, removing all peaks less than a given threshold. On average, approximately 3% of the total area was removed by applying a 1% threshold, with between 0 and 10 peaks removed in individual samples; average number of peaks removed across all samples was 5. The 5% threshold had a

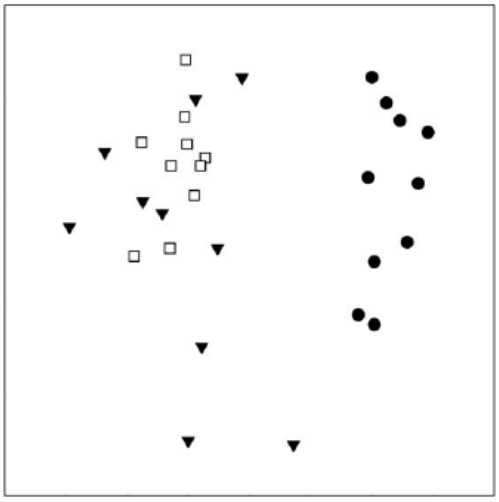

Figure 1. Multidimensional scaling plot based on Bray-Curtis similarities of the T-RFLP data consisting of the presences/absence of the terminal restriction fragments over a 1% threshold. Closed circles (site 1), open squares (site 2) and closed triangles (site 3) are unconnected sites of an ephemeral stream (stress of the plot = 0.16).

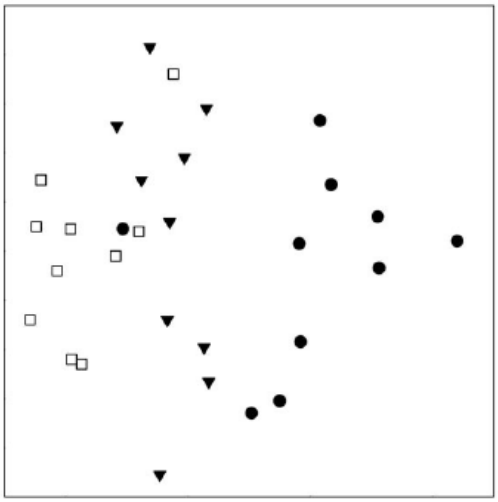

Figure 2. Multidimensional scaling plot based on Bray-Curtis similarities of the T-RFLP data consisting of the presences/absence of the terminal restriction fragments over a 5% threshold. Closed circles (site 1), open squares (site 2) and closed triangles (site 3) are unconnected sites of an ephemeral stream (stress of the plot = 0.13).

much more dramatic effect on the data set, with an average of 40% of the area removed, equating to 1 to 20 peaks being removed from individual samples. For our first analysis we selected a 1% peak threshold and transformed the data to presence and absence of T-RF (Figure 1). The 10 samples from sites 1 and 2 formed tight clusters that were separate from each other in two dimensional space. There was considerably more scatter in the data from site 3 and, represented in two dimensions, it appears to have some overlap with site 2. The integrity of the T-RF groupings was maintained after application of a 5% threshold to the relative fluorescence data although the MDS ordination plot indicates more scatter was apparent in site data (Figure 2). Applying a 5% threshold to our T-RF data generally resulted in sites having similar levels of scatter to each other but gave better visual separation of site 2 from site 3.

An MDS plot of site T-RFs based on 1% threshold and calculated with relative abundance of each peak, i.e., standardized peak areas, rather than the presence and absence of peaks, showed a greater divergence of the site data (Figure 3). Most notably, the T-RFs from sites 2 and 3 showed a greater difference between each other; the MDS map indicating that the sites have distinct microbial communities.

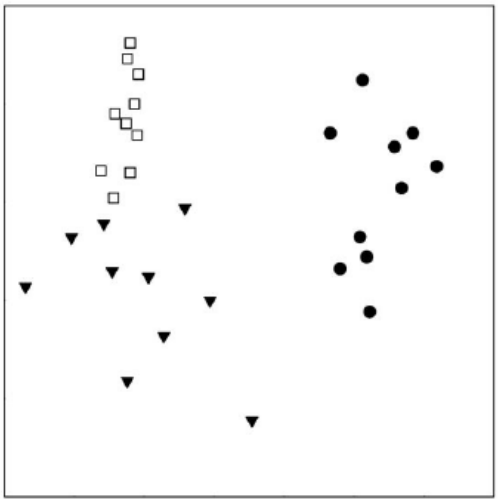

Figure 3. Multidimensional scaling plot based on Bray-Curtis similarities of the T-RFLP data consisting of the relative abundance of the terminal restriction fragments over a 1% threshold. Closed circles (site 1), open squares (site 2) and closed triangles (site 3) are unconnected sites of an ephemeral stream (stress of the plot = 0.14).

Analysis of similarity

The global R statistics from ANOSIM of the 1% and 5 % peak threshold were 0.708 and 0.630 respec-

Table 1. Analysis of similarity (ANOSIM) of the three sites following application of 1% and 5% thresholds. Similarity was calculated based on presence and absence for 1% and 5%, and relative abundance of the 1% threshold data. $p < 0.01$ in all cases for the pairwise comparisons

Site comparison	R statistic of 1% threshold data based on:		R statistic of 5 % threshold data based on:
	Presence/ absence	Relative abundance	Presence/ absence
Site1, Site 2	0.983	1	0.722
Site1, Site 3	0.837	0.902	0.644
Site 2, Site 3	0.317	0.538	0.568

tively, with $p < 0.01$, demonstrating that the overall difference between sites were large and statistically significant. Pairwise comparisons of the sites demonstrated a significant difference between sites 1 and 2 and 1 and 3 ($p < 0.01$ in both cases) when a 1 % threshold was employed (Table 1). The R value for the site 2 and 3 pairwise comparison was 0.317, indicating these sites were more similar to one another than the other two sites, however, p was less than 0.01, indicating the sites were significantly different. When data from the 5% threshold were analyzed, all the sites were significantly different from one another ($p < 0.01$). Furthermore, when the 1 % threshold data were analyzed by relative abundance of the peak areas, the sites also showed a high level of dissimilarity.

Similarity percentage analysis

SIMPER analysis showed similarities between sites were generally very low. When calculated on the presence/absence of peaks, pairwise similarities between sites 1 and 2, 1 and 3, and 2 and 3 were 23%, 21% and 39%. When analyses were based on the relative abundance of peaks, pairwise similarities between sites 1 and 2, 1 and 3 and 2 and 3 were 13%, 14% and 29%. Further analysis (relative abundance of peaks) showed that 5 T-RFs accounted for 75% of the average similarity between sites 2 and 3 and 12 fragments accounted for approximately 50% of the similarity.

Dispersion index

The dispersion indices calculated on presence/absence data with 1% threshold for sites 1, 2 and 3 were: 0.989, 0.571 and 1.44 respectively. Using the similarity matrix of the relative abundance of the 1% threshold data to calculate the dispersion index, the values for sites 1, 2, and 3 were: 0.946, 0.668 and 1.386 respectively. The pattern of dispersion remained the same after each of the treatments, but the actual values for each site did alter.

Discussion

The first step in analyzing T-RF data, often overlooked in the literature, is an appropriate method for aligning peaks. This is particularly important since peak size discrimination is to 1 base pair and has been highlighted in a recent study (Fierer et al. 2003). Manual alignment is time consuming and uses subjective judgment to decide if two T-RFs in different samples, with a size difference close to one base pair, truly reflect similar T-RF. Our rounding approach employing an Excel macro had no impact on the final ordination of our data and differences between sites remained significant, while eliminating possible subjective bias through manual aligning. Furthermore, the aligning procedure within Excel was significantly less time consuming than manual alignment.

In our data set, like others described in the literature, there were T-RFs that were jointly-absent in some samples, while present in other samples. Although often described in the literature, Euclidean coefficients are not an appropriate method to determine similarity within T-RFLP data sets as they do not adequately handle data containing blocks of double zeros, or joint absences of fragments (Legendre and Legendre 1988; Clarke 1993). Instead, we used a Bray-Curtis coefficient to examine similarity in our samples. The characteristics of the Bray Curtis coefficient that have led to its wide application in ecological studies are: identical samples have a similarity value of 100 % and samples with no shared species (or T-RFs in this study) have 0 % similarity, comparisons can be made with data sets not simply limited to species lists (as in this study), the inclusion or exclusion of species (or T-RFs) absent in two

samples does not change the similarity value and inclusion or exclusion of further samples does not change the similarity between other sample comparisons (Clarke and Warwick 2001). The Bray-Curtis coefficient has also been shown to offer a high level of power and robustness (Faith et al. 1991). Given these characteristics, the Bray-Curtis coefficient is ideally suited to T-RFLP data sets, whereas Euclidean methods are not. The Sorensen, or Dice, coefficient has also been described in microbial community analysis (Eichner et al. 1999). It is worth noting that when the Bray-Curtis coefficient is used with presence/absence data, it becomes identical to the Sorensen coefficient, which uses presence/absence data by definition.

A range of techniques are available to visualize similarity data sets. Cluster analysis has been the most popular analytical tool. The dichotomous nature of cluster analysis tends to constrain interpretation of data, with comparisons of groups that occur in different branches not necessarily intuitive. Data transformation, followed by ordination using PCA has been used to show distances in community patterns (Legendre and Gallagher 2001). However, the application of PCA has not been widely accepted as a good tool for T-RFLP analysis. PCA is known to suffer if data is not normally distributed. Logarithmic transformation of data can sometimes overcome this problem but it is still doubtful whether a simple log transformation overcomes the statistical violations in this analysis (Dollhopf et al. 2001). Non-linear data along large gradients can lead to arching in PCA ordinations ('horseshoe effect') which can obscure underlying patterns (Quinn and Keogh 2002).

MDS ordination is conceptually simple; the distance of a given sample from all others, based on values generated in a similarity matrix, determines the position of each point on an MDS map. An MDS ordination does not suffer from the problems associated with PCA, is robust when examining non-linear data along large gradients, nor does it suffer from the shortcomings or difficulties in interpreting results from cluster analysis. MDS has been reported in the analysis of microbial community data derived through denaturing gradient gel electrophoresis (DGGE) studies (van Hannen et al. 1999; Díez et al. 2001), however, its use as an ordination technique in microbial ecology are rare. We have shown that MDS ordination can be applied to sediment data and that the patterns described by our analysis are robust. Although not changing the overall outcome of our results, subjecting our data to different degrees of transformation gave us greater insight into our results. When data is transformed to presence and absence, a small peak has the same impact on the analysis as a large peak. In other words, the role of rarer species in the community data analysis can be highlighted. This compares with the relative abundance data where larger common peaks will dominate the degree of similarity. Differences were seen in the relative abundance and presence/absence MDS plots. In particular, the tighter clustering of site 2 and 3 data in the relative abundance MDS plot (Figure 3), compared with the presence/absence MDS (Figure 10) show that there was some similarity in the actual peaks that were present (shown by some overlap in the presence/absence data), but that when the relative abundance was considered, the site groupings diverged. This is born out in the SIMPER analysis where the degree of similarity in sites 2 and 3 (presence/absence) was 39% and only 29 % similarity when considering the relative abundance of peaks. We showed that applying either a 1 % or 5% peak rejection lead to only minor changes in the ordination. ANOSIM showed the differences were significant in both cases. This result demonstrates the statistical robustness of our overall analysis.

We believe that the current lack of significance testing is a major failing of microbial community analysis and suggest that ANOSIM provides the analysis crucial to understanding ordination data. This is highlighted in Figure 1 where the analysis in two dimensions showed that site 1 appears quite separate, but there is an apparent overlap of the data from sites 2 and 3. ANOSIM showed that sites 2 and 3 are significantly different. This difference is further highlighted when ordination plots are examined in 3 dimensions (data not shown); sites 2 and 3 diverge on the z axis.

Warwick and Clarke (1993) have suggested that the amount of variability in community data may be a consequence of increased perturbation and that comparing dispersion indices can be a useful tool to evaluate environmental perturbations. To date, this type of analysis has mostly been restricted to environmental impacts on macrofauna such as invertebrates, corals and fish. In our study, site 3 was the most heterogeneous site, comprising a variety of shallow pools with a variety of biofilms on rock surfaces and bottom sediments; this site also had the highest dispersion index. Site 2 had fine sediments distributed throughout and had the least degree of

dispersion. It is noteworthy however, that the dispersion index, calculated from a similarity matrix, will depend on the degree of transformation made prior to calculating similarity. Comparison of dispersion indices does suffer from lack of a statistical framework and significance testing is not possible. Although the later caution should be applied to its use, comparison of dispersion indices may prove to be valuable in contaminant or ecotoxicological studies involving microbial populations.

In this paper, we have demonstrated the value of using MDS plots of Bray-Curtis similarity matrices and ANOSIM as primary tools for the analysis of T-RFLP data sets. Data interrogation using tools such as SIMPER and dispersion measures allowed further ecological interpretations to be made. Removal of peaks under a given threshold remains an integral part of T-RFLP analysis. We showed that application of a 1% threshold to our data proved to be a very acceptable level of peak filtering that yielded a large number of peaks that withstood the important statistical rigor required for community analysis. We would suggest a 1% threshold as a good starting point for further analysis. The techniques outlined in this paper do not suffer from many of the assumptions and requirements that exist in a range of commonly-applied statistical procedures. The techniques were robust and given the statistical rigor and validity of our methods, we recommend their wider use in the statistical analysis of microbial population dynamics. Data sets from denaturing gradient gel electrophoresis (DGGE) studies would be one such case where these procedures would be suitable for treatment as we have described. Having demonstrated that differences can be shown unequivocally, it is now possible for microbial ecologists to start exploring mechanisms that may be responsible for differences in microbial populations.

Acknowledgements

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2.3 Manuscript 2 - Exploring The Possible Effects of Catchment Urbanization on Denitrifier Community Structure.

The following manuscript details results from a two site comparison of streams that have measurable differences in levels of catchment urbanization (see Figure 3.0 in Chapter 3 for the location of these sites with respect to the city of Melbourne). These sites were used as a test to determine whether the denitrifying community was different between sites and also whether there were differences between ecologically different locations within each site. The paper discusses the likely determinants of the observed differences. The page numbers have been changed.

An expanded examination of the results follows the manuscript.

Aquat Ecol
DOI 10.1007/s10452-007-9085-3

ORIGINAL PAPER

Analysis of denitrifying communities in streams from an urban and non-urban catchment

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Abstract Urbanization leads to degradation in water quality and has a major effect on the biota of streams, but its effect on microbial communities is not as well understood. DNA-techniques that target functional genes are being used to examine microbial communities, but less frequently applied to freshwater aquatic systems. Our aim was to determine whether terminal restriction fragment length polymorphism and sequence analysis of polymerase chain reaction-amplified (PCR) *nosZ* gene sequences could be used to show if there were measurable differences in the denitrifying community in two urban streams in catchments with contrasting degrees of catchment urbanization. Community structure in the sediments and associated riparian zones were studied at the contrasting sites. We showed that the denitrifying community in the sediments and riparian soils of the two streams were significantly different. There were also significant differences between the sediment and riparian zone communities within each of the sites. Terminal restriction fragment length polymorphism analysis proved to be a valuable technique that could resolve patterns of the denitrifying community in streams of contrasting degrees of urbanization, but sequence analysis was required to confirm the identity of the amplified products.

Keywords Community structure · Denitrification · *nosZ* · Sediment bacteria · T-RFLP

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Introduction

Urbanization has had a major impact on ecosystem structures and functions (Grimm et al. 2000). Large areas of impervious surfaces and high levels of hydraulic connection of impervious surfaces to streams, through stormwater pipes or drains, are two characteristics of urban environments that can lead to major changes in urban streams (Walsh et al. 2005b). These characteristics cause changes in streams through the combined effects of decreased levels of evapotranspiration and infiltration and rapid delivery of waters to streams.

Increased delivery of nitrogen to rivers and streams is a major consequence of human

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activities in landscapes (Seitzinger 1988; Peierls et al. 1991), however, aspects of nitrogen delivery and metabolism in urban systems have only recently been examined (Zhu et al. 2004; Groffman et al. 2005). Nitrate is a key nutrient in urban aquatic systems (Groffman et al. 2005; Grimm et al. 2005), introduced either through direct input of nitrate to streams, or produced within streams through nitrification. Subsequent denitrification can occur in sediments or biofilms, where anoxic conditions result in a switch from aerobic respiration to denitrification, thus removing nitrogen from the system. The critical role played by riparian zones in nitrogen cycling is well recognized (Hedin et al. 1998; Martin et al. 1999; Bardgett et al. 2001) and similarly, urban riparian zones may also be an important site for the transformation of nitrogen compounds.

The advent of DNA-based techniques has lead to significant advances in our understanding of nitrogen transformations. Terminal restriction fragment length polymorphism (T-RFLP) is one such DNA-based technique (Avaniss-Aghajani et al. 1994) and has been used to examine community structure by targetting genes coding for denitrifying enzymes (Braker et al. 1998; Scala and Kerkhof 1998, 2000; Prieme et al. 2002; Rösch et al. 2002). Relatively few studies have examined denitrifying communities in freshwater aquatic ecosystems, particularly urban streams, leading to our limited understanding of the microbial dynamics in such systems.

To address this knowledge gap, we targetted a segment of the *nosZ* gene and tested whether two different DNA-techniques would be able to resolve any potential differences in the denitrifying community of two urban streams. To this end, we selected two streams identified in earlier studies as representing the extremities of an urban gradient (Hatt et al. 2004; Taylor et al. 2004; Walsh et al. 2005a). We tested whether T-RFLP and sequence analysis of cloned DNA fragments could demonstrate whether denitrifying communities differed between the urban and non-urbanized streams. In addition, we compared the communities between sediments and riparian zones within the sites.

Materials and methods

Brushy Creek is located in the outer suburbs of Melbourne, South Eastern Australia (37°46.90′, 145°18.30′), approximately 30 km east of the city centre. Brushy Creek is a highly modified incised stream with a clay bed, over which patchy deposits of gravel, sand, silt and debris collect. The 1,479 ha catchment upstream of the sampling location was 22% impervious, dominated by low to medium density housing (Hatt et al. 2004; Taylor et al. 2004). Of the total impervious surface, 89% was directly connected to Brushy Creek by piped municipal drains.

Lyrebird Creek rises in the Dandenong Ranges 37 km to the east of Melbourne (37°49.82′, 145°23.82′). The sampling site was primarily within a messmate (*Eucalyptus obliqua*) forest, with some Manna Gums (*E. viminalis*) and an understorey of ferns (*Blechnum* sp.) and tree ferns (*Dicksonia* sp.) close to the stream. The stream sediment consisted of sand and silt deposits over a stream bed dominated by cobbles. The 724 ha catchment upstream of the sampling location has 0.1% impervious area not directly connected to the stream (Hatt et al. 2004; Taylor et al. 2004).

The average water quality in each stream was derived from a data set of water quality variables, obtained on a fortnightly basis from the year prior, up to and including the day that samples were collected for microbial community analysis (Table 1). These data represent a subset of an extensive dataset published elsewhere and reflect the degree of urbanization in the two study sites (Hatt et al. 2004; Taylor et al. 2004).

Eight samples from the top 5–10 cm of sediment were collected along an ~20 m section of each stream during September 2002 and transferred to pre-weighed 50 ml centrifuge tubes. Eight samples from the adjacent riparian areas were collected from the top 5–10 cm of the soil profile. For our purpose, we defined riparian soil as un-inundated soil or sediment at the time of sampling (at base flow) at distances ranging from ~30 cm to ~10 m from the stream edge. Samples were frozen for later DNA analysis.

Detailed descriptions of the general methods for DNA extraction and T-RFLP procedures

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Table 1 Average annual water nutrient concentrations and benthic chlorophyll *a* concentrations in Brushy and Lyrebird Creeks in the year preceding microbial community analysis

Parameter[a]	Brushy Creek			Lyrebird Creek		
	Mean	SD	*n*	Mean	SD	*n*
TN (mg l^{-1})	2.5	1.5	190	1.0	0.4	66
NO_x (mg l^{-1})	0.65	0.36	198	0.37	0.12	64
NH_4^+ (mg l^{-1})	0.08	0.10	198	0.02	0.02	63
TP (mg l^{-1})	0.33	0.27	190	0.05	0.03	66
FRP (mg l^{-1})	0.03	0.02	198	0.004	0.002	64
TOC (mg l^{-1})	11	4	5	11	4	14
DOC (mg l^{-1})	7.2	2.9	23	4.2	2.1	50
EC (µS cm^{-1})	408	238	160	84.7	7.7	71
TSS (mg l^{-1})	208	237	188	55	32	72
Benthic chlorophyll *a* (mg m^{-2})	92	200	145	8	13	147

[a]TN—Total Nitrogen, NO_x—Nitrogen Oxides, NH_4^+—Ammonium, TP—Total Phosphorus, FRP—Free Reactive Phosphorus, TOC—Total Organic Carbon, DOC—Dissolved Organic Carbon, EC—Electrical Conductivity, TSS—Total Suspended Solids

have been described previously (Rees et al. 2004, 2006). Polymerase chain reaction (PCR) amplification of an approximately 780 base pair fragment of the *nosZ* gene was carried out in a final volume of 50 µl, using the HotStar *Taq*™ DNA polymerase kit and master mix described by the manufacturer (Qiagen, Clifton Hill, Australia), with the exception that 2 units of *Taq* polymerase were used per reaction. The forward primer *nosZ*f (5′-CGYTGTTCMTCGACAGCCAG-3′) and reverse primer *nosZ*-R (5′-CATGTGCA GNGCRTGGCAGAA-3′) were used for the PCR (Rösch et al. 2002). For T-RFLP analysis, the forward primer was labelled at the 5′ terminus with 6-carboxyfluorescein. Unlabelled forward primer was used for PCRs destined for sequence analysis.

PCR was carried out on a BioRad iCycler thermal cycler (Regents Park, Australia) with the following conditions: 95°C for 15 min to activate the HotStar*Taq*, denaturation at 94°C for 30 s, 35 cycles starting with a touchdown procedure beginning at 58°C for 40 s, decreasing in 0.5°C steps for 9 cycles until a final annealing temperature of 54°C, and extension at 72°C for 1 min 20 s—final extension was 72°C for 7 min. DNA extracted from *Pseudomonas denitrificans* (NCTC 10688) was used as the positive control and amplicon size was confirmed by agarose gel electrophoresis. Amplified samples were purified using the MoBio UltraClean™ PCR clean-up kit following the manufacturer's instructions (MoBio Laboratories, Solena, CA, USA).

A TOPO TA cloning kit (Invitrogen Life Technologies, CA, USA) was used to clone PCR products from *nosZ* PCRs into chemically competent, One Shot TOP10 *Escherichia coli* cells, using the manufacturer's instructions. Colonies that grew well on LB agar plate containing 100 µg ml^{-1} ampicillin were picked and PCR was used to screen clones for the correct sized insert. Clones containing an insert of the correct size were re-amplified using the primers supplied with the TOPO TA kit, purified and sequenced at the Australian Genomic Research Facility (Queensland, Australia).

Terminal restriction fragment length polymorphism data sets were analyzed as described previously (Rees et al. 2006). Raw data sets require a standardization procedure prior to statistical analysis (Dunbar et al. 2001; Sait et al. 2003) and accordingly, we adopted a constant percentage standardization (Sait et al. 2003; Rees et al. 2004), rejecting peaks that made up less than 0.25% of the total fluorescence in each sample. Similarity matrices were calculated using the Bray–Curtis coefficient based on the presence or absence of terminal fragment lengths. Differences between streams (Brushy and Lyrebird) and sediment/soil types (sediment and riparian) were tested using a 2-way crossed analysis of similarity (ANOSIM) (Clarke and Warwick 2001). Non-metric multi-dimensional scaling (MDS) plots with 20 random restarts were used for visual interpretation of community patterns (Clarke and Warwick 2001).

Sequences from the clone library were analyzed using the freeware program *MEGA* version 3.0 (Kumar et al. 2004). Sequences were submitted to the National Centre for Biotechnology Information online BLAST analysis (http://www.ncbi.nlm.nih.gov/BLAST/) to confirm sequence identity. Sequences confirmed to be

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nosZ were aligned with sequences from named organisms retrieved from the NCBI database and a ~760 bp segment common to all sequences was used for phylogenetic analysis, using the neighbour joining algorithm within the default settings of the *MEGA* software. The *nosZ* sequences obtained in this study have been submitted to GenBank under the accession numbers DQ324382 to DQ 324421.

Results

Cloned PCR products, ~780 base pairs (bp), were obtained from all the soil and sediments samples of the two study sites. Of the 50 cloned fragments that were sequenced, 40 were recognized as *nosZ* gene sequences and were used for phylogenetic analysis (Fig. 1). The *nosZ* clones were distributed across three broad clusters; 11 clones grouped

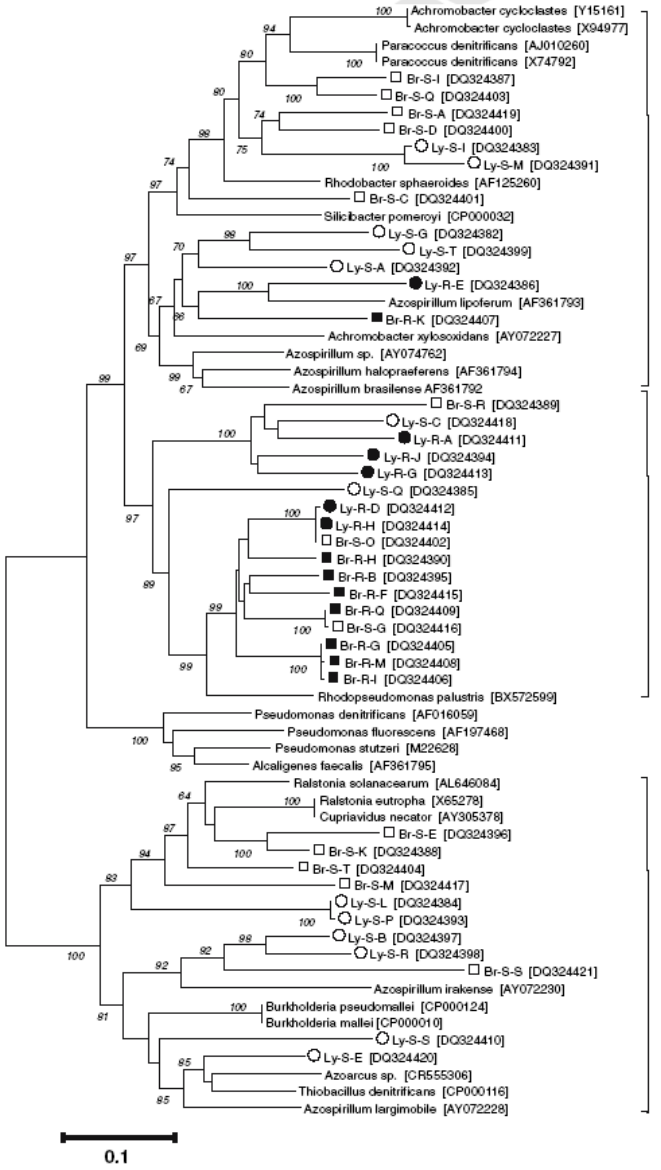

Fig. 1 Neighbour-joining tree for *nosZ*. Clones are indicated by their site (Br = Brushy Creek, Ly = Lyrebird Creek) and the sample type (S = sediment, R = riparian soil. The scale represents 10% nucleotide change and numbers at the nodes indicate bootstrap re-sampling based on 750 replicates

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within cluster 1, 17 clones in cluster 2 and 12 clones in cluster 3. Clones from both sites were present in cluster 1, but all the clones were derived from sediment samples. Six of the clones in cluster 1 aligned most closely to the *Cupriavidus* group. Of the 17 clones in cluster 2, 12 were derived from riparian soil and only 5 from sediments. Cluster 2 clones were also most dissimilar from other known (i.e. named) *nosZ* sequences available in databanks. In cluster 3, 10 clones were derived from sediment samples and only 2 from riparian soil. Five of the clones in cluster 3 could be assigned to the *Azospirillum/Achromobacter xylosoxidans* group and the reminder clustered within the taxonomically diverse group represented by *Paracoccus denitrificans* and *Silicibacter pomeroyi*.

A total of 114 terminal restriction fragments (T-RFs) were derived over all but one Lyrebird Creek sediment sample. Our normalizing manipulations of the T-RFLP raw data gave approximately 15–20 T-RFs for each sample. The MDS ordination (Fig. 2) shows good separation of the denitrifying community groups from the two streams and the two zones within each site (sediment and riparian soil). Notably, Brushy Creek samples appear on the right-hand side of the ordination, with sediments generally in the upper half and riparian soils in the lower half. Lyrebird Creek samples are predominantly on the left-hand side of the ordination, and like Brushy samples, the sediments generally are in the upper half of the ordination and riparian soils in the lower half of the ordination.

The denitrifying communities at the Brushy Creek and Lyrebird Creek sites were significantly different (ANOSIM, $R = 0.516$, $P < 0.001$). There were also significant differences between the riparian and sediment habitat types ($R = 0.754$, $P < 0.001$). Within sites, the difference between the sediment and riparian soil habitats was greater at Brushy Creek ($R = 0.648$, $P < 0.005$) than at Lyrebird Creek ($R = 0.378$, $P < 0.005$). Interestingly, the difference between riparian soil samples ($R = 0.625$, $P < 0.005$) was not as large as the difference between sediment samples ($R = 0.931$, $P < 0.005$).

The average percent (±SD) of sediment greater than 1 mm at Brushy and Lyrebird creeks were 23 (10)% and 6 (5)%, respectively. The average (±SD) of the sediment less than 355 μm were 17 (18)% and 58 (10)% at Brushy and Lyrebird, respectively. Mean (±SD) loss on ignition at Brushy and Lyrebird were 3(1)% and 8(2)%, respectively.

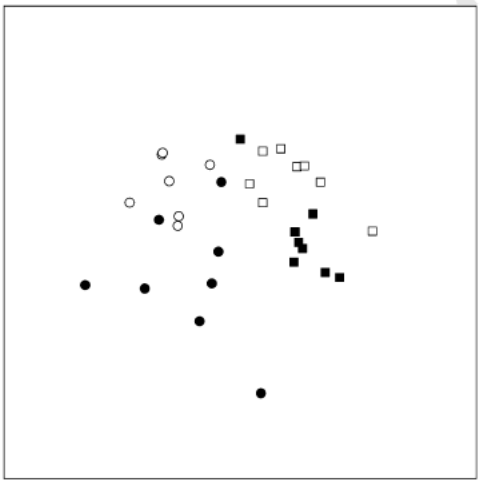

Fig. 2 Non-metric multi-dimensional scaling (MDS) plot of *nosZ* gene community structure, derived by terminal restriction fragment length polymorphisms, in Brushy Creek (*squares*), Lyrebird Creek (*circles*), sediments (*open symbols*) and riparian soil (*closed symbols*). Two Lyrebird sediment points are superimposed. The stress for this plot is 0.21

Discussion

Terminal restriction fragment length polymorphism and sequence analysis of clone fragments showed that the denitrifying community structure was measurably different in two urban streams with contrasting degrees of urban impact, at the time of sampling. Differences were also shown between the sediment and adjacent riparian areas within a given site. Both clone and T-RFLP analyses provided different insights into the denitrifying communities. First, sequence analysis of *nosZ* clones provided an important validation of our PCR reaction. It also showed that a diverse range of organisms with *nosZ* genes was distributed across the two sediments and soils. Although clone analysis is proving to be a very

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2.4 Work Developed from Manuscript 1 & Manuscript 2 - Relating Variables to Community Structure.

In the previous sections of this chapter (2.2 & 2.3) manuscripts were presented that introduced an ordination method (NMDS) and metrics (SIMPER, Dispersion) to describe the community structure, and a test of significance (ANOSIM) for determining the strength of community similarity. The manuscript in section 2.3 utilized the ANOSIM test to demonstrate clear differences between both the sites and, within sites, clear separation between the riparian and sedimentary bacterial communities at each location. The objective of this short section is to explore multivariate methods for determining the likely variables that influence the observed difference in community composition with emphasis on the sedimentary material.

2.5 Methods.

2.5.1 Major and trace elements by X-ray fluorescence (XRF).

Sample preparation and analysis.

For each sediment sample for which a T-RFLP trace was produced, the remaining sediment was dried (105°C) and sieved sediment samples were gently disaggregated and passed through a series of 2 mm, 1 mm and 355 μm mesh-sized sieves and the weight remaining recorded. The smallest fraction was sent for analysis by XRF (CSIRO, Adelaide)[1]. Approximately 1 g of each oven-dried (105°C) sample was accurately weighed with 4 g of 12-22 lithium borate flux and transferred into Pt/Au crucibles. The crucibles were then heated to 1050 °C for 12 minutes then poured into a 32 mm Pt/Au mould heated to a similar temperature. The melt was cooled quickly over a compressed air stream and the resulting glass disks were analysed on a Philips PW1480 wavelength dispersive XRF system (Philips Analytical, Almelo, The Netherlands) using a dual anode Sc/Mo tube. A separate sub sample of this material was ignited at 550 °C to determine loss on ignition (APHA 1995).

[1] CSIRO Land and Water, Waite Rd, Urrbrae, SA 5064. http://www.clw.csiro.au/services/xrf/services.html

Statistical exploration.

Data were screened and corrected for normality with appropriate transformations as necessary. Differences in concentrations between the two streams were assessed using Students t-test.

Principle components analysis (PCA).

Principle Components Analysis (PCA) using Systat v10 (SPSS 2000) was used to reduce the complexity of the screened data set; highly correlated variables were removed. The site scores from the reduced set of environmental variables were plotted using scores from the first two factors (Figure 2.2A) and visually compared to an NMDS plot generated from a reduced set of the *nosZ* T-RFLP data corresponding to the same sediment samples (Figure 2.2B) generated in the same manner as described in Manuscript 2

BIO-ENV.

BIO-ENV is a procedure that searches iteratively through the set of environmental variables to find the best single or combination of variables (represented by a similarity matrix using the Euclidean distance) that provides the best description of the community data (represented by a similarity matrix using an appropriate distance measure) as a rank correlation coefficient between the two similarity matrices (Clarke and Warwick 2001). The procedure as described by Clarke and Warwick (2001) was performed on the XRF data described above using a subset of the *nosZ* community data representing the sediments only, using the PRIMER package (Clarke and Gorley 2001).

2.6 RESULTS.

Sediment chemistry.

There were major differences in the major and minor components within the sediment chemistry (Table 2.1). For the major components, silicate, calcium and magnesium were notably higher in Brushy creek (highly urbanized), whereas alumina was notably higher in Lyrebird creek. Among the minor components, zinc, copper and lead were significantly higher in Brushy creek, although the reverse occurred with zirconia. The particle size fractions of the two sediments were significantly different, with the sediment

from Brushy creek coarser than Lyrebird creek. The carbon fraction lost on ignition was greater in Lyrebird creek.

Table 2.1 The elemental composition, size fractionation and loss on ignition for sediment samples from Brushy and Lyrebird Creek. Elements are presented as the element oxide as determined by XRF. The data presented are significantly different between sites at α = 0.05 using the Students t-test.

	Brushy Creek (n=8)		Lyrebird Creek (n=7)	
Variable	Mean	SD	Mean	SD
SiO_2 [a]	78.3	4.7	65.1	4.0
Al_2O_3 [a]	6.6	1.6	10.3	1.1
MgO [a]	1.2	0.1	0.8	0.1
Fe_2O_3 [a]	5.7	2.0	8.7	2.7
CaO [a]	1.8	0.2	0.5	0.1
Na_2O [a]	0.6	0.1	0.4	0.04
TiO_2 [a]	0.9	0.3	6.3	2.9
P_2O_5 [a]	0.12	0.02	0.08	0.01
MnO [a]	0.03	0.01	0.22	0.09
ZnO [b]	391	108	84	12
CuO [b]	33	11	19	4
SrO [b]	91	9	72	6
ZrO_2 [b]	405	206	1386	383
NiO [b]	53	24	28	2
V_2O_5 [b]	148	44	291	77
PbO [b]	102	17	35	2
Ga_2O_3 [b]	22	4	30	4
ThO_2 [b]	26	6	41	3
% sediment > 1mm [a]	23	10	6	5
% sediment < 355 μm [a]	17	18	58	10
% Loss on ignition [a]	3	1	8	2

a. % by weight;
b. μg/g

Principle components analysis.

Table 2.2 shows the factor load for each retained variable for the first two factors which together account for 72 % of the variation. The factor loadings show that Factor 1 is dominated by Fe_2O_3, Ga_2O_3, V_2O_5, SiO_2, TiO_2 and to a lesser extent Na_2O, SrO, and MnO

whereas Factor 2 is strongly influenced by the two size fraction variables and the group P_2O_5, ZnO, NiO and CuO.

Table 2.2 Factor Loads for retained XRF variables for Lyrebird Ck and Brushy Ck sediment samples.

Variable	Factor 1 (38.8%)	Factor 2 (33.4%)
Fe_2O_3	0.96	0.08
Ga_2O_3	0.84	-0.12
V_2O_5	0.94	-0.19
SiO_2	-0.82	0.21
Na_2O	-0.71	0.21
SrO	-0.76	0.39
MgO	-0.22	0.41
ThO_2	0.74	-0.50
TiO_2	0.80	-0.51
MnO	0.77	-0.55
PbO	-0.46	0.57
ZrO_2	0.80	-0.58
CaO	-0.61	0.62
% sediment > 1mm	-0.28	0.76
P_2O_5	-0.44	0.81
ZnO	-0.37	0.86
NiO	-0.10	0.94
CuO	0.01	0.87
% sediment < 355 μm	0.19	-0.86
% Loss on ignition	0.33	-0.54
Al_2O_3	0.52	-0.18

BIO-ENV analysis.

Results from this procedure are shown in Table 2.3 where the best correlation for single variables, then pairs of variables and finally triplets of variables are presented. Also included is the association of individual variables with the PCA Factors 1 & 2 described above. The variable with the single largest BIO-ENV correlation to the community data was ZnO (0.667), closely followed by TiO_2 (0.656), CaO (0.636), SiO_2 (0.632) and ThO_2 (0.626).

Table 2.3 The correlation (Spearman coefficient) between the *nosZ* community similarity matrix (Bray-Curtis) and XRF sediment composition using BIO-ENV.

Number of Variables	Correlation between sets	Variables included (in order)	Factor on which each variable loads
1	0.667	ZnO	2
1	0.656	TiO_2	1
1	0.636	CaO	=
1	0.632	SiO_2	1
1	0.626	ThO_2	1
1	0.625	MnO	1
1	0.620	PbO	=
1	0.559	ZrO_2	1
1	0.550	P_2O_5	2
1	0.525	SrO	1
2	0.775	ZnO,Ga_2O_3	2, 1
2	0.773	SiO_2, ZnO	1, 2
2	0.761	SiO_2, P_2O_5	1, 2
2	0.744	P_2O_5, Ga_2O_3	2, 1
2	0.744	ZnO, ThO_2	2, 1
2	0.741	SiO_2, > 1 mm	1, 2
2	0.738	Ga_2O_3, < 355 um	1, 2
2	0.735	TiO_2, ZnO	1, 2
2	0.732	P_2O_5, ThO_2	2, 1
2	0.727	SiO_2, < 355 um	1, 2
3	0.815	SiO_2, P_2O_5, > 1 mm	1, 2, 2
3	0.814	P_2O_5, Ga_2O_3, >1 mm	2, 1, 2
3	0.797	SiO_2, ZnO, ThO_2	1, 2, 1
3	0.793	ZnO, Ga_2O_3, > 1 mm	2, 1, 2
3	0.792	ZnO, SrO, Ga_2O_3	2, 1, 1

2.7 DISCUSSION.

Linking environmental variables to community data: PCA of environmental variables compared to NMDS of the *nos*Z community data.

It is unclear from the raw XRF data (Table 2.1) which if any (or a combination) of the variables are influencing the observed community data. Also, comparing the means between sites may not be an appropriate method of comparison as the variability of individual elements, and there relationship with each other is lost. Principle Component Analysis (PCA) is an ordination method commonly used to simplify complex data sets,

and to retain the original variability of the data with the minimum number of new derived variables (Legendre and Legendre 1998, Tabachnick and Fidell 2001). In ideal cases the new variables, can be interpreted by considering the relative contribution of source variables. The main assumptions of the method are that variables are approximately normal and linear relationships exist between groups of variables (Tabachnick and Fidell 2001). These two assumptions also explain to some extent why PCA is an effective tool for ordination of continuous environmental variables, but is inadequate when applied to community data (see Chapter 3).

It is clear that both sets of data (Figure 2.2 A & B) identify two groups (keeping in mind that NMDS plots can be freely rotated). A reasonable interpretation of the combination of transition and alkali metals represented by the PCA ordination (Table 2.2) could be that Factor 1 describes the basic mineralogy of the catchment. Factor 2, on the other hand, by incorporating information about the particle size and the transition elements Zn, Ni, Cu (heavy metals) is suggestive of the influences expected from an urban environment. These coincidental associations, although being plausible, offer only speculative possibilities as to the causative agents of the community differences observed in the *nos*Z ordination. While PCA accounts for correlations between related variables, there is no formal measure of the degree of relationship between these two sets of data other than visual inspection.

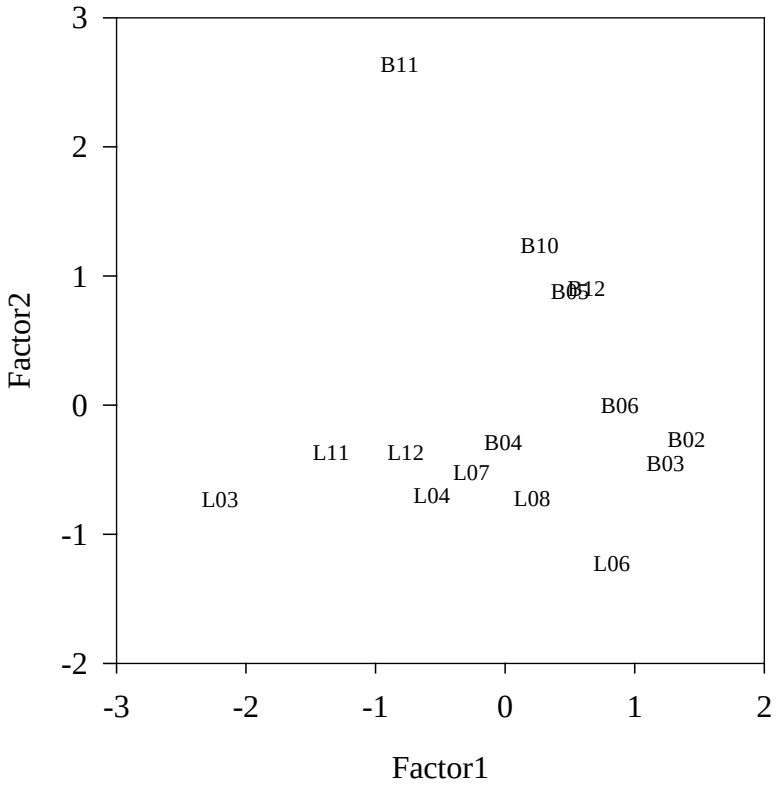

Figure 2.2A PCA plot of factor scores derived from XRF date in Table 2.2. B = Brushy L= Lyrebird.

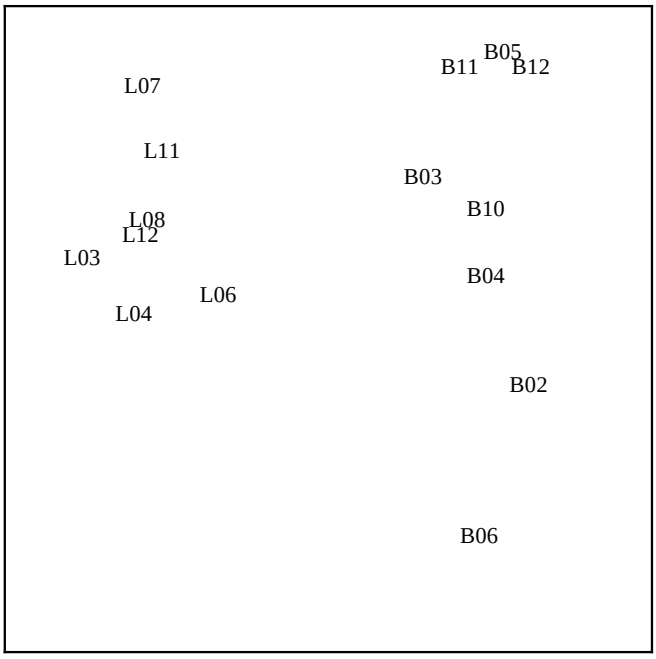

Figure 2.2B NMDS plot of T-RFLP data for the same sediment samples – a reduced set of the data presented in manuscript 2. B = Brushy L= Lyrebird.

Linking environmental variables to community data; the BIO-ENV procedure.

The PRIMER procedure known as BIO-ENV (Clarke and Gorley 2001) attempts to find the 'best match' between two sets of data: the similarity matrix representing the community data and a set of environmental variables. As noted by the originators of the technique (Clarke and Gorley 2001, Clarke and Warwick 2001), this should be achieved in a way that does not depend "*on the dimensionality one happens to choose to view the two patterns*". What they are referring to here is that an NMDS could in theory be plotted in as many dimensions as there were species (or descriptors) in the original matrix. The final output, typically a two or three dimensional plot, is chosen to summarize the data in an orientation that provides simple interpretation. Likewise, a PCA ordination is an attempt to find those 'factors' (i.e. related variables) that may be interpreted in a manner that is consistent with the observed distribution of samples and known properties of the system under study.

These valid theoretical concerns aside, it is hard to imagine that ThO_2 or Ga_2O_3, materials that are only present in trace amounts (Table 2.1), have any meaningful deterministic effect on the community structure. It is more likely, that there position in this table merely reflects correlation with the major compositional variables SiO_2, TiO_2 and Fe_2O_3, as indicated by the strong common association of these variables with Factor 1 described above (Table 2.2). Although Bio-Env does calculate a measure of relatedness (Spearman coefficient), as is evident from Table 2.3, two problems in interpretation arise. By chance alone, a co correlate may appear to explain more of the variation than more plausible explanatory variables (see above). Secondly, the inclusion of more variables will automatically increase the amount of explained variance. Coupled with the problem of chance co correlation already mentioned, some of the latter entries in Table 2.3 are possibly less informative than the single variable correlations.

The strong effect that the single variable ZnO appears to have, coupled with the knowledge that this heavy metal has been documented to effect changes in community structure (Kelly et al. 1999, Hill et al. 2003, Moffett et al. 2003, Mertens et al. 2006) and function (Hemida et al. 1997, Duarte et al. 2004) at concentrations similar to the those reported in Table 2.1, informed the decision to use it in the experiments described in Chapters 5 & 6.

2.8 Concluding Remarks.

Manuscript 1 introduced the basic methodology and descriptive statistics for analyzing bacterial community structure using T-RFLP fingerprints of the 16s rDNA gene and demonstrated that clear differences could be seen from ecologically distinct sites. Manuscript 2 demonstrated that the metabolic gene *nos*Z could also be used to demonstrate differences in the communities of (potential) denitrifiers from urban and non urban sites. Both papers used the ANOSIM statistic to provide a clear and meaningful test of the differences in community structure. In addition, manuscript 2 demonstrated that the differences seen in the T-RFLP derived MDS plot were to a large extent mirrored in the phylogenetic tree derived from sequences extracted from these sites. However, the number of *nos*Z sequences from described organisms is limited, thus hindering the extent to which this phylogenetic information can be used to infer ecological preferences of unnamed environmental clones.

To overcome our limited ability to make ecological inferences from the phylogenetic data, a preliminary investigation was made to link the T-RFLP fingerprint data with some overall measures of sediment chemistry. Overall the correlation and factor association data of Table 2.3 do not, as should probably be expected, clearly point to any one variable describing the overall variation in the community data, only likely candidates for further experimentation. The significant factor/s contributing to variation in the community data may not in fact be present in these tables, but may have been captured through some other relationship with the variables present. From the data presented here the tentative conclusion is that some property of the sediment, or catchment processes that structure the sediment, is responsible for explaining approximately 70% of the community at these two sites. Or in other words, those factors that determine the sediment composition (both physically and chemically) have an effect on the final community structure as measured here using T-RFLP fingerprints of the *nos*Z gene. These effects and limitations to the above analysis will be explored further in the next chapter using an expanded set of sites to test this initial hypothesis.

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CHAPTER 3

VARIATION IN THE DENITRIFYING COMMUNITY STRUCTURE ALONG AN ECOLOGICAL GRADIENT – AN EXPLORATION OF ENVIRONMENTAL VARIABLES.

OVERVIEW.

The previous chapter introduced methods that form the basic starting point for the analysis of bacterial community structure used throughout this thesis. Manuscript 2 and the later sections of Chapter 2 dealt with a two site (4 habitats) comparison that demonstrated unambiguous site differences using two independent methods, Non metric Multi Dimensional Scaling (NMDS) and Principle Components Analysis (PCA). This initial study was used to determine whether the adopted methods were able to distinguish sites known to be at opposite ends of an ecological milieu.

The sensitivity of the method established, this chapter presents work that expands on the structural differences noted in Chapter 2 through the examination of changes in the *nos*Z community from streams on a previously described ecological gradient and the application of direct or restricted ordination to investigate the relationship between sediment properties and the observed community structure. In other words, this chapter aims to explore the question of whether there are systematic changes in community structure and the extent to which these are related to sediment variables and geographic variables of the underlying ecological gradient.

To this end, this chapter first presents a brief introduction to the concept of the 'urban gradient' as described in the literature with particular reference to studies previously conducted at these sites. Canonical Correspondence Analysis (CCA) will be introduced as a method of direct gradient analysis that has many properties suitable to the analysis of T-RFLP community pattern data and its use demonstrated for sites representing a gradient of urban sites. After presentation of the data, the discussion will explore and critique the generality of the relationships suggested by this technique followed by an in-depth exploration of some of the likely mechanisms structuring the bacterial community.

As a further guide, the following questions will be considered:

Does the microbial community structure represented by the *nos*Z gene demonstrate that different urban environments contain different communities of denitrifiers?

Does the evidence suggest that the bacterial community structure varies along an ecological gradient?

What variables do the techniques used in this chapter suggest are responsible for structuring any observed systematic changes and are these plausible?

Carbon is examined in more detail in the following chapter.

3.1 INTRODUCTION.

Results presented in Chapter 2 suggested that observed differences in community structure may be related to the properties of the sediment. However since the results in Chapter 2 were from a two site comparison only, little could be said about the strength of any relationship between the highly significant (ANOSIM) differences in community structure between sites and those measured differences in physico-chemical properties adjudged statistically significant or different by students t-test. The purpose of the work in Chapter 2 was to establish whether the *nos*Z community signal from recognizably different sites contained enough genetic information/variability to differentiate sites. The significant result from that chapter is that genetic/community information resulting from T-RFLP analysis of the *nos*Z gene appears to be a conservative indicator of particular environments. This is inferred from the fact that significant differences were found between *nos*Z T-RFLP communities from the 4 habitats tested via ANOSIM, the observation of noticeable physico-chemical differences between habitat variables and thirdly, that there were consistent groupings between habitat type and known/described organisms at the sequence level (see Figure 1 from Manuscript 2, Chapter 2). This last result suggests that the *nos*Z motif amplified and sequenced in this study appears to reflect the particular evolutionary, and therefore 'habitat' specific requirements of the organisms. If this result holds more generally then examination of a range of sites from established environmental gradients would allow us to delineate the factors/variables that

determine the likely presence of particular organisms or conversely, DNA sampling may allow a broad inference of the probable 'conditions' at a site. Studies such as that presented here are the first steps towards this larger goal of understanding the factors determining the distribution of 'uncultured' organisms.

3.1.1 The ecologically distinct changes in urban streams in response to the process of urbanization – relating indices of urbanization to consistent ecological responses.

Urban streams have become a focus of research as communities attempt to prevent further degradation of stream conditions as a result of the surrounding built environment, or to target specific impacts when undertaking restorative work (Lee and Bang 2000, Pickett et al. 2001, Kolpin et al. 2002, Hatt et al. 2004, Taylor et al. 2004, Grimm et al. 2005, Groffman et al. 2005, Meyer et al. 2005, Walsh et al. 2005a). Comprehensive reviews of the effects of urbanization are available (Paul and Meyer 2001, Walsh et al. 2005b) and the effects of urban environments on streams are summarized in Tables 3.1 and 3.2 (adapted from these sources). To date, the emphasis of these studies has been on the hydrological consequences of urbanization and subsequent ecological effects with a focus on macro invertebrates and the larger 'charismatic' biota (fish, birds and mammals) as indicators of stream health. The symptoms categorized in the studies from which Table 3.1 is derived were divided into two groups depending on whether the symptom was observed to be a consistent indicator or response to urbanization for that particular feature or, whether the described symptom has not clearly been attributed to urbanization. For example, from Table 3.1 and depending on the exact nature of the urbanizing process, base flow could increase due to increased runoff as a consequence of increased impervious surface area, or it could decrease if the runoff is directed to piped sewer treatment facilities. Similarly, suspended sediments could increase due to increased flow in urbanized streams, or might decrease once all fines have been washed out of the system, and new sediment is not delivered as the surrounding catchment is sealed beneath urban structures. The focus on hydrology rests on the simple fact that urban areas have increased impervious surface cover resulting in decreased infiltration and increased surface runoff (Paul and Meyer 2001). This has led to the adoption of Impervious Surface Cover (ISC) as a general index or measure by which to group the observed effects of catchment urbanization – where streams with an ISC around 10 - 20% (see

Table 3.2) show measurable decreases in measures of ecological integrity and streams with an ISC of ~ 45% are generally considered degraded (Paul and Meyer 2001).

Table 3.1 Observed symptoms associated with urbanized streams (adapted from Walsh et al 2005b). Arrows pointing up indicate an increase in that symptom due to the effects of urbanization.

Feature	Consistent Response	Inconsistent Response
Hydrology	↑ Frequency of overland flow ↑ Frequency of erosive flow ↑ Magnitude of high flow ↓ Lag time to peak flow ↑ Rise and fall of hydrograph	Base flow magnitude
Water Chemistry	↑ Nutrients (N, P) ↑ Toxicants ↑ Temperature	Suspended sediments
Channel Morphology	↑ Channel Width ↑ Pool Depth ↑ Scour ↓ Channel complexity	Sedimentation
Organic Matter	↓ Retention	Standing stock/inputs
Fish	↓ Sensitive Fish species	Tolerant fish species Fish abundance/biomass
Invertebrates	↑ Tolerant Invertebrates ↓ Sensitive Invertebrates	
Algae	↑ Eutrophic Diatoms ↓ Oligotrophic Diatoms	Algal biomass
Ecosystem Processes	↓ Nutrient Uptake	Leaf breakdown

"*A dominant paradigm in fluvial geomorphology holds that streams adjust their channel dimensions (width and depth) in response to long-term changes in sediment supply and bankfull discharge…*" (Paul and Meyer 2001). Due to alterations in sediment supply, which in many cases becomes dominated by in stream processes, changes in sediment texture are observed as fine sediment fractions are removed and coarse sand fractions increase. Large woody debris (LWD) is also decreased – presumably due to changes in

LWD supply from the catchment and removal by municipal authorities to increase drainage efficiency. The inconsistent response noted in Table 3.1 for suspended sediments and sedimentation is possibly due to observations being made on streams at different points in the development cycle and is discussed in some detail in Paul and Meyer (2001). During initial development of the catchment, hill slope erosion can lead to large increases in catchment sediment supply to streams leading to bed aggradation. Once development has concluded, and once pervious surfaces have been capped by impervious structures, this source of sediment is reduced, but bank full flows have been increased leading to greater channel incision (Paul and Meyer 2001). In a separate review (Walsh et al. 2005b) it is noted that although "*… stormwater impacts are the primary driver behind the often-reported correlations between stream condition and catchment imperviousness*" it is observed that "*small events likely also explain[s] the common observation of disproportionate increases in channel erosion with only minor increases in discharge*". Walsh et al. (2005b) suggest that attempts to manage large infrequent flow events may have less ecological benefit than attempting to manage the smaller more frequent increases resulting from increased catchment imperviousness.

Elevated metal concentrations are another common feature of urban streams – especially lead, zinc, chromium, copper, manganese, nickel and cadmium (Wilber and Hunter 1977, 1979b). Ignoring the possible contribution of industrial point sources, perhaps the single largest source of many metals is the automobile (Forman and Alexander 1998, Pratt and Lottermoser 2007) which accumulate on roads as components such as brake linings (containing Ni, Cr, Pb and Cu), tyres (containing Zn, Pb, Cr, Cu) and other engine parts (e.g. bearings, catalytic converters…) degrade with use. Other sources of metals in the urban environment include, for example, zinc from metal cladding and roofs, the metal oxides used for some paint pigments, copper-chrome-arsenate treated pine timber and organo tin compounds used as a stabiliser in PVC plumbing (Brooks 1996, Weis and Weis 1996, Davis et al. 2001, Hoch 2001, Solimini et al. 2006). These metals are then delivered to urban streams through the storm water drainage system.

The range of organic contaminants found in urban streams is a very large topic in its own right and only a cursory mention of probable contaminants will be made. Pesticides including insecticides, herbicides and fungicides are frequently found at high levels in urban streams (Daniels et al. 2000, Foster et al. 2000).

Table 3.2 Effects of impervious surface cover (ISC) resulting from urbanization on physical and biological stream variables[1]

Study Subject	Findings	Reference
Physical Responses: hydrology	Peak discharge/ bank full discharge/ surface runoff increases and lag time decreases with ISC.	Espey et al. 1965 Leopold 1968 Arnold & Gibbons 1996
Physical Responses: geomorphology	Channel enlargement increases with ISC. Dramatic changes in channel dimensions. Channel enlargement begins at 2 - 6%.	Hammer 1972 Morisawa & LaFlure 1979 Dunne & Leoplod 1978 Booth & Jackson 1997
Physical Responses: temperature.	Stream temperatures increase with increasing ISC.	Galli 1991
Biological responses: fish	Fish diversity decreased above 12 – 15% ISC and absent above 30 – 50%. Fish IBI* decreased above 10% ISC; streams with high riparian forest cover less affected.	Klein 1979 Steedman 1988
Biological Responses: Invertebrates	Invertebrate diversity decreases from 1 – 25% ISC (various studies). Insect IBI decreases between 1 – 6% ISC, except in streams with intact riparian zones.	Klein 1979 Jones & Clark 1987 Schueler & Galli 1992 Horner er al. 1997

* Index of Biological Integrity

Poly-cyclic aromatic and aliphatic hydrocarbons have many sources in the urban environment, including natural aromatic hydrocarbons derived from plants and trees, but also from industrial sources (e.g. solvent spills) and the complex mixture that is spent/used lubricating oil; the main source being leaks from car engines (Whipple and Hunter 1979a). Other organics detected in urban streams include pharmaceuticals, legal stimulants (e.g. caffeine, nicotine) and detergents (Kolpin et al. 2002, Ellis 2006, Haggard

[1] Adapted from Table 1 Paul and Meyer 2001: references in original

et al. 2006). The probable main source of these in urban streams is from faulty sewerage connections or as overflows in response to flooding.

Although in stream carbon retention is generally decreased (Table 3.1), the standing stock and rate of supply of organic matter to the stream is a function of the type and quantity of riparian vegetation in the surrounding catchment – as some studies have found that deciduous trees commonly planted in urban settings can increase the rate of supply through the storm water drainage system (Miller and Boulton 2005). Generally ecosystem processes (primary production, nutrient cycling) have not been studied extensively in urban streams (Paul and Meyer 2001). Some studies have considered organic carbon, but base flow concentrations of carbon (either natural or anthropogenic) are not known well. "*Removal of added nutrients and contaminants is an ecological service provided by streams and relied upon by society. Although nutrient uptake in flowing waters has been extensively studied in forested ecosystems (Meyer et al. 1988, Workshop 1990, Marti and Sabater 1996), urban settings have been largely ignored*" (Paul and Meyer 2001). Measures of nutrient uptake lengths in urban streams are longer than in non urban streams of similar size, suggesting that nutrient removal efficiency (i.e. retention) is reduced (Grimm et al. 2005, Meyer et al. 2005), however the effect of measurement error on these calculations needs to be considered (Hanafi et al. 2006).

Bacterial densities may be higher in urban streams (refs within Paul and Meyer 2001), but this is mainly attributed to coliform bacteria from mandated culture based tests of water quality and may not represent a true measure of bacterial density. See the discussion on the use of molecular methods as a means to overcome the selective nature of culture methods in Chapter 2. There is evidence that the increased delivery of antibiotics mentioned above, has led to the development of antibiotic resistance in some urban bacterial populations (Goni-Urriza et al. 2000) and that this resistance can also be positively correlated to heavy metal concentrations (Wei and Morrison 1992, McArthur and Tuckfield 2000)

The general response of algae to urbanization is a decrease in diversity and an increase in biomass – especially in urban settings with elevated light and nutrient levels. The higher water turbidity and frequent bed disturbance can mitigate against algal growth, as can the higher metal and herbicide concentrations mentioned above (Chessman et al. 1992,

Davies et al. 1994, Dodds and Welch 2000, Héctor F. Olguín 2000). In the review of Walsh (Walsh et al. 2005b), although the authors discuss ecosystem processes such as nutrient processing (with the specific example of denitrification), little attention was paid to the effect that the symptoms noted in Tables 3.1 & 3.2 and mentioned above might have on the microbiological community. Instead this is considered indirectly through references to changes in the type and nature of carbon sources and possible reduction in hyporheic flow as a result of habitat simplification.

Currently, debate has focussed on the relationship between indices of biological condition as a function of some measure of urbanization. In particular, the question of whether or not biological condition decreases monotonically or whether there is some threshold of urbanization beyond which rapid decline or collapse of the biological community is observed. For the sites studied by Walsh and others (Walsh et al. 2001, Hatt et al. 2004, Taylor et al. 2004, Walsh 2004, Harbott et al. 2005, Hanafi et al. 2006) and relevant to this manuscript, the best descriptor for the response of a wide range of ecological descriptors was the effective imperviousness, defined by Walsh (Walsh 2004, Walsh et al. 2005a) as the proportion of impervious surfaces connected to a stream by pipes. In the models used by Walsh to compare indices of urbanization (Walsh et al. 2005a), connection was found to be a better predictor of taxonomic richness and composition than Impervious Surface Cover (ISC) alone, particularly for the bulk of sites that fell in the narrow range of 2 – 12 % imperviousness. Additional studies on the same set of sites by Hatt et al (2004) and Taylor et al (2004) (details in Section 3.2.1 and 3.2.2) indicate that the connection and imperviousness indices are a reliable description/measure of an ‘urban gradient’ and that it should be possible to extend the observations contained in Tables 3.1 and 3.2 with a better description of the effect of urbanization on the bacterial community, or at least the effect on the community described by T-RFLP fingerprints of the *nosZ* gene.

3.1.2 Canonical[1] correspondence analysis (CCA): a brief introduction to direct gradient analysis.

As the examples in the previous chapter (and section 3.4 this chapter) show, ordination methods are useful in grouping community data such that samples that are similar appear close together and samples that are dissimilar appear apart (e.g. NMDS, CA, PCA), or for reducing the complexity of related continuous environmental variables by maximising the amount of variance explained while minimising the number of new variables required (e.g. PCA). Species ordination invites the researcher to suggest what effect or environmental gradient leads to the observed arrangement of the species/sites in the ordination space, whereas PCA for example, invites the interpretation/labelling of the axis by plausibly accounting for the observed relationship of variables contributing to each axis and thus producing the distribution of sites. In the happy case where species and environmental data are available which produce clear and 'identical' patterns in the two ordinations that conforms to the known (or suspected) response of the species and the known properties of the variables, interpretation can be straight forward. The strength of the relationships can be tested by regression of the species site scores with either the PCA axis scores (for synergistic effects) or with original variables when testing individual effects. Relating species and environmental variables in this way is known as indirect gradient analysis.

Although NMDS has found wide application as a species ordination method, chiefly as it places few restrictions on the data and is capable of handling joint absences (Clarke and Gorley 2001, Clarke and Warwick 2001, Rees et al. 2004), it is unclear how multidimensional species/site scores that have usually undergone a polar transformation (Bray-Curtis similarity) with projection onto two dimensions, can be related to environmental variables as "*…it is unclear what response models multidimensional scaling can cope with*" (Jongman et al. 1987) And although some consider MDS as "*… the best technique for recovering complex gradients*" (Legendre and Legendre), whether NMDS can "*…detect a particular underlying data structure depends in an unknown way on the chosen dissimilarity coefficient and on the initial ordinations supplied*" (Jongman

[1] The "*canonical form is the simplest and most comprehensive form to which certain functions, relations, or expressions can be reduced without loss of generality*" (Legendre and Legendre 1998)

et al. 1987). The ordinations produced via the underlying similarity matrix have been found to show clear separation of groups when such separation is known to exist (Clarke and Gorley 2001) and the accompanying ANOSIM procedure is useful for testing the strength of *a priori* group differences, but the orientation of the ordination itself is completely arbitrary.

Correspondence analysis (CA) is an ordination method that maximises the dispersion of site scores along a series of hypothetical environmental gradients (ter Braak 1986, Jongman et al. 1987). The underlying model or assumption of this method is that the abundance of each species is highest where the optimum suite of environmental variables for each species intersect, thus producing a unimodal distribution[1] along each hypothetical axis. This kind of distribution is frequently observed in nature (Palmer 1993, ter Braak et al. 2004). In their treatise '*Data analysis in community and Landscape Ecology*', Jongman, Ter Braak and van Tongeren (Jongman et al. 1987) state that *"[u]nimodal models are more general than monotonic ones, so it makes sense to start by using unimodal models and to decide afterwards whether one could simplify the model to a monotonic one.*" Since CA (and its variants) produces axes "*scaled in units of the standard deviation (SD) of species turnover*" (Legendre and Legendre 1998), axes with a SD less than about 2 will be monotonic and PCA (and related techniques) could be tried (Jongman et al. 1987). However, if axes have lengths greater than approximately 4 SD, then the data is highly non linear and PCA (and related methods) should not be used (Jongman et al. 1987). Like NMDS, CA can be performed with presence absence data whereas this form of data is generally not suitable for PCA for which the assumption is that data are quantitative species abundances (Jongman et al. 1987). 'Problems' arise with CA due to the underlying assumptions of the method that can lead to compression of scores at the ends of axes and to the so called arch effect where the second (and subsequent) axes appear to exhibit a polynomial relationship to the first[2]. Although techniques exist to suppress these problems, these solutions are best described as "*inelegant and arbitrary*" (Palmer 1993).

[1] Here meaning having one peak or maximum and not necessarily limited to the normal distribution.
[2] It should be noted that the arch effect is not limited to CA and can occur in PCA analysis (Palmer 1993).

Canonical correspondence analysis (CCA), an extension of CA, was introduced as a direct gradient analysis[1] technique (ter Braak 1986) which attempts to relate the changes in the entire community composition (not just one species at a time) with changes in the measured environmental variables (ter Braak 1986, Palmer 1993, Legendre and Legendre 1998). Palmer (1993) states that, "*the statistical model underlying CCA is that a species' abundance or frequency is a unimodal function of position along environmental gradients. CCA is an approximation to Gaussian Regression under a certain set of simplifying assumptions...*" According to its developer (ter Braak 1986) one of the possibilities of the technique is to estimate the significance of the measured environmental variables in the case where they "*… cannot explain the main variation, [but] they may still explain some of the remaining variation, which can be substantial…*" In other words, "*[c]anonical ordination techniques are designed to detect the patterns of variation in the species data that can be explained 'best' by the observed environmental variables*" (ter Braak 1986). Apart from the assumption of unimodal distribution cited above, two other important assumptions are made. One is "*that the species distributions are under environmental control … [and] the mode of each species is at its optimum along the various environmental variables*" Legendre & Legendre (1998). The second is "*that the gradient under study is long enough to allow each species to go from some less-than-optimum low frequency to its high-frequency optimum, and back…*" (Legendre and Legendre 1998), a point related to the discussion of axis length and the choice of ordination method mentioned above. This point clearly illustrated in Figure 1.7 (Chapter 1).

In essence, the method constructs axes that are linear combinations of the observed environmental variables in a way that dispersion of the species scores is maximised for that axis; subsequent axis are constructed with the usual proviso of being unrelated (orthogonal) to all previous axes (ter Braak 1986). The important distinction between CCA and CA is that the later absolutely maximises the dispersion of the site scores based on the species, whereas CCA restricts or constrains the dispersion to the variables included in the analysis. An important caveat to the technique is that the number of variables must be less than the number of sites (ter Braak 1986, Legendre and Legendre 1998). As the number of variables approaches the number of sites, the 'constraints' on

[1] This method is aso called "*constrained gradient analysis*".

the method are relaxed; when the number of variables is one less than the number of sites the method is no longer a constrained ordination method. One property of the method useful for examining T-RF data, as T-RF traces can contain many peaks, is that the number of species for each site is not restricted (ter Braak 1986). In addition, the arch effect[1] discussed above may not necessarily be a problem, as it *"…can be removed more elegantly by dropping superfluous environmental variables*" (ter Braak 1986, Palmer 1993, Legendre and Legendre 1998).

With the above assumptions in mind, CCA is advantageous for the initial exploration of data when the global response of species to variables is unclear. But like all correlation methods it must be kept in mind that variables demonstrating a strong relationship with canonical axis and therefore site scores (and through these the underlying community structure) can not be interpreted as causative. These variables should be viewed as related to those variables that do in fact structure the population, and the CCA method as an efficient way to screen for a reduced set of likely variables in an iterative procedure that eventually incorporates individual species-variable correlations and manipulation experiments.

[1] Detrending techniques to remove the 'arch effect' can also destroy curved/nonlinear structures that should legitimately remain for interpretation (Legendre and Legendre 1998).

3.2 SITE DESCRIPTIONS.

3.2.1 Site selection.

The sites used in this study (see Figure 3.1 & Table 3.3) are a subset of sites used in an extensive study titled "*Project D210 - Urbanization and the ecological function of streams*"[1] (Walsh et al. 2001, Hatt et al. 2004, Taylor et al. 2004, Walsh 2004, Walsh et al. 2005a, Walsh et al. 2005b) to determine which factors in the process of urbanization have the largest effect on stream degradation. This was a primary reason for the specific sites selected as it should enable the research conducted here to be interpreted as an 'urban gradient' within the overall framework of ecological changes established by previous work and discussed above (3.1.1).

Results detailed in two studies from Project D210 (Hatt et al. 2004, Taylor et al. 2004) form the basic background description of the ecological gradient chosen for the current study. The main findings from Taylor et al (2004) are that the benthic algal biomass was positively correlated with those variables jointly correlated with measures of catchment urbanization, but that independent of this altitude and drainage connection also had a significant correlation with the benthic chlorophyll' *a* concentration. Interestingly, they found that light levels only explained a small proportion of the variation in chl*a* concentrations. The study by Hatt et al (2004), a corollary to the study of Taylor et al (2004), explored the relationship between measures of water quality and the main measures of urbanization identified for these streams. The data shown in Table 3.3 is a subset of the results presented in Hatt et al (2004) and Taylor et al (2004) for the streams considered in this study. Hatt et al (2004) concluded that the measured water quality parameters are significantly correlated with the measures of urbanization and that several parameters are independently correlated with drainage connection after the effects of joint variation were accounted for by hierarchical partitioning.

[1] http://www.sci.monash.edu.au/wsc/urbanwater/d210/index.html

3.2.2 Description of the urban ecological gradient from which stream sediment DNA was extracted.

The gradient identified by Walsh and co-workers is perhaps best visualized by the interaction of the indices Catchment Imperviousness (i.e. the percentage of the catchment covered by hard, impervious surfaces) and Drainage Connection (i.e. the percentage of drainage infrastructure directly connected to urban streams) as shown in Figure 3.2 A. The response is not linear; drainage connection increases rapidly as imperviousness increases before reaching a plateau where virtually 100% of drainage infrastructure is connected directly to urban streams once catchment imperviousness reaches 50%. Figure 3.2 B represents the general response of many chemical variables to either of these urbanization indices after transformation of the data (see Hatt et al. Table 3 for a more detailed analysis). An exception to this general trend was found in the distribution of septic tanks which had a higher density in catchments of low to intermediate urbanization. Although ammonium concentrations were correlated with indices of catchment urbanization, total nitrogen (TN) and nitrate were significantly correlated with the septic tank density.

Another potentially confounding factor is that altitude generally increases in going from the more urban sites to the less urban sites. In Figure 3.1 the sites a-i are clustered at various altitudes around the Dandenong Ranges located to the east of Melbourne. Although areas of these Ranges have been impacted by urban development, the vegetation on the Ranges is different from that located within Melbourne proper. The studies on which these sites were selected suggest that DOC concentrations were related to urbanization, or specifically to the connection index (Hatt et al.), DOC was also independently correlated with elevation (Hatt et al. Table 3).

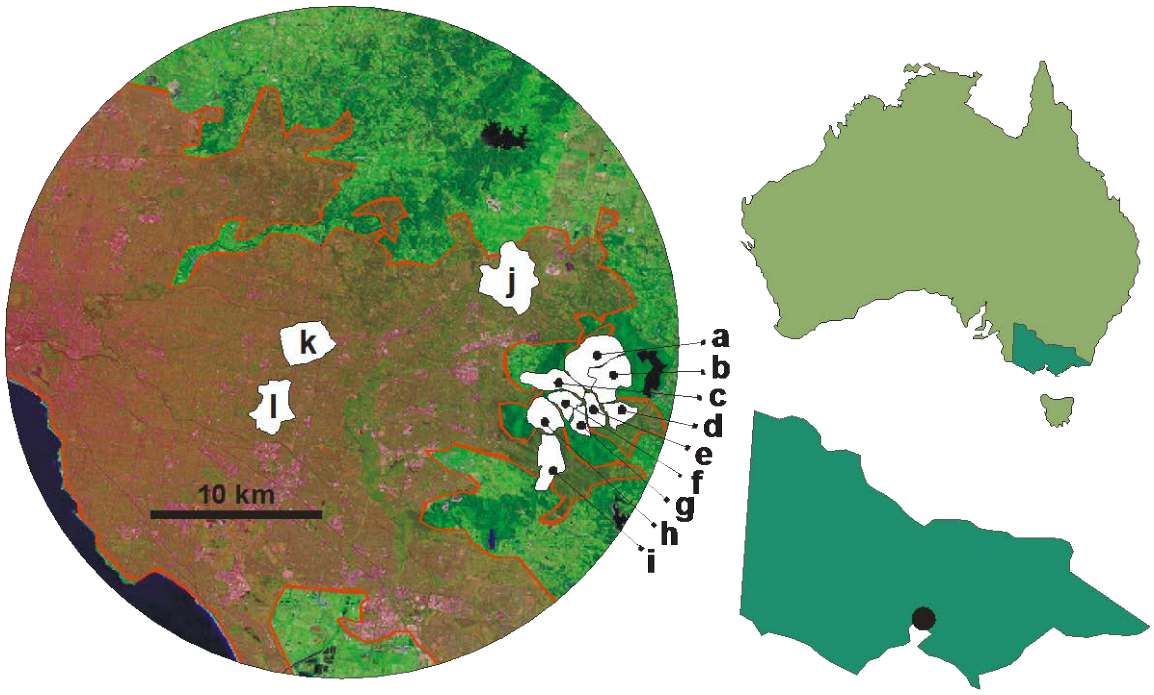


Figure 3.1 Locations of catchments in relation to the city of Melbourne. Outlined area in orange ■ indicates the approximate extent of the metropolitan area. The maps to the right indicate the location of Melbourne in relation to the state of Victoria and to continental Australia. The indicated stream catchments are a: Olinda, b: Lyrebird, c: Dandenong, d: Emerald, e: Perrins, f: Hughes, g: Sassafras, h: Dobsons, i: Ferny, j: Brushy, k: Gardiner and l: Scotchmans. Further information in Table 3.3

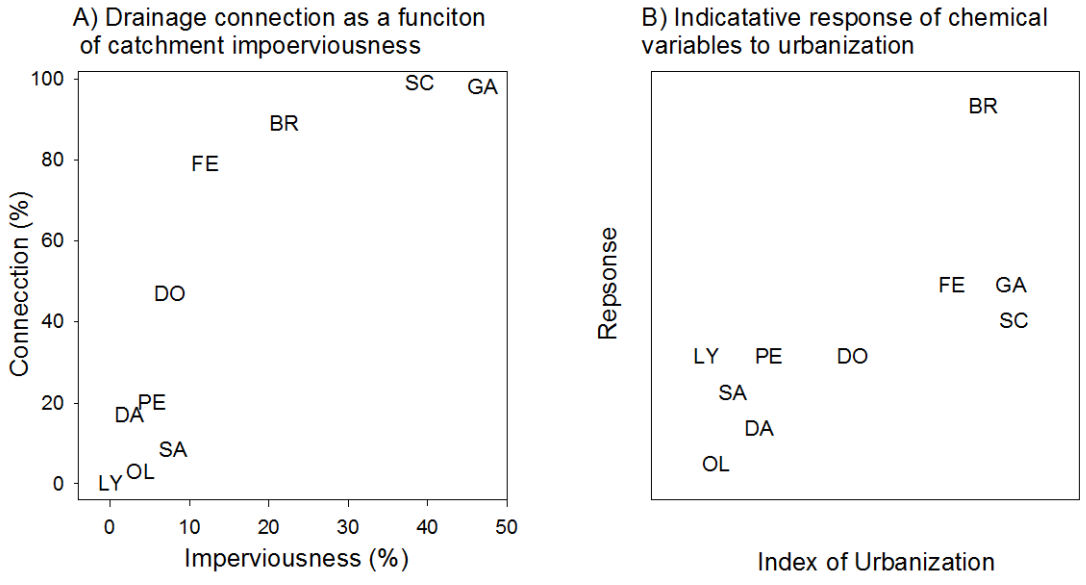


Figure 3.2 The 'urban gradient' as indicated by two indices of urbanization - connection and imperviousness (A) and the common response of chemical variables (after appropriate transformations) to these indices (B).

Table 3.3 Summary of the major geographic, physical and chemical attributes of streams used as field sites in this study: data is a subset of published values[1] recorded from Sept 2001 until March 2003.

Creek	Abr[2]	Map Label	Area (ha)	CBD[3] (km)	Elevation[4] (m)	Depth[5] (cm)	Imp.[6] (%)	Conn.[7] (%)	Septics[8] (N/km^2)	TP[9] (mg/L)	TN (mg/L)	FRP (mg/L)	DIN (mg/L)	DOC (mg/L)	Chla (mg m^{-2})	EC (mS /cm)
Brushy	BR	j	1479	29.1	82	26	22	89	37.8	0.11	0.97	0.027	0.6	7.00	25	561
Dandenong	DA	c	424	30.7	154	7.7	2.5	17	61.5	0.02	1.20	0.006	1.1	2.55	9.6	124
Dobsons	DO	h	365	30.7	168	11	7.6	47	43.8	0.04	1.65	0.008	1.4	4.00	8.7	190
Ferny	FE	i	642	32.3	163	23	12	79	80.4	0.06	1.55	0.013	1.4	5.75	28	417
Gardiners	GA	k	982	15.0	81	25	47	98	0	0.05	0.79	0.009	0.4	6.00	17	310
Lyrebird	LY	b	724	37.0	222	8.1	0.1	0	0	0.04	0.82	0.003	0.3	2.85	4.0	83
Olinda	OL	a	907	36.9	222	13	3.9	3.0	88.2	0.01	1.90	0.002	1.6	2.15	5.1	110
Perrins	PE	e	218	35.1	327	6.6	5.3	20	74.9	0.04	1.90	0.010	1.7	1.80	2.0	99
Sassafras	SA	g	189	34.2	370	6.8	8.0	8.3	141	0.03	2.10	0.004	1.8	2.00	1.8	110
Scotchmans	SC	l	812	15.5	78	42	39	99	0	0.05	0.61	0.012	0.3	5.90	47	357

[1] See Taylor et al. (2004) Table 1 and Hatt et al. (2004) Table 1 for the original data.
[2] Abreviated name.
[3] Distance to Central Business District (CBD)
[4] Height above sea level
[5] Median depth at various points in stream reach over period of study: see Taylor et al. (2004) for details.
[6] The percentage of each catchment covered by impervious surfaces.
[7] The percentage of impervious surfaces directly or intimately connected to stream via drainage infrastructure.
[8] The density of septic tanks in the stream catchment.
[9] All nutrient data expressed as the median concentration as found in the original references, except DOC and EC. These medians were calculated after access to the original database was obtained from C. Walsh.

3.3 METHODS.

3.3.1 Sample collection.

Ten streams were sampled at the beginning of spring, end of winter 2004 approximately one year after the study described in Chapter 2 and using the same methods. Coring devices were constructed by removing the ends from 25 mL syringes. At each stream, four samples from the top 5-10 cm of sediment were collected along a ~20 m section of each stream and transferred to 50 mL centrifuge tubes. Where sediment depth was limited, two or more cores were taken to give a total sediment volume of approximately 40 – 50 mL. All samples were held on ice until returned to the laboratory where they were frozen. No riparian samples were taken for this comparison and replication was limited to 4 per stream due to logistical and financial constraints.

Immediately upstream of the point from which each sediment sample was taken, stream water was sampled using a 25 ml syringe and filtered through a 0.2 µm nucleopore membrane filter held in a Swinnex filter holder into a clean acid washed 10 ml sample tube. These water samples were also kept on ice until returned to the laboratory where they were frozen for later analysis.

3.3.2 DNA extraction and analysis.

DNA Extraction.

As stated in Chapter 2 (Manuscripts 1 & 2), the methods used for DNA extraction and T-RFLP procedures have been described previously (Rees et al. 2004). The samples were thawed at room temperature and homogenized by mixing the sample with a clean, flamed spatula. After thawing and mixing samples in the 50 mL centrifuge tubes, 0.25 – 1 g of material was used for DNA extraction, using the MoBio UltraClean™ Soil DNA isolation kit, as described by the manufacturer (MoBio Laboratories, USA)

DNA amplification.

Amplification of an approximately 780 base pair fragment of the *nosZ* gene was carried out in a final volume of 50 µL, using the HotStarTaq™ DNA polymerase kit and master

mix described by the manufacturer (Qiagen, Clifton Hill, Australia), with the exception that 2.5 units of *Taq* polymerase were used per reaction. The forward primer *nosZ*-F (5'-CGYTGTTCMTCGACAGCCAG-3') and reverse primer *nosZ*-R (5'-CATGTGCAGNGCRTGGCAGAA-3') were used for the polymerase chain reaction (PCR) (Rösch et al. 2002). For T-RFLP analysis, the forward primer was labelled at the 5' terminus with 6-carboxyfluorescein.

PCR was carried out on a BioRad iCycler thermal cycler (Regents Park, Australia) with the following conditions: 95 °C for 15 min to activate the HotStar*Taq*, denaturation at 94 °C for 30 s, 35 cycles starting with a touchdown procedure beginning at 58 °C for 40 s, decreasing in 0.5 °C steps for 9 cycles until a final annealing temperature of 54^{o}C, and extension at 72 °C for 1 min 20 s - final extension was 72 °C for 7 min. DNA extracted from *Pseudomonas denitrificans* (NCTC 10688) was used as the positive control and amplicon size was confirmed by agarose gel electrophoresis. Amplified samples were purified using the MoBio UltraClean™ PCR clean-up kit following the manufacturer's instructions (MoBio Laboratories, Solena, CA, USA).

T-RFLP analysis.

Purified amplicons from PCRs with the forward-labelled primer were digested with *Hin6I* (Geneworks, Adelaide, Australia) for 3h at 37 °C. The 25 µL digestion reactions contained between 80 -100 ng DNA, 2.5 µL of incubation buffer (prepared from the provided 10x stock solution: included with the *Hin6I* enzyme), 3 µL of endonuclease (3 U) and autoclaved Milli Q water. Post-digestion restriction fragments were precipitated in 3 volumes of 100% ethanol at -20 °C overnight. The precipitate was washed in 70% ethanol to remove excess salts and dried for final analysis. Dried pellets were resuspended in 10 µL water. A 2.5 µL volume was added to 0.3 µL Genescan Rox size standard (Applied Biosystems, Foster City, CA, USA), followed by 0.4 µL dextran Blue (50 mg ml^{-1} in 25 mmol L^{-1} EDTA, pH = 8), 1.8 µL formamide then denatured at 95 °C for 3 min. Final separation was carried out with a 377 DNA sequencer (Applied Biosystems)[1].

The final output files for each sample consisted of a list of fragment lengths and fluorescence values for each fragment, which were cross tabulated and aligned both

[1] This analysis was performed at the Walter and Eliza Hall Institute (WEHI) by the Australian Genome Research Facility: reagents and standards were prepared or obtained by this laboratory.

manually and 'automatically' using a Visual Basic routine that rounded lengths to the nearest integer, to counter subjective bias in the assignment of integer values for fragment length data[1]. Variations in the amount of DNA analyzed affect the number of peaks that appear in electropherograms and consequently, raw data sets require a standardization procedure prior to statistical analysis (Dunbar et al. 2001, Sait et al. 2003). A constant percentage standardization (Sait et al. 2003, Rees et al. 2004), rejecting peaks that made up less than 0.25 % of the total fluorescence in each sample. Sets or runs that had less than 10 peaks after these transformations were not included in further analysis.

3.3.3 Chemical analysis.

Stream nutrient analysis.

Nutrient analysis for Dissolved Phosphorus, Nitrate (NO_3^-) and Ammonia (NH_3) were all undertaken at the NATA[2] certified Analytical Laboratory of the Monash University Water Studies Centre using Flow Injection Analysis (FIA) using the automated phenate colorimetric method for NH_3, molybdenum blue colorimetry for phosphorus and the cadmium reduction method for nitrate–nitrite. Flow injection analysis was performed on a Lachat Quick Chem 8000 analyzer using standardized QA procedures (WSC 2001)

XRF, size fraction analysis and loss on ignition (LOI).

After sub samples had been extracted for T-RFLP analysis, a sub sample was removed and ground in a mortar and pestle for XRF analysis. Approximately 1 g of each oven-dried sample (105°C) was accurately weighed with 4 g of 12-22 lithium borate flux and transferred into Pt/Au crucibles. The crucibles were then heated to 1050 °C for 12 minutes then poured into a 32 mm Pt/Au mould heated to a similar temperature. The melt was cooled quickly over a compressed air stream and the resulting glass disks were analysed on a Philips PW1480 wavelength dispersive XRF system (Philips Analytical, Almelo, The Netherlands) using a dual anode Sc/Mo tube. A separate sub-sample of the ground material was ignited at 550 °C to determine loss on ignition (APHA 1995). The remainder of the dried sediment samples were gently disaggregated and passed through a series of 2 mm, 1 mm and 355 μm mesh-sized sieves and the weight remaining recorded.

[1] Code for this routine, referred to in Manuscript 1 as treeflap.xls can be found in Appendix 1
[2] The National Association of Testing Authorities (NATA, http://www.nata.asn.au/) is Australia's national laboratory accreditation authority.

3.3.4 Statistical analysis.

Ordination of T-RFLP data using NMDS.

Similarity matrices were calculated using the Bray-Curtis coefficient, on presence-absence transformed T-RFLP fingerprints using PRIMER (Clarke and Gorley 2001, Clarke and Warwick 2001). Differences between stream sites were tested using a one-way analysis of similarity (ANOSIM) (Clarke and Gorley 2001, Clarke and Warwick 2001). Non metric multi-dimensional scaling (MDS) plots with 50 random restarts were generated for visual interpretation of community patterns.

Ordination and data reduction of the XRF and size fraction data sets.

Mean results for XRF, sediment sizes and LOI are shown in Table 3.6. These data (including replicates) were screened for normality, skewness and kurtosis using the 'Descriptive Statistics' routine in SYSTAT (Version 10: SPSS Inc). Variables that were non-normal or highly skewed were transformed (log) to remove this effect before all variables were standardized to zero mean and unit variance. XRF and 'Size' variables were used in a Principle Components Analysis (PCA) ordination using varimax rotation as an exploratory means of determining whether the same patterns revealed through NMDS of the community data were evident in the sediment composition data using the 'Factor Analysis' tool in SYSTAT. Varimax rotation was used to produce simple orthogonal factors (Tabachnick and Fidell 2001). Since the number of factors is unknown, the default settings (i.e. eigenvalues greater than 1) were used to select factors for retention (Legendre and Legendre 1998, Tabachnick and Fidell 2001, Wayland et al. 2003, Fitzpatrick et al. 2007) and scores from the first two factors used for ordination.

In order to perform CCA the number of variables in the second matrix needs to be less than the number of sites (see introductory discussion). To achieve this goal while producing interpretable factors, the data was split into three groups (Major Elements, Minor Elements and Size Classes) that were separately factored using SYSTAT (Vaughan and Ormerod 2005, Fitzpatrick et al. 2007).

Canonical correspondence analysis (CCA) of the *nos*Z T-RFLP community restricted by sediment and environmental variables.

The presence-absence data set was screened using Detrended Correspondence Analysis (DECORANA) in PC-Ord 4.41 (McCune and Mefford 1999) using the default settings to determine whether, under the assumption of a unimodal species distribution, the length of the hypothetical environmental gradient(s) met the length requirement of being greater than 2 standard deviation units. Restricted ordination was then performed on the T-RFLP data with the factorized XRF and environment variables as the second matrix using CCA as implemented in PC-Ord. The analysis was setup such that Site Scores were linear combinations of the variables with a compromise scaling of the ordination scores between Sites and T-RFLP fragments. A Monte Carlo test with 500 random restarts was performed to test the hypothesis that there was no relationship between the matrices. Rows and columns were additionally centered and normalized during this procedure. A second CCA was performed using 8 additional variables that described the nutrient status (i.e. [NOx], [NH_4^+] and [PO_4^{3-}]) of the stream water and the geographic status of the site locations (i.e. Altitude, % Catchment Imperviousness, % Drainage Connection, Catchment Area, and the distance from the central business district of Melbourne).

3.4 RESULTS.

3.4.1 Congruence of Community NMDS and XRF PCA ordinations.

Figure 3.3 A is a two dimensional representation of the T-RFLP community data using NMDS. The sites have been colored according to the assumed *a priori* grouping based broadly on levels of urbanization as per Figure 3.2 A and Table 3.3. From this plot and referring to Figure 3.2 A, it can be seen that sites (e.g. Lyrebird Ck; LY and Olinda Ck; OL) with the lowest levels of urbanization (i.e. low impervious area and low connection; see Table 3.3) appear to form a distinct group separate from both intermediate groups (e.g. Perrins Ck; PE, Sassafras Ck; SA), which in turn are distinct from the most urbanized sites (e.g. Brushy Ck; BR, Scotchmans Ck; SC). The stress for the 2-dimensional representation is 0.15, however a 3-dimensional representation (not shown) exhibited a stress of 0.09. The differences observed in Fig 3.3 A, are also evident in the results of the ANOSIM test shown in Table 3.4. An R value approaching 1 indicates a large effect (i.e. large difference between sites) while the significance level is determined

from a Monte Carlo re-sampling of the available data. The Global R estimate (10,000 permutations) of 0.858 (significance level 0.01%) indicates that highly significant differences exist within the total set of sites. The remainder of the table shows the results of tests between respective pairs of sites limited by the number of replicates at each site.

Table 3.4 Pair wise one-way ANOSIM analysis of the *nosZ* data shown in Figure 3.1. The Global R estimate from 10,000 permutations was 0.858 with a significance level of 0.01%. The global test strongly indicates that significant differences exist between sites, the strength of these differences are suggested in the table below.

Groups		R Statistic	Significance Level %	Possible Permutations	Actual Permutations	Number >= Observed
BR	DA	1.00	10	10	10	1
BR	DO	1.00	10	10	10	1
BR	FE	0.75	10	10	10	1
BR	LY	1.00	2.9	35	35	1
BR	OL	1.00	10	10	10	1
BR	PE	1.00	2.9	35	35	1
BR	SA	1.00	2.9	35	35	1
BR	SC	1.00	10	10	10	1
DA	DO	0.75	10	10	10	1
DA	FE	1.00	33.3	3	3	1
DA	LY	0.79	6.7	15	15	1
DA	OL	0.46	20	10	10	2
DA	PE	1.00	6.7	15	15	1
DA	SA	0.96	6.7	15	15	1
DA	SC	0.92	10	10	10	1
DO	FE	0.67	10	10	10	1
DO	LY	0.91	2.9	35	35	1
DO	OL	0.93	10	10	10	1
DO	PE	0.98	2.9	35	35	1
DO	SA	0.82	2.9	35	35	1
DO	SC	1.00	10	10	10	1
FE	LY	1.00	6.7	15	15	1
FE	OL	1.00	10	10	10	1
FE	PE	1.00	6.7	15	15	1
FE	SA	1.00	6.7	15	15	1
FE	SC	1.00	10	10	10	1
LY	OL	0.22	28.6	35	35	10
LY	PE	0.91	2.9	35	35	1
LY	SA	0.92	2.9	35	35	1
LY	SC	0.88	2.9	35	35	1
OL	PE	1.00	2.9	35	35	1
OL	SA	1.00	2.9	35	35	1
OL	SC	0.82	10	10	10	1
PE	SA	0.44	2.9	35	35	1
PE	SC	1.00	2.9	35	35	1
SA	SC	1.00	2.9	35	35	1

The general pattern observed in this table is that the effect size (R) is large between dissimilar sites (i.e. Urban , Intermediate, Non Urban) but that the significance level is never lower (i.e. better) than 3%. Pairs[1] with noticeably low R values (i.e. similar) are LY – OL, DA – OL and PE – SA, while the significance level for LY – OL and DA – OL suggests these sites are indistinguishable, there is evidence that the *nosZ* community between PE & SA are significantly different.

The results of a PCA Ordination using Systat v10 (SPSS 2000) with Varimax rotation of all sediment XRF and size class data (also see mean results in Table 3.6) are shown in Figure 3.3 B and Table 3.5. Sites in this 2-dimensional representation have been color coded in the same way as Figure 3.3 A and the separation of sites indicates a similar pattern. The coincidental orientation of these two plots must be ignored as NMDS plots can be freely rotated (see introductory section for elaboration).

The sediment components driving this separation of sites are shown in Figure 3.3 C and Table 3.5. This table indicates that variance in the data can be represented by at least 4 Factors. Factor 1 is a compound variable consisting mainly of those variables describing the size distribution of the sediment combined with several major and minor co variables. The relationship between these variables is not clear. With the exception of SiO_2, Factor 2 could be interpreted as transition metals.

[1] See Table 3.3 for abbreviation names.

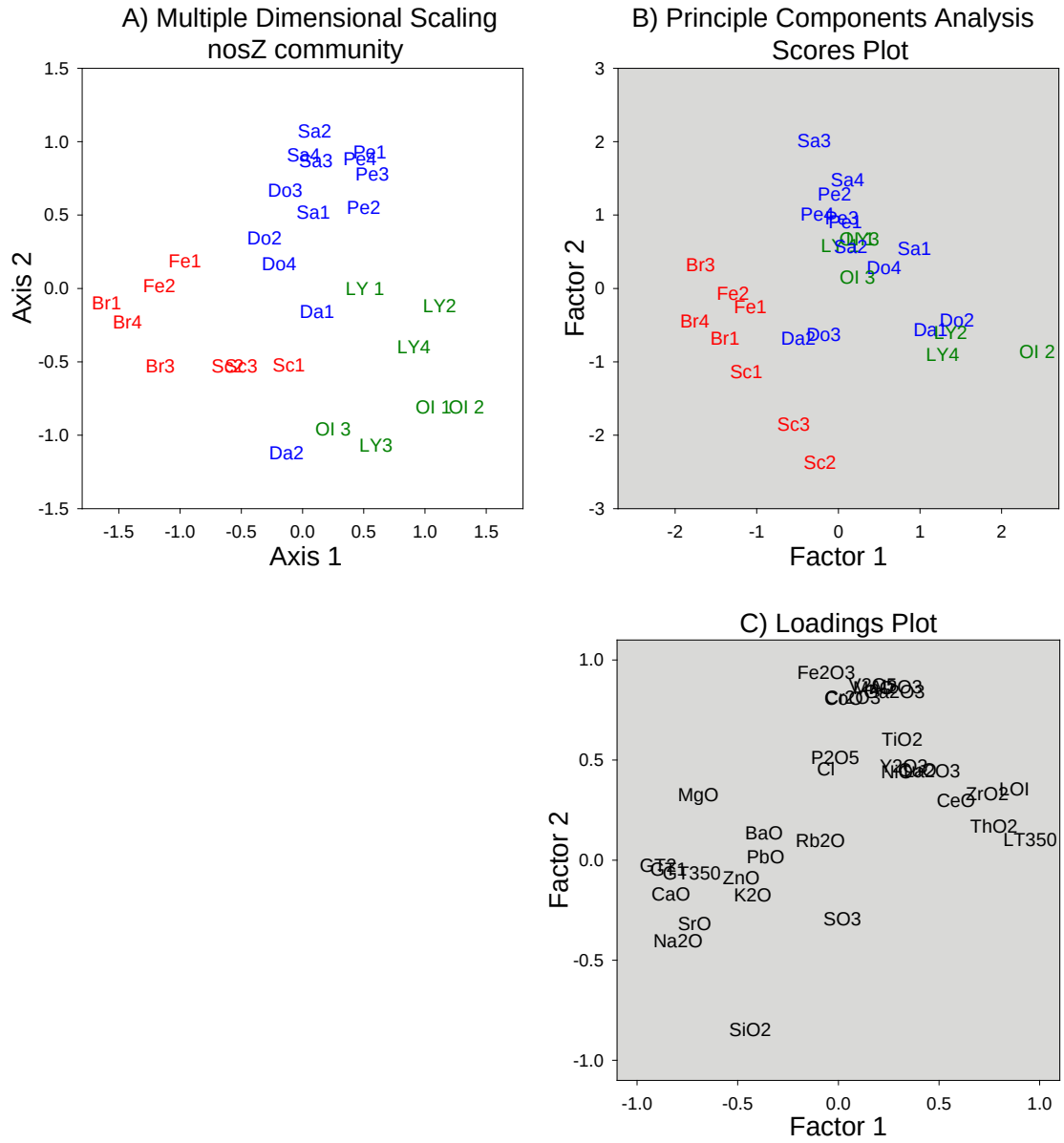


Figure 3.3 Ordination plots of the *nos*Z community data by **A**) NMDS (50 restarts; stress = 0.15) and sediment element composition (XRF) including particle size classes using **B**) Principle components analysis showing PCA scores (top) and **C**) PCA loadings (bottom). Site abbreviations as per Table 3.3; numbers represent replicates. Colors highlight those sites that can be broadly classified as urban (■), intermediate (■) and non urban (■). Symbols in C) refer to variables included in the PCA analysis see Table 3.5 for complete list.

Table 3.5 Sorted factor correlations/loadings for XRF, sediment size class and LOI variables. Numbers underlined highlight variables with a loading greater than 0.6 on each extracted factor[1]. The variance explained, percent variance explained and cumulative variance explained by inclusion of each factor is shown at the bottom of the table.

Variable	Factor				
	1	2	3	4	5
Fines	0.953	0.101	-0.045	0.204	0.108
2 mm	-0.895	-0.025	0.111	0.044	0.022
LOI	0.874	0.353	-0.024	0.018	0.165
1 mm	-0.843	-0.045	0.381	-0.052	-0.137
CaO	-0.832	-0.17	0.285	-0.171	0.201
Na_2O	-0.797	-0.401	0.410	0.022	-0.004
ThO_2	0.773	0.17	0.286	0.198	0.394
ZrO_2	0.739	0.331	0.076	0.451	0.141
355 um	-0.728	-0.064	-0.367	-0.314	-0.270
SrO	-0.714	-0.316	0.496	-0.106	0.139
MgO	-0.696	0.327	0.145	-0.392	0.245
CeO_2	0.585	0.297	0.620	-0.167	0.269
Fe_2O_3	-0.060	0.940	-0.149	-0.035	0.062
V_2O_5	0.171	0.874	0.072	0.125	-0.154
Al_2O_3	0.287	0.865	0.038	-0.198	-0.171
MnO	0.175	0.863	-0.323	0.179	-0.118
SiO_2	-0.438	-0.849	-0.040	0.041	-0.106
Ga_2O_3	0.279	0.840	0.299	0.109	0.174
Cr_2O_3	0.072	0.813	-0.026	-0.079	0.437
CoO	0.028	0.810	0.210	-0.286	-0.004
TiO_2	0.317	0.605	-0.087	0.601	-0.180
P_2O_5	-0.015	0.514	0.228	-0.770	0.095
Rb_2O	-0.088	0.097	0.958	0.049	-0.080
K_2O	-0.425	-0.174	0.857	0.094	-0.056
BaO	-0.369	0.134	0.841	0.080	0.198
Y_2O_3	0.325	0.471	0.589	0.149	0.410
PbO	-0.362	0.018	-0.149	-0.828	-0.117
ZnO	-0.482	-0.090	-0.173	-0.797	0.069
CuO	0.392	0.450	-0.178	-0.631	0.044
SO_3	0.020	-0.294	0.280	-0.134	0.761
NiO	0.292	0.442	-0.229	0.075	0.666
La_2O_3	0.466	0.448	0.448	0.355	0.196
Cl	-0.062	0.456	0.241	-0.110	0.043
Variance (%)	28.2	25.6	14.5	11.1	6.5
Cumulative (%)	28.2	53.8	68.3	79.3	86.0

[1] Loadings greater than 0.71 imply a 50% overlap in variance with the factor and are considered excellent; 0.63, 40% (considered very good) and 0.55, 30% (considered good) (Tabachnick and Fidell 2001). 0.6 was selected as a reasonable cutoff for strong association with a factor.

The results in Table 3.7 show the result of factoring the XRF and size class variables separately. Table 3.7 A indicates that the variance in the Major Elements can be represented by three factors. In this case, Factor 1 consists of Aluminium, Silica, Iron and Phosphorus whereas Factor 2 consists of Na, K, Mg and Ca. Unlike the results from Table 3.5, these factors can be easily interpreted as 'Alumino-Silicates' and 'Major Cations' with the remainder of the variance from this set contained in Factor 3. The second set, 'Minor Elements' (Table 3.7 B), consists of 4 Factors that also lend themselves to interpretation. Factor 1 from this set, consisting of Pb, Cu and Zn can clearly be called 'Heavy Metals', while Factor 2 consisting of Ba, Rb and Sr is an analogue (group I and II elements) of the 'Major Cations' group from the 'Major Elements' set. The two remaining factors from the 'Minor Elements' set consist of two sets of transition elements. The last set of variables consists of the 'size classes' and Loss on Ignition (LOI). The result of PCA on this set is one factor with LOI and Fines negatively correlated with the larger size fractions but highly correlated with each other. This single factor can be interpreted as the sediment 'Texture'. The mapping of these compound factors to new variable names is shown in Table 3.8.

Table 3.6 The mean composition of elements from ground samples as determined by XRF and distribution of particle sizes. Upper panel includes mean elemental composition of the Earths upper crust (Hawkesworth and Kemp 2006)[1]. Note that the upper panel is in units of weight percent (wt%) while the lower panel is in parts per million (ppm).

	Major Element (wt%)										Size Class & LOI (wt%)				
Site	SiO_2	Al_2O_3	MgO	Fe_2O_3	CaO	Na_2O	K_2O	TiO_2	P_2O_5	MnO	> 2mm.0	> 1 mm	> 355 μm	Fines	LOI
Upper Crust	66.6	15.4	2.48	5.04	3.59	3.27	2.8	0.64	0.15	0.1					
Br	73.0	9.0	1.2	6.2	3.0	1.4	1.8	0.5	0.1	0.0	19.5	24.0	47.4	8.5	2.3
Da	70.9	11.5	0.8	5.5	0.7	0.8	1.6	2.1	0.1	0.1	7.6	12.2	33.3	46.3	7.5
Do	65.5	11.2	0.8	6.4	0.8	0.6	1.8	2.6	0.1	0.1	6.7	13.7	21.8	56.5	12.5
Fe	65.5	8.9	0.9	11.0	1.4	1.2	1.6	9.2	0.1	0.3	11.8	17.6	33.7	36.6	2.2
Ly	62.5	11.6	0.7	6.8	0.6	0.3	0.8	3.8	0.1	0.2	4.3	4.0	18.3	72.2	16.2
Ol	59.2	13.9	0.6	8.0	0.5	0.3	0.8	3.7	0.1	0.1	2.4	5.4	24.0	67.3	16.2
Pe	56.4	17.7	1.2	9.4	0.8	0.4	1.0	1.6	0.2	0.2	7.6	13.6	38.3	39.9	13.2
Sa	56.0	18.3	1.1	9.5	0.7	0.4	1.0	3.1	0.2	0.2	5.8	10.8	33.3	49.5	11.8
Sc	84.0	5.2	1.0	4.5	1.2	0.9	0.9	0.4	0.1	0.0	14.7	13.5	49.1	22.2	2.2

	Minor Element (ppm)														
Site	ZnO	CuO	SrO	ZrO_2	NiO	Rb_2O	BaO	V_2O_5	Cr_2O_3	La_2O_3	CeO_2	PbO	Y_2O_3	CoO	Ga_2O_3	ThO_2
Br	200.8	9.4	167.8	229.4	38.7	85.0	1350.0	149.5	121.5	60.7	86.9	50.3	42.2	18.2	23.7	17.2
Da	81.8	6.7	101.7	724.5	20.7	91.3	1008.0	182.2	90.8	70.6	86.3	25.6	35.0	11.0	25.2	23.8
Do	144.2	14.8	117.4	828.3	39.2	110.0	921.6	236.0	98.4	78.0	104.6	32.0	47.9	17.0	28.2	35.0
Fe	165.6	13.7	156.1	1129.8	17.2	78.8	848.3	402.3	125.9	79.5	76.1	37.5	30.7	16.6	25.4	24.0
Ly	77.5	11.9	80.5	984.4	60.0	59.2	678.4	192.0	121.9	74.2	87.2	21.4	40.6	15.3	29.0	39.7
Ol	108.1	15.9	70.4	734.9	41.9	53.8	654.9	225.6	114.8	63.9	86.5	29.1	37.2	16.3	31.1	32.3
Pe	195.8	17.7	89.3	521.6	46.3	79.1	791.2	250.3	135.3	65.8	97.6	37.8	36.3	23.0	34.3	25.0
Sa	208.8	22.8	86.1	699.7	44.8	67.3	723.1	258.0	131.9	70.7	89.5	45.7	37.1	24.6	34.1	25.7
Sc	296.3	14.4	133.6	134.6	31.0	41.5	515.3	104.2	76.3	43.0	50.4	49.7	18.2	6.5	14.6	10.6

[1] See Table A3.1 Appendix 1

Table 3.7 Loadings of constructed variables (i.e. factors) using PCA data reduction of XRF element composition, sediment size variables and LOI after separating variables into related groups with identical units to simplify interpretation. Factor loadings greater than 0.6 are underlined to guide interpretation[1].

A) Major element factor loadings

Element oxide	Factor 1	Factor 2	Factor 3
P_2O_5	0.858	0.104	0.331
Al_2O_3	0.820	-0.262	-0.347
Fe_2O_3	0.809	-0.004	-0.477
SiO_2	-0.794	0.363	0.335
MnO	0.583	-0.269	-0.713
MgO	0.531	0.742	0.183
Na_2O	-0.358	0.897	0.201
CaO	-0.013	0.896	0.291
K_2O	-0.190	0.765	0.076
TiO_2	0.152	-0.272	-0.874
SO_3	-0.017	0.107	0.613
Variance (%)	31.83	28.29	21.66
Cumulative (%)	31.83	60.12	81.78

B) Minor element factor loadings

Element oxide	Factor 1	Factor 2	Factor 3	Factor 4
PbO	-0.910	0.009	0.035	-0.289
ZnO	-0.892	0.049	-0.085	-0.319
CuO	-0.611	-0.391	0.441	0.345
BaO	0.099	0.931	0.202	0.062
Rb_2O	0.081	0.871	0.091	0.324
SrO	-0.241	0.782	-0.298	-0.289
Cr_2O_3	-0.040	-0.061	0.891	0.165
CoO	-0.215	0.155	0.846	0.091
Ga_2O_3	0.187	0.125	0.840	0.380
V_2O_5	0.105	-0.042	0.805	0.162
NiO	0.103	-0.338	0.542	0.330
Y_2O_3	0.212	0.384	0.526	0.605
ThO_2	0.275	-0.098	0.163	0.890
CeO_2	-0.034	0.265	0.296	0.859
ZrO_2	0.485	-0.278	0.273	0.707
La_2O_3	0.396	0.150	0.417	0.676
Cl	-0.226	0.258	0.368	0.233
Variance (%)	16.13	17.33	25.13	21.88
Cumulative (%)	16.13	33.47	58.59	80.47

C) Size factor loadings

Classification	Factor 1
Fines	-0.989
LOI	-0.917
1 mm	0.895
2 mm	0.891
355 um	0.776
Variance (%)	80.34

[1] See footnote bottom of Table 3.5

Table 3.8 Mapping of derived factors to new compound variable names; the names in square braces are the abbreviations used in later analyses.

Variable origin	Associated original variables					Compound variable name
Major Components						
Factor 1	P_2O_5	Al_2O_3	Fe_2O_3	SiO_2		Alumino-Silicates [Al-Si]
Factor 2	MgO	Na_2O	CaO	K_2O		Major Cations [MCations]
Factor 3	MnO	TiO_2	SO_3			Residual Majors [ResMajor]
Minor Components						
Factor 1	PbO	ZnO	CuO			Classic Heavy Metals [HMetals]
Factor 2	BaO	Rb_2O	SrO			Major Cation Analogues [Cat-Anal]
Factor 3	Cr_2O_3	CoO	Ga_2O_3	V_2O_5		Transition Metals [TMetals]
Factor4	Y_2O_3	ThO_2	CeO_2	ZrO_2	La_2O_3	Residual Minors [ResMinor]
Size Class						
Factor 1	Fines	LOI	1 mm	2 mm	355 um	Texture [Texture]

3.4.2 Canonical Correspondence Analysis of the *nos*Z T-RFLP community with environmental variables.

The length of the hypothesized environmental gradient in the *nosZ* T-RFLP matrix was screened using DECORANA as implemented in PC-Ord (default settings). The axes were rescaled (30 segments) without down weighting of rare species. Before rescaling the first axis had a standard deviation score of 2.66 and the total variance of the community data was 2.7404. After rescaling the standard deviation score was 2.821. Both of these standard deviation scores suggest that a gradient of sufficient length (such that species composition can vary through a unimodal optima) is present in the community data. The second and third axes had standard deviation scores after rescaling of 2.876 and 1.650 respectively. Ordination of the species data by DECORANA is shown in Figure 3.4 F where the pattern of separation is similar to that seen in the NMDS, although inverted along the first axis.

The results of CCA using the *nosZ* T-RFLP data and the factored XRF data set is shown in Table 3.9 and Figure 3.4 A – E. The total variance explained by all the environmental variables included in the analysis was ~ 30% (28.8). The first axis accounted for half of the explained variation or ~15% (14.4) of the total variance in the species data. The Monte Carlo test returned a 'p value' of 0.002 after 500 iterations[1]. The Pearson correlation between the species and environment data was 0.940 and also had a p value of 0.002. From the intraset output (table not shown but see Figure 3.4 B for Bi-Plot scores), variables that were strongly correlated with this axis were 'Major Cations' (r = 0.828), 'Texture' (r = 0.809) and 'Major Cation Analogues' (r = 0.644). Separation on the second axis, accounting for ~8 % of the explained variance was mainly due to weak positive correlations with 'Alumino-Silicates' (r = 0.682) and 'Transition Metals' (r = 0.613). Separation on the third axis (only 6% of the explained variance, see Figure 3.4 C & D) was due to a weak negative correlation with the 'Residuals Major' (r = -0.687) and 'Heavy Metals' (r = 0.622). The significant feature of the scores plot (Figure 3.4 A & C) is the positive relationship between Axis 1 and the more urbanized sites and the negative relationship with the less urbanized sites.

Repeating the CCA ordination with the inclusion of the 8 additional variables describing the nutrient and geographic status of the streams produced the results in Table 3.10 and Figure 3.5. For this second run the compound variables 'Transition-Metals' and 'Residual-Minor' were removed as these variables added little extra explanation to the data. Including the additional set of nutrient and geographic variables increased the amount of explained variance by 4% from 28.8 to 32.9 %. The species environment correlation of axis 1 increased from 0.940 to 0.989 (p = 0.002). The recalculated first axis had strong correlations with Elevation (r = -0.851), Area (r = 0.875), [PO_4^{3-}] (r = 0.828) and [NH_4^+] (r = 0.778) while the correlations with 'Major Cations' (r = 0.715), 'Texture' (r = 0.692) and 'Major Cation Analogues' (r = 0.568) were reduced. The correlation of individual explanatory variables on the remaining axes was not strong; 'Major Cations' (r = 0.569) and NO_x (r = 0.561).

[1] 0.002 is equivalent to 1/500. For 500 permutations this is the lowest calculable probability.

Table 3.9 Decomposition of variance explained & correlation of species data with canonical axes as determined by CCA of composite XRF and size class variables.[1]

Total variance ('inertia') in the species data:	2.7404	Axis 1	Axis 2	Axis 3
Eigenvalues		0.394	0.225	0.171
Mean		0.227	0.147	0.116
Min.		0.118	0.103	0.086
Max.		0.375	0.236	0.161
p**		0.002		
Variance in species data				
% of variance explained		14.4	8.2	6.2
Cumulative % of variance explained		14.4	22.6	28.8
Pearson Correlation* Spp-Envt		0.940	0.905	0.949
Mean		0.810	0.841	0.841
Min.		0.679	0.651	0.653
Max.		0.943	0.949	0.954
p**		0.002		

* Correlation between sample scores for an axis derived from the species data and the sample scores that are a linear combination of the environmental variables. Set to "0" if the axis is not canonical.
**p is not reported for axes 2 and 3 because using a simple randomization test for these axes may bias the p values.

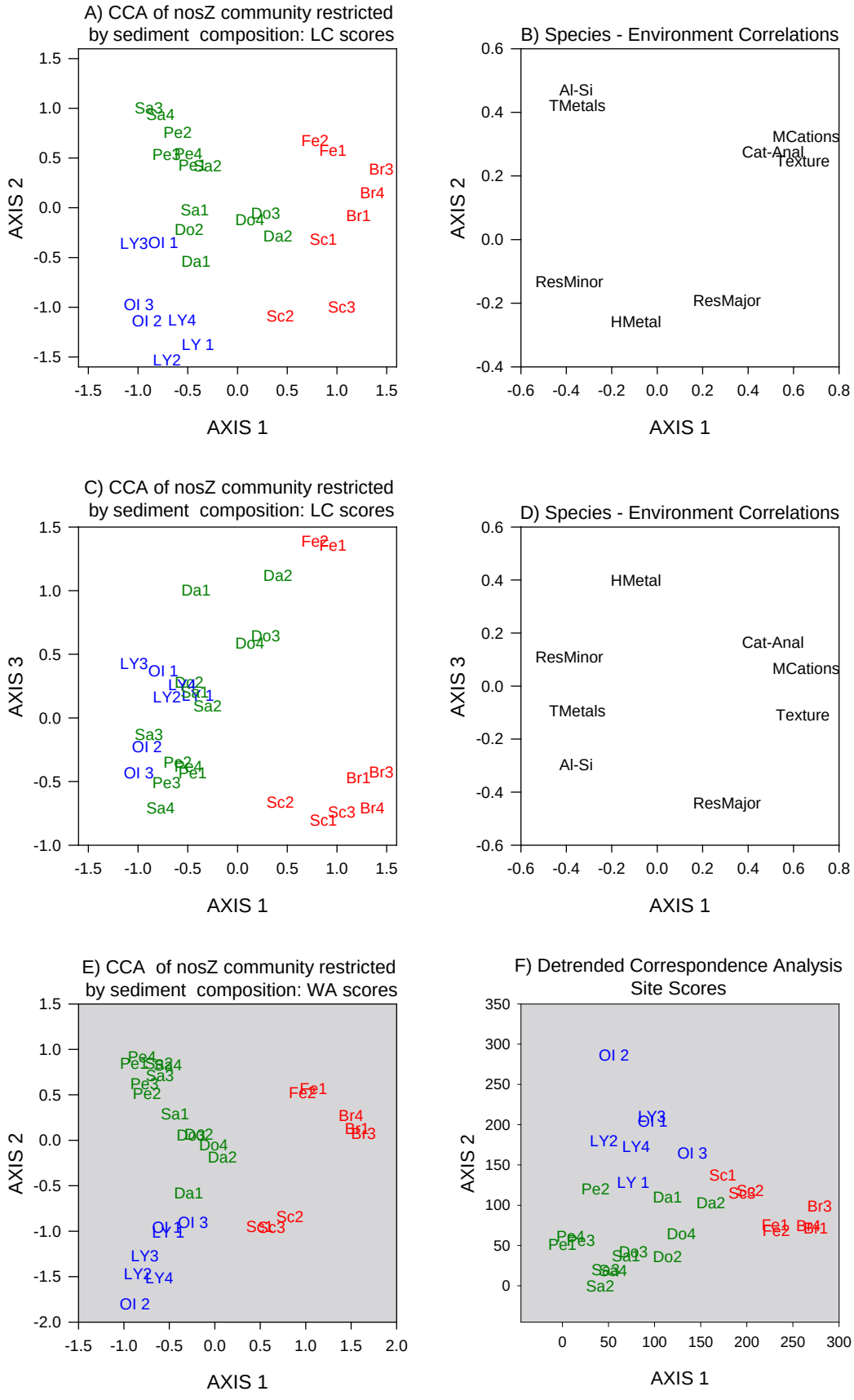
Table 3.10 Decomposition of variance explained & correlation of species data with canonical axes as determined by CCA of selected composite XRF and size class variables with the inclusion of stream nutrient data and geographical indices.

Total variance ('inertia') in the species data:	2.7404	Axis 1	Axis 2	Axis 3
Eigenvalues		0.446	0.274	0.181
Mean		0.302	0.195	0.154
Min.		0.195	0.139	0.121
Max.		0.414	0.287	0.191
p**		0.002		
Variance in species data				
% of variance explained		16.3	10.0	6.6
Cumulative % of variance explained		16.3	26.3	32.9
Pearson Correlation* Spp-Envt		0.989	0.959	0.973
Mean		0.871	0.890	0.921
Min.		0.775	0.742	0.740
Max.		0.964	0.988	0.985
p**		0.002		

* Correlation between sample scores for an axis derived from the species data and the sample scores that are a linear combination of the environmental variables. Set to "0" if the axis is not canonical.
**p is not reported for axes 2 and 3 because using a simple randomization test for these axes may bias the p values.

[1] Earlier versions of PC-Ord calculated a p value for the second and third axis.

Figure 3.4 Canonical correspondence plots of the denitrifying community restricted by factored environmental variables describing the stream sediment condition.



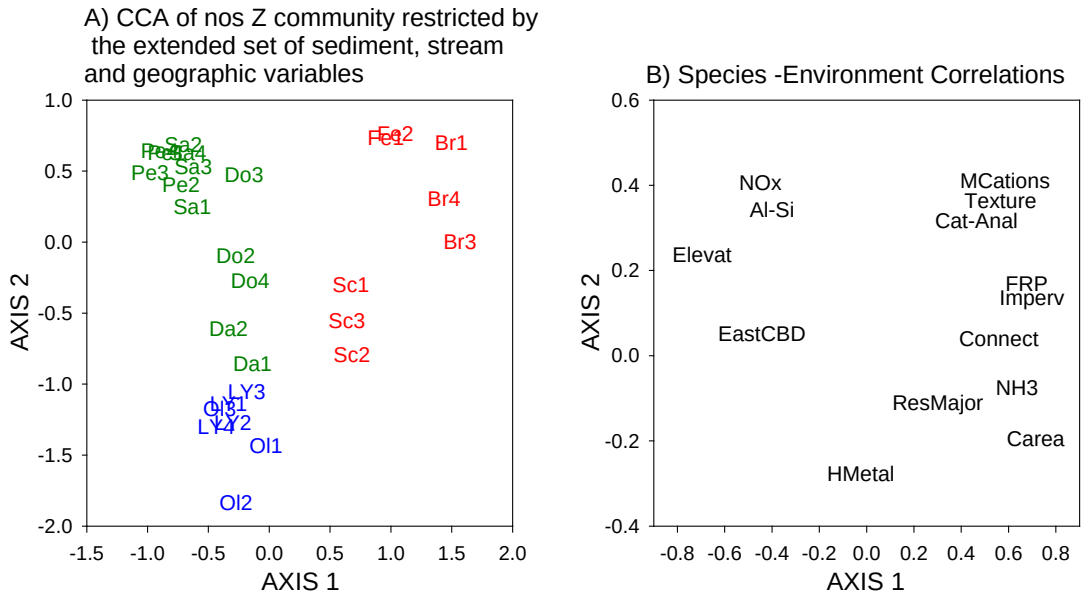


Figure 3.5 Canonical correspondence plots of the *nos*Z T-RFLP community restricted by PCA factored environmental variables describing the stream sediment condition after removing 'Transition-Metals' and 'Residual-Minor' compound variables but including additional variables representing stream nutrients and geographical indices of the sites.

3.5 DISCUSSION.

3.5.1 Ordination of the T-RFLP community data and comparison to the physico chemical variables.

Non metric multidimensional scaling.

The NMDS results and accompanying ANOSIM test presented here show unequivocal differences in the community structure between sites sampled along a continuous environmental gradient. However, unlike in Chapter 2 where the *a priori* assignation of groups was uncontroversial (i.e. urban stream vs. non urban stream; riparian soil vs. stream sediment), when sites are located on a gradient the only valid *a priori* grouping for the ANOSIM test was grouping replicates by site. Thus, although the ANOSIM analysis can, at the global level, detect that there are strong differences somewhere in the community data set (Table 3.4), the strength of individual differences between sites is often less clear cut and limited by the level of replication that was achievable within each site. This was particularly the case for the comparison between Dandenong Ck (DA) and Ferny Ck (FE). After screening out samples for which PCR was unsuccessful or where the resulting T-RFLP fingerprint had fewer than ten fragments, only 3 possible

permutations were available for generating the test statistic. Although the NMDS ordination shown in Figure 3.3 A has the same orientation is the adjacent PCA ordination of the XRF elemental composition data, this is purely coincidental. An NMDS ordination, especially one shown in 2 dimensions, is the 'best' projection of a multidimensional data set (with theoretically as many dimensions as species) via a polar transformation into the reduced space whilst maintaining the underlying relationships between samples. For this reason, the interpretation of the generated pattern is fluid; other than to indicate overall or general site differences.

Relying solely on the ANOSIM test (Table 3.4), it is possible to interpret the community data as suggestive of a threshold in some variables beyond which the community changes. As the only significant differences between groups was found between samples from the far ends of the urban – non urban gradient presented here, this might appear to be a reasonable interpretation. However, due to the nature of the test, incremental changes in the community structure often return non significant test statistics for the intermediate cases. The upshot of this is that the ANOSIM test, although well suited to testing a priori groups, primarily those of planned experimental treatments (Chapter 2), is not suited for gradient analysis – beyond establishing that there are differences *somewhere* in the data.

Principle components analysis of the XRF data.

Using PCA, the approach adopted was to be flexible in its application, but principally as a data reduction method while also attempting to establish interpretable factors. In the initial attempt at factoring the XRF data, the general relationship of the sites in the PCA ordination of the XRF elemental composition data Figure 3.3 B (not withstanding the coincidental orientation), suggested that there are differences in the sediment properties that are reflected in the general pattern observed in the NMDS ordination. The distinguishing feature of Factor1 (Table 3.5 & Figure 3.3) was the separation of sites based on the size factions and the major cations calcium and magnesium. This factor clearly separates the known urban sites (Brushy Ck, Scotchmans Ck and Ferny Ck) from the non urban sites (Lyrebird Ck & Olinda Ck). Some of the variables making up this factor can be related to the effects of urbanization noted in Tables 3.1 and 3.2. More urban sites have lower carbon retention; perhaps indicated here by the negative correlation with LOI (for urban sites) due primarily to increased discharge. A related effect of the increased discharges of urban sites is the retention of larger sediment size

classes. The review of Paul and Meyer (Paul and Meyer 2001) reports several studies that showed a correlation between increased concentrations of Ca, Mg, Na and K in urban streams. Further separation of sites was achieved by including the second factor which was dominated by Aluminium and the major first row transition elements. Interestingly, Factor 4 from this ordination was dominated by the heavy metals Pb, Zn and Cu. Although increased metal concentrations have been well documented in urban environments (Wilber and Hunter 1977, 1979a), the influence of other metals in this preliminary ordination has been spread across several factors.

Although ordination of data in this way simplified the data set, the correlations of relatively minor constituents with major constituents made factors difficult to interpret in a purely physical or chemical sense. This is partly due to the final zero mean – unit variance transformation employed but mainly as a result of mixing variables of significantly different magnitudes. The original unit of the XRF major elements was weight percent (wt %), whereas that of the minor elements was parts per million (ppm) and the Size Classes and LOI units are in percent (%). On performing the PCA, although legitimate correlations exist between major and minor components, the relative scales of these correlations are now potentially mismatched[1].

As stated in the introductory section, when performing CCA it is necessary for there to be fewer variables in the second restriction matrix than the number of sites for the method to more reliably indicate which of the selected environmental variables have important correlations with the community data (ter Braak 1986, Jongman et al. 1987, Jongman et al. 1995, Legendre and Legendre 1998). For this reason, the second PCA data reduction method was adopted to produce interpretable factors whilst reducing the total number of variables used to perform the CCA with a small loss of information. In this case, the 33 individual variables of Table 3.7 were reduced to three sets of factors, each containing 80% of the total variance in the original data, but now constituting a total of only 8 variables. An additional advantage of splitting the data set in this way is that individual factorizations become statistically more robust. As a means of simply reducing the data set and exploring correlations PCA can be performed on data where there are more

[1] This situation may also exist in the PCA results shown in Table 3.5 without this transformation as PCA uses scaled linear combinations of variables to produce the final factors.

variables than cases, but the results can be unreliable. Splitting the data set helps resolve this issue allowing factors to be more readily interpreted[1] when the number of cases exceeds the number of variables.

Variables aggregated under the composite factor 'Alumino-Silicates' are here interpreted as representing the major composition of the sediment matrix encountered in these streams, accompanied with Iron. Phosphorus is also located within this factor most likely as it forms associations with the oxides of both Iron and Aluminium (Richardson 1985). The second factor, 'Major-Cations' indicates that the major cations, which are also common constituents of minerals, are highly correlated: not surprising as Na & K and Ca & Mg have similar chemistries coming from groups I and II in the periodic table (Brown et al. 1991). Similar relationships can be seen in the minor elements where the heavy metals Zn, Cu and Pb form one factor ('HMetals'), the biologically significant transition metals (Wackett et al. 2004) Cr, Co and V (with Ga as a correlate) form a second factor ('Tmetals') and the elements Rb, Sr and Ba (also from groups I and II in the periodic table) form a factor. This later group shares some of the chemical properties of the elements that make up the 'Major-Cations' factor (Brown et al. 1991).

Although this second slightly more protracted PCA ordination removes some of the interesting correlations between variables suggested by the first round 'omnibus' approach, it does allow a more internally consistent interpretation of the newly derived variables in that variables related by similar chemical (or physical) properties are now generally grouped together. As a means of reducing complex data sets and avoiding risky data dredging using automatic variable selection, this approach is similar to the pre-clustering approaches adopted by Vaughan and Ormerod (Vaughan and Ormerod 2005) and Fitzpatrick (Fitzpatrick et al. 2007). The data used here is conceptually simple and can easily be seen as three blocks of related variables. In the situation where multiple habitat/environmental descriptors are available, using clustering approaches to establish groups before applying PCA to each group separately may be a fruitful approach (Vaughan and Ormerod 2005).

[1] Strictly speaking the 'factors' produced by PCA are called 'components' and represent purely empirical relationships among variables. True 'factors', as used in Factor Analysis are thought to 'cause' the distribution of variables (Tabachnick and Fidell 2001).

Canonical correspondence analysis.

The direct ordination (CCA) of the community data shown in Figure 3.3 A – E and Table 3.9 demonstrate strong support for at least one gradient (Axis 1, p = 0.002: Table 3.9 & 3.9), but that the total variance in the community data explained by this gradient is less than 20%. It can be seen that this gradient is derived mainly from positive correlations with the 'Major-cations' (Na, K, Mg, & Ca) and the analogues Ba, Sr & Rb ('Cat-Anal') and the sediment 'Texture', and weaker negative correlations with 'Alumino-silicates', transition metals and the likely co-correlates Ti and Mn ('Res-Minor') which are also transition metals. Table 3.7 shows that a strong positive correlation with 'Texture' suggests a strong relationship with the larger class sizes in the sediment and a strong negative relationship with the 'fines' (silt and clay like particles less than 355 um) and carbon (as measured by LOI). This data suggests that a possible structuring mechanism of the denitrifying community between sites is the coarseness or sandiness of the stream sediments.

Repeating the CCA ordination after removing two variables with little influence on the above ordination ('Transition-Metals' and 'Residual-Minor'), and including additional nutrient and geographic variables did not increase the explained variation in the community data much (Table 3.10). However, the new variables are correlated with the existing variables and the community data in a suggestive way (Figure 3.4). The concentration of PO_4^{3-} & NH_4^+, 'Connection', 'Imperviousness' and 'Catchment Area' are all positively correlated with the first axis, while the variables [NOx] and 'Elevation' 'EastCBD' and 'Alumino-Silicates' are negatively correlated with this axis. This alignment of the environmental-community ordination mirrors the results of Walsh et al. (Walsh et al. 2001, Hatt et al. 2004, Taylor et al. 2004, Walsh et al. 2005a). The more urbanized sites appear to the right of the ordination along with both the indices 'Connection' and 'Imperviousness' and direct measures of urban impacts 'Catchment Area' and concentrations of the nutrients PO_4^{3-} & NH_4^+. To the left of this axis are the sites affected to a lesser extent by urban processes. Again, 'Texture' and 'Alumino-Silicates' are located at either end of this axis, which could be explained by a greater retention or input of finer particles in the less urban streams and the coarser sedimentary material found in the urban streams as a result of the flow regime.

Although it cannot be interpreted as causative, the concordant orientation of the indices 'Imperviousness' and 'Connection' with nutrient data (both positive and negative correlations), independently shown to correlate with measures of urbanization, suggests there are processes associated with urbanization that are, at least in part, responsible for structuring this community. To elaborate, the sediment bacterial community does not directly sense these constructed or abstract indices ('Imperviousness' and 'Connection') measuring the degree to which individual streams have been impacted by urban processes, so that in principle the relationship between the *nos*Z community and these indices could be arbitrary. However, the strong association between these indices and plausible structuring variables (e.g. sediment texture, nutrients) suggests an indirect relationship between the urban gradient and the distinct separation of urban and non urban sites along this gradient. This pattern also demonstrates that the denitrifying community described by the *nos*Z gene is distinctly different between the urban and non urban environments studied, thus confirming the initial findings of Chapter 2.

3.5.2 Possible mechanisms and factors structuring the stream sediment bacterial community.

It is almost cliché to observe that the list of variables responsible for structuring bacterial communities is as large as the number of environments from which species have been isolated. To date however, many studies have focused on those key limiting nutrients (Schut et al. 1993, Bussmann et al. 2001, Connon and Giovannoni 2002) and metabolic substrates (Locher et al. 1989, Evans et al. 1991, van Schie and Young 1998) or anti microbial agents (among others) that have proven effective in isolating species in culture (Myers and Varela-Diaz 1973, Ferris and Hirsch 1991). Although interesting as amenable demonstrations of ecological theory and as a practical tool for obtaining and maintaining pure cultures, the extent to which some of these variables affect community structure in extant environments has not been as clearly demonstrated (Madigan et al. 2000, Gray and Head 2001).

Texture.

Generally, grain size is recognized as a controlling factor in the ecology of the hyporheos (Brunke and Gonser 1997, Boulton et al. 1998, Johnson et al. 2003, Ulrich and Becker 2006). In a review of the functional significance of the hyporheic zone, Boulton (Boulton

et al. 1998) comments that "*[f]ine scale granulometric features (size, shape, and composition of sediments) derive from catchment scale geological processes and determine most physical and chemical processes in the [hyporheic zone]. Interstitial flow patterns are a product of hydraulic gradient (direction and strength of flows…) and streambed porosity*". Brunke is of the opinion that "*[g]rain size and grain size distribution are the most influential ecologically*" (Brunke and Gonser 1997), Ulrich and Becker found that texture "*showed only a partial relation between the soil texture and the community structure*", but they also note that other studies found "*[s]oil texture was especially proved to have a decisive influence on the composition of the bacterial communities*" (Ulrich and Becker 2006). Sediment particle size was also shown to be a strong determinant of bacterial biomass, ranked ahead of sediment DOC concentrations in a study of four streams with different catchment land use (Bott and Kaplan 1985b).

Previous research has also found that denitrification rates are associated with sediment texture (Pinay et al. 2000, Vidon and Hill 2004, Inwood et al. 2005) among other factors (Hedin et al. 1998). Vidon and Hill (2004) found that "*the location of 'hot spots' of denitrification within riparian areas can be explained by the influence of key landscape variables such as slope, sediment texture and depth*" as these control the delivery and interaction of the electron donors (nitrates) and electron acceptors (carbon substrates). In the systems studied, Pinay (2000) and Inwood (2005) found that a positive relationship exists between the percentage of silt and clay and the denitrification rate; below a threshold of 65% silt and clay content, denitrification rates where negligible (Pinay et al. 2000).

Texture has two primary influences on the bacterial community. The first is that bacterial biomass is highly correlated with the smaller sized silt and clay fractions (Bott and Kaplan 1985b, Boulton et al. 1998, Pinay et al. 2000, Sessitsch et al. 2001, Inwood et al. 2005), principally as this fraction has the greatest surface area. The second is by structuring the interaction of the electron donors and acceptors (i.e. delivery mechanisms) (Brunke and Gonser 1997, Vidon and Hill 2004) and by setting the stage for the creation of redox gradients by the bacterial community (Boulton et al. 1998). These interactions are represented conceptually in Figure 3.6 where changes in texture are shown in two dimensions as a change in the size distribution from gravel to silt (left to right) along the x axis, which corresponds to an increase in the total particle surface area of sediment.

The vertical axis represents a change in the packing density or pore volume of the sediment particles which is influenced by the shape of the particles (Boulton et al. 1998). The interplay of these parameters affects the delivery of nutrients by advective flow within the sediment. Coarser grains allow greater flow than fine grains; a tighter packing reduces the pore space which reduces the flow.

Bacteria that require the efficient delivery of nutrients (e.g. oxygen, organic substrates) may colonize these sediments to a greater depth, but at lower overall cell densities than fine sediments, since the coarser grained sediments have a lower overall surface area than fine grains. As the fine grained sediments restrict advection, oxygen will be rapidly depleted leading to the development of a steep redox gradient (see also Figure 3.7). Although the surface area available for colonization is greater, bacteria that reside in these sediments may have less access to the delivery of material via advective flow through the sediment. This is shown in Figure 3.6 by the development of three hypothetical bacterial communities (**A**, **B** & **C**) at different depths in the sediment depending on each community's requirement for the delivery of nutrients. For example, community **A** (Figure 3.6) could represent heterotrophic aerobes; community **B** could represent denitrifiers or facultative anaerobes and community **C** obligate anaerobes.

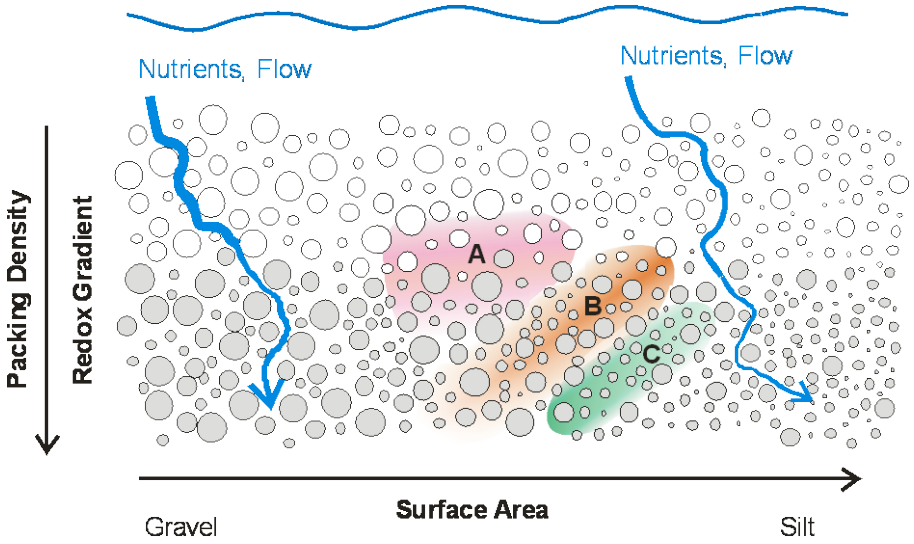


Figure 3.6 The interaction of particle size distribution and packing density determines the effect of texture on bacterial community location and activity. Blue arrows represent two hypothetical flow paths delivering 'nutrients'. The total surface area of the sediment particles increases in the direction of an increase in the proportion of silt. For a given size distribution the packing density can vary (e.g. due to shape and grading). **A**, **B** and **C** represent the locations of three hypothetical bacterial communities, positioned at some optimal point depending on the delivery of nutrients which is determined by the interaction of Surface Area and Packing Density, which together represent the texture.

As mentioned above, since the velocity of fluid passing through a bed of smaller particles is lower (Figure 3.7), this increases the contact time of reactive chemical species with the colonizing bacteria. This increased contact time, coupled with the greater surface area available for bacterial colonization, facilitates the removal or transformation of reactive species. This is represented in Figure 3.7 (lower graphs) by the greater rate of change of the interstitial concentrations with depth in to the bed. Implicit in this argument is the idea that texture has, at least in part, a moderating effect on the concentrations of nutrients experienced or sensed by bacterial communities.

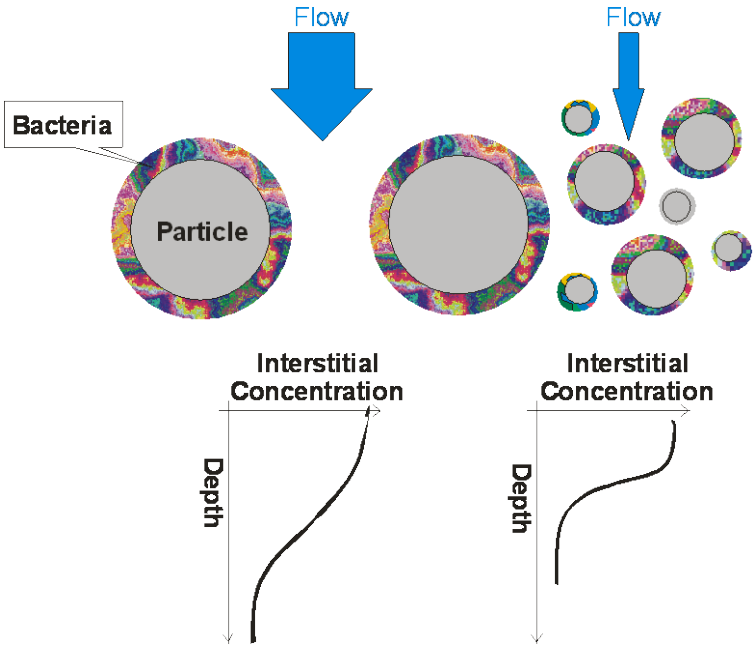


Figure 3.7 The effect of particle size and associated attached bacteria on the chemical composition of the interstitial pore water. The blue arrows indicate the directions and relative magnitude of flows resulting from the different sediment textures (gravel left, silt right). Graphs indicate the changes in interstitial concentrations of nutrients with depth in the different sediments[1].

Sediment parent material.

The parent material from which soils and sediments are formed is a significant factor in determining the structure of microbial communities (Boulton et al. 1998, Girvan et al. 2003, Ulrich and Becker 2006). Fungal communities involved in the process of rock dissolution are strongly influenced by the surface chemistry of the minerals that they have

[1] Both Ficks Law of diffusion and Darcy's Law of flow in porous media have a linear realtionship with porosity: an increase in porosity (ie an increase in particles size) leads to an increase in both diffusive fux and discharge.

colonized (Gleeson et al. 2005, Gleeson et al. 2006). Using the major element chemistry to classify rock samples (Gleeson et al. 2005) demonstrated that the mineralogy of granitic rocks determined the fungal community structure, going so far as to postulate "*that individual chemical elements exert selective pressure on fungal populations in situ*." Heavy metals were also found to affect fungal populations on granite outcrops, with community responses possibly being moderated by the surface reactions and exchange processes between the measured heavy metals and the underlying parent mineral (Gleeson et al. 2006). Direct manipulations of the soil mineral composition have clearly demonstrated substantial changes in bacterial community composition (Carson et al. 2007).

Table 3.9 shows that one axis (Axis 1, Figure 3.4 B & D) of the species - environment ordination is significant, although it explains a relatively small proportion of the total variance in the data. Two groups of variables have high loadings on this axis. Texture and the two Cation Groups (MCations, Cat Anal) have a positive correlation while the factors describing the alumino-silicate and transition chemistry of the sediments (Al-Si, TMetals and ResMinor) have a negative correlation with this axis. This separation is similar to the results reported by Gleeson (Gleeson et al. 2005, Gleeson et al. 2006) as shown in Figure 3.8 (see also Appendix 1 Figures A3.1 and A3.2). These differences could be viewed as a division between the more exchangeable elements (Na, K, Ca and Mg) found in many mineral phases (e.g. Calcite, Dolomite, Biotite etc) and the less readily weathered elements Silicon (e.g. quartz), Iron (e.g. goethite) and Aluminium (e.g. Gibbsite)[1]. The surface chemistries of Silicon, Aluminium and Iron oxides lead to the production of pH dependant charges that affects the anion exchange capacity of soils and sediments (Barrow and Bowden 1987, Froelich 1988, Jacobson 2000).

[1] See Table A3.2 Appendix 1

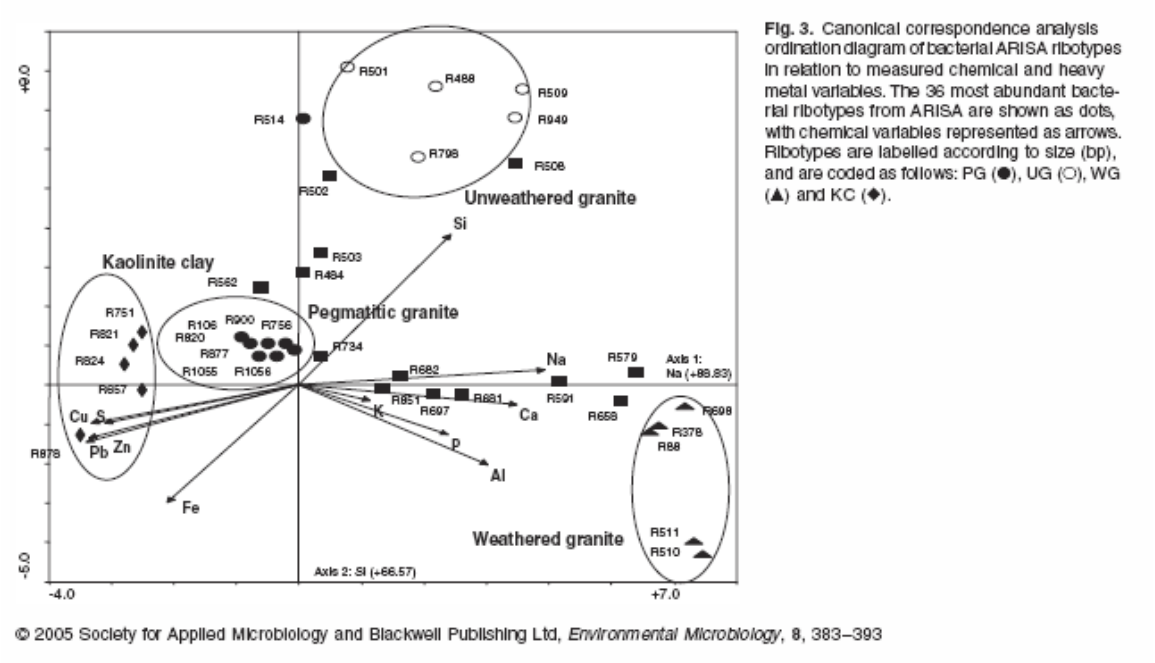


Fig. 3. Canonical correspondence analysis ordination diagram of bacterial ARISA ribotypes in relation to measured chemical and heavy metal variables. The 36 most abundant bacterial ribotypes from ARISA are shown as dots, with chemical variables represented as arrows. Ribotypes are labelled according to size (bp), and are coded as follows: PG (●), UG (○), WG (▲) and KC (♦).

Figure 3.8 Reproduction of Fig. 3 from Gleeson et al (2006).

In view of the above, a plausible mechanistic scenario accounting for the bacterial community structure might simply view these changes as a response to weathering processes of the source material derived in the upper catchment areas, for example by erosion of the Dandenong Ranges (Section 3.2), overlaid on the pre existing geologic formations. Using this template, material derived from the upper catchment is gradually reduced in size as it moves through the landscape. In the process, leaching and other weathering processes alter the mineralogy of the source material. At the macro level this interpretation might account for the distribution of sites observed in Figure 3.5 A reflecting the general geographic layout seen in Figure 3.1.

Tempting as it is to attribute much of the observed variation to the source rock and changes due to weathering processes, at least two issues need resolving. Why are larger particles in the lowlands, and why are silicon, and the major cations group (Ca, Mg, Na & K) slightly higher in concentration (Table 3.6) and correlated (Table 3.7) in the urban lowlands? As mentioned in the introductory section, one of the consistent responses of streams subject to increased urbanization is increased and rapid changes in discharge (Table 3.1). Whereas streams and rivers in lowlands typically have finer textured sediments, the flashy flows encountered in urban streams flush these fractions, leaving only the coarser materials. The observed pattern of larger particles is therefore most likely a result of the increased flows in these sites as a direct result of the increased

imperviousness found in these catchments. The above hypothetical delivery and weathering mechanism should produce mineral phases in the lower catchment with reduced concentrations of the more easily exchanged metals. The most likely simple and expedient explanation for this may be that the urban gradient, the altitude gradient and the distance of sites measured from the CBD are all also correlated with distance from Port Phillip Bay, and the elevated and correlated levels of these particular cations may at least partly be due to there presence in wind borne sea spray.

3.6 CONCLUSION.

The ordination techniques (NMDS & CCA) clearly show that different communities of denitrifiers are present at the different sites and, as in Chapter 2; these changes mirror changes of physico chemical variables as represented by ordination using PCA (Figure 3.3 B). Although the similarity matrix underlying the NMDS ordination allows a robust test using the ANOSIM routine (Section 3.4.1), this test is only valid for *a priori* groupings. As such, while gross changes are readily detectable, incremental change was not. Given the inconclusive results of indirect gradient analysis found in Chapter 2, this chapter explored the alternative of direct gradient analysis as implemented in CCA. To simplify the data set, the environmental variables were used to construct new aggregated variables using PCA; combining those variables that were highly correlated into single variables. This simplification of the data avoids some of the problems encountered using the BIOENV routine. The CCA analysis suggested that the *nos*Z bacterial community structure did vary in a manner consistent with the existence of at least one gradient (Axis 1 Figure 3.5, Table 3.10) although the total amount of variance explained was low (16.3%) but significant ($R = 0.989$, $p = 0.002$). While at least one gradient appears to be present, and this gradient could potentially be explained by processes resulting from the phenomena of urbanization, the confounding factors of altitude and possible oceanic influence (sea spray) can not be ruled out as important structuring agents; as both of these factors can be related to some of the observed changes in key variables.

One group of variables was identified as important for structuring the bacterial community and a mechanism as to why proposed. Sediment texture has previously been identified as an important property of soil and sediment influencing the bacterial community and the results here suggest the same. As a master variable, texture

influences both the physical (i.e. the surfaces), and the chemical (delivery of nutrients) environments in which bacteria exist. The interactions of these are not only key structuring factors of bacterial communities, but also, changes in texture can be related to the changes resulting from the effects of urbanization.

In this Chapter, PCA was employed to aggregate variables and simplify the data set used as input for CCA. Although appearing intimidating, this procedure usefully summarized the complex correlations with the T-RFLP data in a manner that was interpretable. Although this step involves more work, in situations where it is unclear which individual variables are responsible for driving bacterial community structure, or for exploratory studies like this one, this procedure may be preferable to 'cherry picking' potential master variables: especially when there is the need to limit 'over fitting' (i.e. the case where there are more descriptors than cases). Adoption of this procedure might also highlight those variables which should consistently be included by all researchers so that future studies are more directly comparable.

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CHAPTER 4

CORRELATIONS BETWEEN CARBON COMPOSITION & THE DENITRIFYING BACTERIAL COMMUNITY STRUCTURE ALONG AN ECOLOGICAL GRADIENT.

OVERVIEW.

Chapter 3 presented the conceptual framework behind CCA and used this technique to investigate relationships between catchment urbanization and environmental variables. The previous chapter showed very broadly that carbon had a role in structuring the community and could explain some of the differences seen along the urban gradient. This chapter further explores the type of carbon in the streams and tests the hypothesis that the observed changes in denitrifying community structure along the urban gradient are related to changes in the carbon composition of the sediments from streams along a gradient of urbanization. Two methods are used to qualitatively measure the carbon composition of the sediment samples and to then relate changes in carbon composition to changes in the bacterial community structure. Fourier Transform Infrared (FTIR) spectra are produced when materials in a sample absorb infrared radiation that corresponds to energy transitions due to the oscillations (stretching, bending, scissoring, wagging and spinning) of chemical bonds. Excitation Emission Matrix (EEM) spectra are generated from the fluorescence emitted when a sample is excited over a range of wavelengths of light. As sample data for both the EEM and FTIR spectra can easily contain thousands of response variables, easily exceeding the number of samples taken, a method of qualitatively reducing the data set is employed. As in Chapter 3, these new variables can then be compared to the bacterial community data, using the Canonical Correspondence Analysis (CCA) technique described in Chapter 3.

4.1 INTRODUCTION.

4.1.1 Carbon composition in aquatic environments.

Carbon is an important determinant of bacterial communities in aquatic environments (Bott and Kaplan 1985a, O'Connell et al. 2000, Eiler et al. 2003, Lennon and Pfaff 2005, Wawrik et al. 2005, Docherty et al. 2006). Augmenting lotic systems with available carbon can dramatically alter the food web of an entire stream (Wilcox et al. 2005). The concentration of dissolved organic carbon (DOC) is also an important factor controlling nitrogen transformations and in carbon limited streams DOC stimulates bacterial growth (Hedin et al. 1998, Bernhardt and Likens 2002, Groffman et al. 2002). Concentration is not the only control; the bioavailability of DOC also exerts selective pressure on the microbial community (Foreman et al. 1998, Sobczak and Findlay 2002) and the fate of nitrogen in streams (Sobczak et al. 2003, Arango et al. 2007). Denitrification rates in different ecosystems are variable in part due to differences in the supply and availability of DOC (Piña-Ochoa and Álvarez-Cobelas 2006, Arango et al. 2007, Inwood et al. 2007, Groffman et al. 2009). When nitrate is not limiting easily absorbed and assimilated carbon sources can increase denitrification rates (Constantin and Fick 1997). Distinguishing the source and availability of carbon is in important step in evaluating the effects of DOC on microbial processes and community composition (Garland and Mills 1991, Drenovsky et al. 2004, Lennon and Pfaff 2005, Sachse et al. 2005).

In small streams, catchment-derived allocthonous carbon is (usually) the dominant source of carbon, and the process by which this material is assimilated and ultimately mineralized (i.e. oxidised to CO_2) depends on a host of biological and physical factors (Bilby and Likens 1979, Webster et al. 1999, Harmon et al. 2004). Coarse material that enters the stream is colonized by a host of fungal and microbial species, reduced in size and composition by the action of macro invertebrates or physical disruption, abrasion and leaching that are a function of stream hydrology (Bilby and Likens 1979, Petersen et al. 1989, Webster et al. 1999, Hieber and Gessner 2002, Harmon et al. 2004). Organic mater in course and fine particulate states is both metabolic substrate and physical habitat for microbial species (Romaní et al. 2004). While coarse material constitutes the bulk of the carbon entering streams, especially headwater streams, fine and dissolved organic

fractions are the forms in which they are exported (Bilby and Likens 1979, Meyer et al. 1998, Webster et al. 1999).

The biogeochemical processing of organic carbon yields a variety of high and low molecular weight compounds of different but sometimes poorly characterized structure (Kalbitz et al. 2000). In the headwater stream (White Clay Creek) studied by Volk et al. (1997), 75% of the DOC consisted of humic substances with the remainder being composed of material greater than 100 kDalton. On average, carbohydrates made up 30% of the stream DOC with most present as polysaccharides. Amino acids composed only 4% of stream DOC. Of the carbohydrates ~66% was humic bound but only 55% of this was available[1]. For the amino acid component of DOC, 80% was associated with humic substances and 48% of this was available to hetertrophic bacteria. The humic substances were found to be refractory compared to the fraction of DOC greater than 100 kDalton in mass (Volk et al. 1997). Processing of carbon changes the chemical composition. Engelhaupt and Thomas (2001) found that the percentage composition of aliphatic, carbohydrate, aromatic and carboxyl carbon in stream water high molecular weight DOC (41%, 33%, 10% 16%) was different to that found in either the source riparian vegetation (28%, 48%, 15%, 10%) or laboratory prepared leachates (36%, 32%, 12%, 20%) from the source vegetation. The size and structure of an organic molecule in turn determines the relative availability of the molecule to microbes (Amon and Benner 1996, Docherty et al. 2006).

Some of the more common components of cell tissue include carbohydrates like starch and sugars; polysaccharides like cellulose and hemicellulose, chitin, lignin, proteins and lipids (Madigan et al. 2000, Moat et al. 2002, Adl 2003). Proteins, lipids and nucleic acids are the most readily decomposed cellular components as due to there high nutrient content (i.e. the N in protein and P in nucleic acids) all microorganisms have enzymes to degrade these substrates (Adl 2003). The energy storage molecules (starch, lipids and glycogen) are also readily decomposed but since plants translocate starch (and nutrients) before leaf senescence, it is usually a small percentage of the carbon entering streams (Adl 2003). Cellulose, the main structural component of plants is degraded only by certain species of bacteria and fungi (Manahan 1999, Carey 2000, Moat et al. 2002, Adl

[1] Calculated from Table 2 Volk et al. (1997).

2003). Lignin is the most common cell wall component after cellulose and hemicellulose, and is made from monomers of phenyl propanoid units. It is hard to digest and is normally only decomposed by fungi, with bacterial decomposition limited to anaerobic environments (Moat et al. 2002, Adl 2003). Lignin can have a controlling effect on the biodegradation of plant matter with low lignin tissues decomposing more rapidly, however nitrogen fertilization can increase the rate of lignin decomposition (Adl 2003). Chitin consists of polymers constructed from the monomer *N*-acetyl glucosamine and is the main cell wall component of fungi and invertebrate exoskeletons (Carey 2000, Moritz et al. 2009). *N*-acetyl glucosamine is also a component the peptidoglycan found in the cell walls of gram positive bacteria (Moat et al. 2002, Moritz et al. 2009).

The physiological constraints imposed by the bioavailability of different carbon substrates can be used to fingerprint the microbial functional diversity based on substrate utilization patterns (Degens and Harris 1997, Schutter and Dick 2001, Bucher and Lanyon 2005). While microbes actively take up low molecular weight compounds[1], the labile moieties of high molecular weight compounds are cleaved by the release of extra cellular enzymes (Findlay et al. 1997, Vetter et al. 1998). Measurements of the activity of these enzymes can be used to infer which general class of organic matter is present (Findlay and Sinsabaugh 1999, Kirchman et al. 2004, Rulík and Spácil 2004, Harbott and Grace 2005, Harbott et al. 2005). While substrate usage patterns and enzyme activity measurements clearly reveal changes in carbon availability and therefore changes in microbial metabolism, since heterotrophic bacteria can express a broad spectrum of extra cellular enzymes, predictions about community composition based solely on enzyme activity (or substrate utilization patterns) are problematic (Findlay et al. 1997, Preston-Mafham et al. 2002, Kirchman et al. 2004).

Most of the early literature on carbon in lotic systems derives from headwater forested streams (Fisher and Likens 1973, Meyer et al. 1981). As stated in Chapter 3, carbon concentrations, retention, processing and sources in urban streams are all influenced by the dramatic anthropogenic changes in the surrounding catchment (Foster et al. 2000, Hook and Yeakley 2005, Miller and Boulton 2005, Izbicki et al. 2007). The range of potential pollutants is also high and can inlcude; pesticides (Daniels et al. 2000, Foster et

[1] Less than 600 Daltons (Adl 2003).

al. 2000); polycyclic aromatic hydrocarbons (PAH), solvents and oils and polychlorinated biphenyls (PCBs) (Whipple and Hunter 1979b, Schorer and Eisele 1997); pharmaceuticals, stimulants and detergents (Kolpin et al. 2002, Ellis 2006, Haggard et al. 2006, Kasprzyk-Hordern et al. 2009); sewage (Leeming et al. 1998, Daniel et al. 2002, Ellis 2006). The concentration of these contaminants is variable with higher concentrations frequently found during storm flows (Whipple and Hunter 1979b, Hook and Yeakley 2005). Increased flushing of carbon into urban streams during rain events is a direct consequence of the greater impervious surface areas and the greater connection of these surfaces directly to the stream: this also increases the flashiness of stream flow (Jennings and Jarnagin 2002, Hatt et al. 2004). However, increased storm flow delivery may be counterbalanced by reduced retention of organic matter in urban streams (Walsh et al. 2005b).

In the case of extreme contamination the analysis and determination of a single, or a class of related organic pollutants, easily demonstrates the effects of carbon source on microbial community structure (Fahy et al. 2005, Joynt et al. 2006). But, like cultivation studies where the carbon substrate is controlled in order to isolate microbial species based on their substrate preferences (Stevenson et al. 2004, Davis et al. 2005), the type of carbon present is essentially known in advance[1]. This is also true for experimental manipulations employing soil or sediment perturbed with known carbon additions (Stevenson et al. 2004, Nakatsu et al. 2005, Chénier et al. 2006). Knowing the type of carbon in advance makes method selection easy; justifying the time and cost of sophisticated methods (e.g. Nuclear Magnetic Resonance, Mass Spectrometry, and Gas Chromatography) optimized for that class of contaminant. However, useful general compositional information can be obtained using more widely available (and cheaper) spectrometric methods (Abbt-Braun et al. 2004, Pons et al. 2004, Fuentes et al. 2006, Madari et al. 2006, Fellman et al. 2008).

[1] I.e. examining sediment adjacent to an oil refinery immediately suggests the likely type of carbon contamination and therefore the kind of carbon analysis.

4.1.2 Describing the carbon present in aquatic environments: a review of two spectroscopic methods.

Infrared spectroscopy.

Infrared (IR) spectra result from the absorption of radiation that has the same energy as the rotational, vibrational and deformational energies of molecular bonds (Silverstein et al. 1997, Stuart 2004, Pansu and Gautheyrou 2006). The most commonly observed absorptions occur at energies that correspond to radiation in the mid infrared (4000 – 400 cm^{-1}). In diatomic molecules absorption of radiation can only occur if there is a change in the dipole moment of the molecule (Silverstein et al. 1997, Günzler and Gremlich 2002, Stuart 2004). The elemental diatomic gases (e.g. N_2, O_2 etc) do not produce an infrared spectrum but polar molecules like HCl do. The tri-atomic linear molecule CO_2 which is non polar in its resting state, is IR active due to asymmetrical stretching and bending vibrations. The exact location (i.e. wavelength) of IR absorption bands depends on the masses of the atoms forming each bond, the type and strength of each bond (single, double or triple) and the particular vibrational mode that the bond undergoes[1]. For example oxygen singly bonded to carbon has a much lower vibrational energy than oxygen doubly bonded to carbon. This principle is demonstrated in Table 4.1 and Figure 4.1 for the model molecules CO_2 OCS and H_2O. For the CH_2 group, the C – H stretch vibrational mode has a different energy to the C – H scissoring, wagging, rocking or twisting modes of vibration. In complex molecules additional coupling and overtone absorptions are observed and the spectrum becomes increasingly complex. For a nonlinear molecule, the number of normal[2] vibrational modes is given by the relationship;

$$\text{Number of normal modes} = 3N - 6$$

where N = the number of molecules (Coates 2000, Günzler and Gremlich 2002). The actual number of bands is often less than this as some modes are degenerate; having the same wavelength. Vibration modes 3 and 4 (collectively v_3) for both CO2 and OCS (Table 4.1 and Figure 4.1) are an example of this.

[1] The analogous mechanical model for these vibrations is a system of masses connected by springs.
[2] A normal mode is the "minimum set of fundamental vibrations, based on a threefold set of coordinate axes" (Coates 2000).

Table 4.1 The vibrational modes and absorption wavelengths for three model compounds. See the accompanying Figure 4.1 below the table.[1]

Vibration		Wavenumber (cm^{-1})	Mode	Wavenumber (cm^{-1})	Mode	Wavenumber (cm^{-1})	Mode
		CO_2		**OCS**		**H_2O**	
1	v_1	1285/1288*	Symmetric C=O stretch	859	C=S stretching	3657	Symmetric stretch
2	v_2	2349	Anti-symmetric C=O stretch	2062	C=O stretching	1595	Deformation
3	v_3	667	Deformation	520	Deformation	3756	Anti-symmetric stretch
4							Rotational fine structure

* infrared inactive

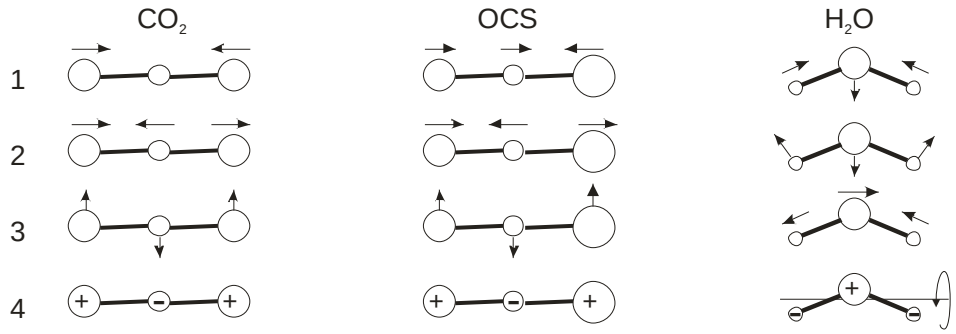

Figure 4.1 Schematic representation of the vibrational modes of the molecules of Table 4.1. Arrows indicate direction of initial motion, + & - signs indicate motion in and out of the page. For CO_2 and OCS, vibrations 3 and 4 occur at the same wavelength and are represented by v_3.

Although IR spectroscopy is a common qualitative technique in identifying organic functional groups of organic molecules, infrared absorption also occurs in inorganic molecules (Lawson 1961, Nyquist and Kagel 1996, Günzler and Gremlich 2002, Madejová 2003, Pansu and Gautheyrou 2006). As a result of this, interpretation of soil and sedimentary spectra must take into account the overlapping spectral contributions of both organic carbon and the inorganic matrix that makes up the bulk of most soils and sediments (Linker et al. 2005, Madari et al. 2006). A summary of the major mid-IR organic absorptions with the potential ecological interpretation is show in Table 4.2. Included in this table is a partial listing of the many overlapping inorganic or mineral absorption bands.

[1] Table 4.1 and Figure 4.1 adapted from (Günzler and Gremlich 2002).

Table 4.2 Infrared features found in the spectra of organic and inorganic samples*.

Wavenumber (cm^{-1})	Assignments	Comments	Overlapping Mineral Bands
3695, 3620			Suggests kaolinite like minerals; two sharp peaks
3380	O – H stretch	phenolic OH	OH stretch of water (Vermiculite)
3340	O – H stretch	Cellulose	
3400 – 3300	O – H stretch N – H stretch	Two well separated bands at 3335 in primary amines	OH stretch of water (Halloysite, Montmorillite, Gibbsite) NH_4^+ (3335 – 3030)
3100 - 2600	$-NH_3^+$stretch of amino acids		
3000	C – H stretch		
2920	Antisymmetric CH_2	Fats, wax & lipids	HCO_3^- (3300 – 2000)
2850	Symmetric CH_2	Fats, wax & lipids	
2940 – 2900	Aliphatic stretch		
2600 2600–2550	O – H stretch of -COOH Thiols, S – H stretch		
2200 – 2000	Cumulated double bonds (– C=C=C –, – C=C=N– , – C=C=O, -N=C=O etc.)		
1820, 1750	Anhydrides		
1725 – 1700 1740 – 1685	C=O stretch of COOH or COOR and ketones. Aldehydes.	Carboxylic acids, aromatic esters. Cellulose.	AlH_4^- (~1785)
1660 – 1630 1660 – 1600	C=O stretch of amide (amide I) groups, quinine C=O. C=N stretch.	Proteinaceous matter. Lignin and other aromatics	OH- bending of water molecules water (Halloysite, Montmorillite, Gibbsite) HCO_3^- (1700 – 1600)
1550	N – H in plane (amide II)	Proteinaceous matter.	
1590 – 1517	COO- symmetric stretch, N–H deformation + C–N stretch (amide II)	Proteinaceous matter.	
1525 – 1510	Aromatic C=C stretch	Lignin/Phenolic backbone.	
1426	Symmetric C – O stretch from COO- or stretch and OH deformation (COOH)	Carboxylate/Carboxylic structures (humic acids).	CO_3^{2-} (1320 – 1530)
1460 – 1450	Aliphatic C – H	Phenolic (lignin) and aliphatic structures	NH_4^+ (1485 – 1390)
1270 – 1265	C – OH stretch of phenolic OH or arylmethylethers	Indicative of lignin backbone	
1300 - 900	C – O or C – N stretch	Aliphatic amines (1220 – 1020) Aromatic amines (1360 – 1250)	
1170 – 950	Combination of C – O stretch and O – H deformation.	Polysaccharides Alcohols Alkyl ketones	Si – O of silicates (various clay minerals) PO_4^{3-} (1100 – 950) SO_4^{2-} (1130 – 1080)
1000	C – C stretch of aliphatic groups		Si – O of silicates (various clay minerals: SiO_4^{4-} 860 – 1175). SiOH broad band near 970
900 890 – 820	Out of phase ring stretch Peroxides, C-O-O- stretch	Cellulose	Al_2OH bending bands of kaolinite and dickite near 915 and 935. Strong Al_2O_3 band near 850
830 – 775	Aromatic CH out of plane bending	Lignin	CO_3^{2-} (880 – 800), Fe_2OH band at 817. AlMgOH bending bands near 842
720	CH_2 wagging	Long chain alkanes	Mg_3OH bending mode of chrysotile near 600
680 - 550			SO_4^{2-} (680 – 610) Fe–O out-of-plane vibration at 676 Strong Fe_2O_3 band from 650 – 550 Strong Al_2O_3 band near 600
560 – 510 500 – 470	Polysulfides (S – S stretch)		Strong CaO band at 560 – 550 Strong MgO band at 520 – 510
400			SiO_4^{4-} 470 – 540

* Adapted from (Nyquist and Kagel 1996, Coates 2000, Günzler and Gremlich 2002, Madari et al. 2006, Artz et al. 2008).

The IR technique has found widespread application in areas where precise identification of constituent compounds is not required but correct identification of samples or assessment based on some sample parameter is needed. For example, the identity and

purity of edible oils in the food industry using mid IR and multivariate methods was demonstrated by Sato (1994). In forensic investigations, Cox (2000) showed that soils of identical color can be identified from the difference spectra obtained between whole soil samples and samples after pyrolysis. Of more ecological interest, is the use of this technique to follow the decomposition process of forest soil in different soil horizons (Haberhauer et al. 1998, Haberhauer and Gerzabek 1999) or to find correlations between spectral components and either measures of soil constituent concentrations, indices of biological condition or heavy metals (Chapman et al. 2001, Dupuy and Douay 2001, Cohen et al. 2006, Janik et al. 2007). These and other examples in the literature demonstrate the potential of IR techniques with or without PCA decomposition in assigning samples to distinct groups based on qualitative data.

Excitation – emission fluorescence matrices (EEMs).

Valence electrons excited by light can jump to a higher electronic state when the quanta of energy contained in the absorbed light matches the band gap from the ground state (S_0) to the excited state (Silverstein et al. 1997, Skoog et al. 2004, Lakowicz 2006). This process is illustrated in Figure 4.2. The adsorption of light ($h\nu_a$ or $h\nu_b$) excites an electron from the ground state (S_0) to a higher electronic level (S_1 or S_2). The excitation of the electron is virtually instantaneous. In molecules, each electronic level is split into finer vibrational levels (0, 1, 2… etc) producing a band of possible absorption wavelengths. Internal processes and vibrational relaxation occurs rapidly before the electron returns to the ground state emitting light of longer wavelengths via fluorescence ($h\nu_f$) in a band. The fluorescence emission spectrum resulting from the $S_0 \rightarrow S_1$ excitation is often a mirror image of the absorption spectrum, shifted to longer wavelengths. This shift is called the Stokes shift and is generally greatest for polar molecules in polar solvents (Lakowicz 2006).

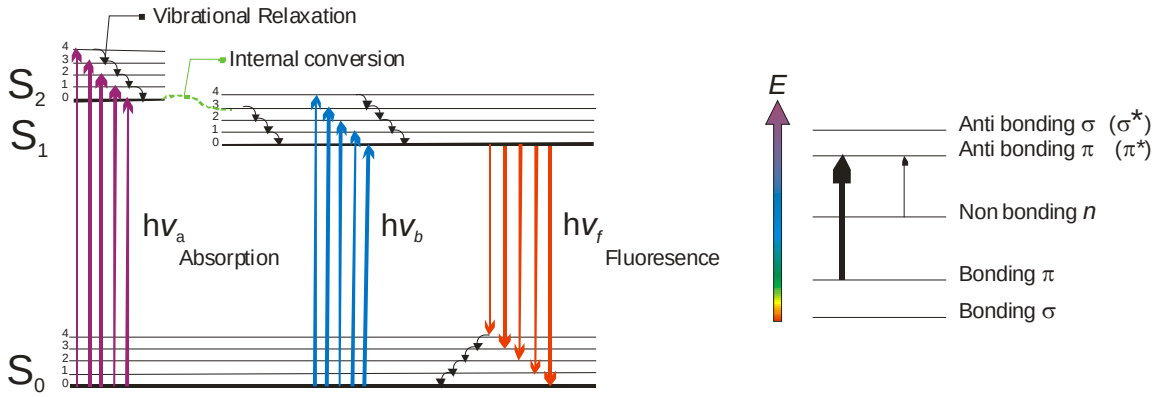

Figure 4.2 Electronic transitions after the absorption of light of different wavelengths. On the left is an energy level (S_0, S_1 & S_2) diagram with band splitting (0, 1, 2…). An electron excited and to a higher state by absorbed light goes through a series of energy relaxation processes and conversions before returning to the ground state emitting light. Non fluorescing relaxation processes are not shown. The relative energy required and probability of electron excitation is shown on the right. Although the $\pi - \pi^*$ transition requires more energy it has a higher probability (thick line) than the $n - \pi^*$ transition. σ = single bonds; π = double bonds; n = non bonding electrons[1].

The relative energy required for electron transitions in different bonds is shown to the right in Figure 4.2. Fluorescence from absorption at single bonds ($\sigma \rightarrow \sigma^*$) is rarely observed as the required energy is usually sufficient to break bonds (Skoog et al. 1998). This fact also puts a lower limit on excitation wavelengths in fluorescence spectroscopy of about 200 nm. Most fluorescence is the result of excitation of electrons in double bonds ($\pi \rightarrow \pi^*$) with a much smaller fluorescence signal from the excitation of non bonding electrons ($n \rightarrow \pi^*$) of atoms adjacent to a double bond (e.g. C=O or C–N=N–C) (Silverstein et al. 1997, Skoog et al. 1998). All molecules that absorb light have the potential to fluoresce but most do not as there exist faster non radiative ways for the excited electron to return to the ground state (Skoog et al. 2004). Molecular factors that favor fluorescence are the presence of aromatic rings or conjugated double bonds and structural rigidity of the molecule (Willard et al. 1988, Silverstein et al. 1997, Skoog et al. 2004).

The absorption processes described above are also observed in UV-Vis spectroscopy, but unlike IR transitions, adjacent atoms have little influence on the wavelength at which absorption occurs (Silverstein et al. 1997, Skoog et al. 1998). Thus a UV-Vis spectrum

[1] Figure adapted from Silverstein (1997), Skoog (1998) & Lakowicz (2006).

has many fewer features, and the absorbing sites (chromophores) from different molecules in a sample basically overlap at the same wavelength in the spectrum. The fluorescence spectra produced by excitation at a single excitation wavelength is also correspondingly simple (Figure 4.2). However, by producing an emission spectrum by incrementally increasing the excitation wavelength, a total fluorescence spectra or excitation emission matrix (EEM) is produced (Figure 4.3). The fluorescence produced by light absorption at similar chromophores (or at similar wavelengths) can be shifted to longer wavelengths or decreased in intensity depending on intramolecular interactions (Willard et al. 1988).

Figure 4.3 shows an idealized EEM with regions representing general classes of organic matter as reviewed by Chen et al (2003b). The two diagonal lines indicate the position of the Rayliegh scattering bands. Fluorescence signals appearing in regions I and II suggest protein signals while region IV suggests proteinaceous breakdown products of microbial origin. Regions III and V are indicative of plant derived carbon from different sources. As these large macro molecules contain many chromophores and possible fluorophores (Thomsen et al. 2002, Abbt-Braun et al. 2004, Fuentes et al. 2006), there is the potential for relaxation processes to proceed through a longer chain of broad overlapping excitation bands: emission at longer wavelengths is the result.

Using the EEM technique researchers have been able to use the fluoresence carbon spectra to track the discharge from landfills (Baker and Curry 2004), sewage (Baker 2001, Paul et al. 2001, Westerhoff et al. 2001, Sheng and Yu 2006), wetlands (Mariot et al. 2007) and rivers (Kowalczuk et al. 2003) in receiving environments and the movement or classification of coastal oceanic water masses (Persson and Wedborg 2001, Boehme et al. 2004, Zepp et al. 2004, Hall and Kenny 2007, Gonsior et al. 2008). Other studies interpreted the EEM spectra to determine the origin of particular carbon types in river systems by catchment land use (Baker 2002, Stedmon et al. 2003) or within aquatic systems, the relative contribution of different allocthonous and autocthonous carbon inputs (McKnight et al. 2001, Chen et al. 2003a, Hudson et al. 2007). The technique has also been used to monitor chemical processes: the interaction of metals and natural organic ligands (Wu and Tanoue 2001); the photodegradation of natural organic mater in river waters (Howitt et al. 2008); the oxidation state of fulvic acids (Klapper et al. 2002).

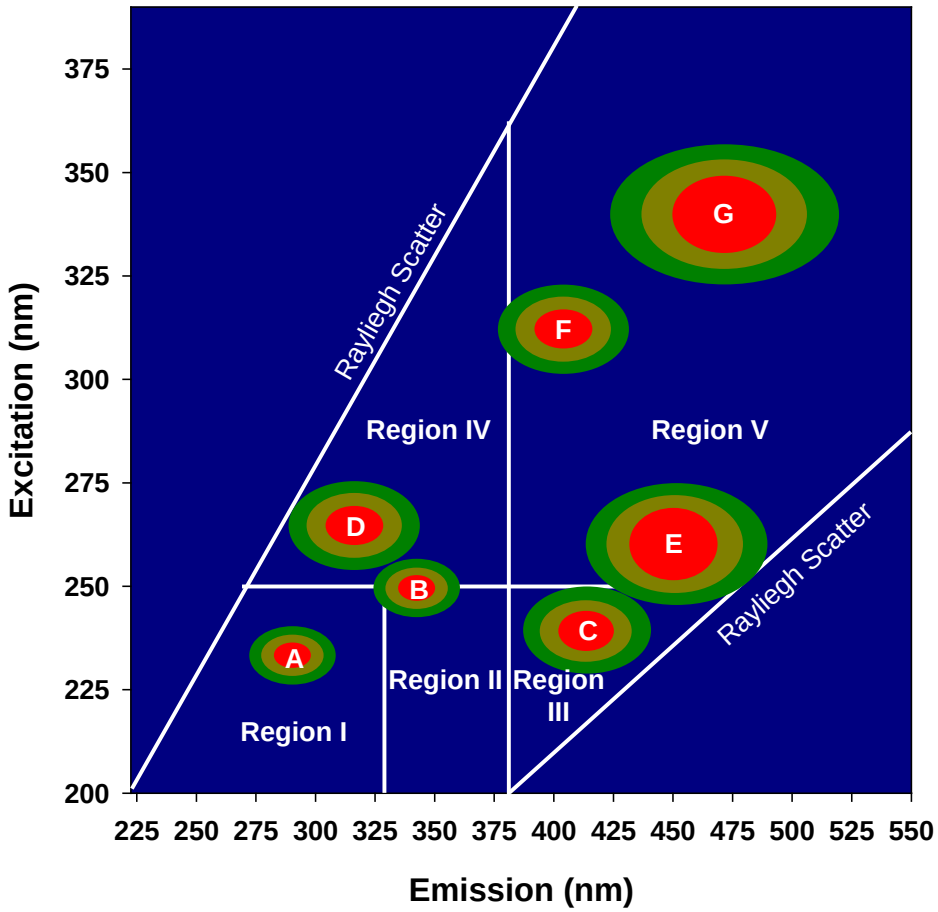

Figure 4.3 Regional representation of the fluorescence landscape from carbon naturally occurring in aquatic environments. Region I is aromatic protein similar to or containing tyrosine (A). Region II is aromatic protein similar to or containing tryptophan (B). Region III represents fulvic acid like compounds (E) but which may also be more hydrophobic (C). Region IV contains microbial by products of tryptophan containing proteins (D). Region V represents more humic like compounds with marine humus (F) distinct from freshwater and model humic compounds (G)[1].

4.2 METHODS.

4.2.1 Fourier transform infra red (FT-IR) spectra of dried whole sediment samples.

Sample preparation.

Sediment sample (~ 100 mg) was weighed into a clean glass test tube. Approximately 0.5g FT-IR grade KBr (Sigma-Aldrich) was also placed into each sample test tube, following which all samples were dried at 110 ^{o}C for 12 hours (i.e. overnight). Samples were cooled and stored in a desiccating chamber. The contents of each tube were poured

[1] Figure 4.3 adapted from Chen (2003).

into an agate mortar and ground quickly before being transferred to a stainless steel KBr pellet press. Pellets were formed under vacuum for 5 minutes after which they were returned to the desiccating chamber until FT-IR spectra were measured. The KBr pellets were placed in a Shimadzu FT-IR 8400S spectrometer. Infra red absorption spectra were accumulated from 16 scans at a resolution of 2 cm in the 400 – 4000 cm^{-1} range using Triangular Apodization. This procedure produced 2700 data points.

Manipulation and statistical analysis of the FT-IR data.

Each spectrum was translated to a common baseline by subtracting the minimum value, followed by normalizing the spectra by dividing each datum by the total absorbance for respective spectra. This process puts each spectrum into a common unit less space removing the differences in concentrations due to the differences in masses of both the sample and path length due to the amount of KBr used. This process means that differences in the relative heights of features of the spectrum will be extracted using the PCA method of factorization. This was performed using SPSS 15 using Varimax Rotation as the final extraction method.

4.2.2 Excitation emission matrices from hexane extracts of dried sediment samples.

Extraction and measurement of hexane extracts.

Sediment samples (~ 0.2 g) were placed in 3 mL of BDH Spec Grade Hexane in clean glass centrifuge tubes, mixed for 10 seconds on a vortex mixer and allowed to stand for 12 hours (over night) while kept at 4 ^{o}C in a refrigerator. Excitation Emission Spectra were collected using a Hitachi F 4500 spectrofluorimeter in a quartz cell with the excitation wavelength varying from 200 – 700 nm in 5 nm windows, while emission data was collected over the wavelength range of 200 – 800 nm. The scan speed was 0.25 s per excitation-emission pair using hexane as the background scan. The raw data sets contained over 3300 data points. Samples were kept at room temperature, and the instrument was stationed in an air-conditioned room (~22 ^{o}C). On the completion of each scan, the spectra were inspected for high (over 1000 fu) fluorescence values, not including the Rayleigh scattering lines. If high values were noted, to minimize the effect of self absorption the sample was diluted by half with hexane and a new spectrum recorded. This procedure was repeated until a suitable low fluorescence spectrum (i.e. peak values below 1000fu) was obtained.

Manipulation and statistical analysis of the EEM data.

For each sample, the lowest fluorescence point was located using the MIN function in Excel and all data points for that sample adjusted with the minimum value taken as a baseline. The data were further reduced by only including data points from the regions between the two Rayleigh scattering bands, by eliminating those regions common to all EEMs that did not contain any signal by removing the Rayleigh scattering bands and reducing the resolution from 5 nm to 10 nm by averaging adjacent cells. These manipulations produced a spectral data set with only 740 data points (compared to the original 3300). For plotting purposes the regions outside the Rayleigh bands were filled with the value zero, but for analysis by PCA these data were removed completely. This simplified the data reduction process greatly by reducing the number of data pairs and by eliminating 'noise' associated with the Rayleigh bands. Subsequently, the data for each sample was normalized by dividing individual data pairs by the sum of the total fluorescence scores for that sample thus putting each spectrum into a common unit less data space. The spectral data were arranged into excitation – emission pairs using Excel (see Figure 4.4)[1].

Data reduction using PCA was performed using Systat 10. Satisfactory factorization was achieved using Quartimax rotation. Site scores and loading scores were saved and the loading scores 'rewrapped', reversing the process described above, to give a visual representation of the spectral regions in the EEM contributing most to the total variation in the data. The site scores were used as new variables, along with those obtained from the FTIR data (see below) to compare the variation in this spectral data with the variation in the community data using the technique of Canonical Correspondence Analysis (CCA) as described in the previous chapter.

[1] Extensive data manipulation/rearrangements were necessary as the Hitachi F 4500 spectrofluorimeter outputs the data as a list of numbers. A macro written by Julia Howitt (2003) was used to partly rearrange data but many of the processes were performed 'by hand'.

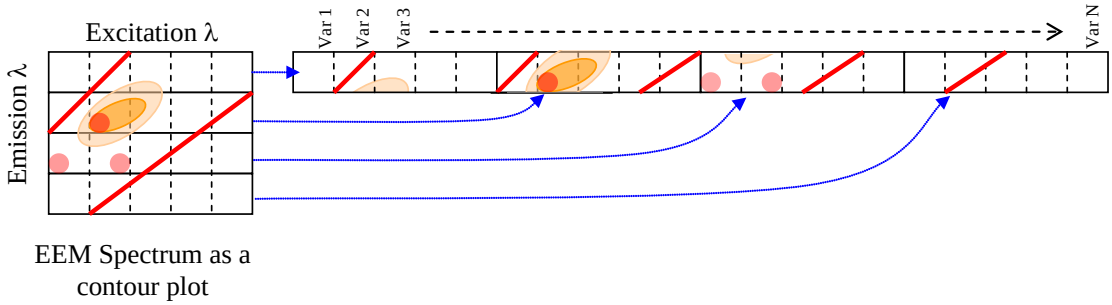

Figure 4.4 Simplified schematic showing the process of unwrapping a three dimensional EEM data set to produce a linear array of N data points suitable for analysis by Principle Components Analysis (PCA). Each sample spectrum is treated this way. Factor Loads can be 're-wrapped' to produce an EEM plot of the extracted factors. The two red lines indicate the Rayleigh scattering bands.

4.2.3 DNA extraction and analysis

As the community data is the same as that used in Chapter 3, see section 3.3.2 DNA Extraction and Analysis for complete details.

4.2.4 Canonical correspondence analysis of the *nos*Z T-RFLP community restricted by variables that qualitatively describe the sediment carbon.

Site scores from both the EEM and FTIR spectral data were used as variables that qualitatively represent the carbon present in the sediment samples. The first 5 principal components from each set (containing >95% of the variance of the original data) were retained for subsequent use in Canonical Correspondence Analysis using the presence/absence of *nosZ* T-RFLP community data. Restricted ordination was performed on the T-RFLP data with the factorized carbon variables as the second matrix using CCA as implemented in PC-Ord. The analysis was setup such that Site Scores were linear combinations of the variables with a compromise scaling of the ordination scores between Sites and T-RFLP fragments. A Monte Carlo test with 500 random restarts was performed to test the hypothesis that there was no relationship between the matrices. Rows and columns were additionally centered and normalized during this procedure

4.3 RESULTS.

Interpretation of the FTIR spectra and PCA loading plots.

The transmission FTIR spectra of several representative dried sediments are overlaid in Figure 4.5. Overall, the spectra show several regions that appear to be broadly similar (or featureless) with most of the obvious variation in the data confined to a few wavelength regions. The obvious broad peak centered on 3400 cm^{-1} is due to the stretching vibrations of bonded and non bonded hydroxyl groups in both organic and inorganic substances (see Table 4.2 above). The band at 1600 – 1650 cm^{-1} is probably due to the C=O stretch of carboxylic acids, aldehydes and ketones but amide groups and the C=N stretch can also contribute to IR absorbance at these wavelengths. There is evidence of a small signal at ~1400 cm^{-1} possibly due to a combination of the C – O stretch and aliphatic C – H signals: this could suggest humic material. The main feature of the spectra is the variable region from 1200 – 800 cm^{-1}. This highly variable portion of the spectra is composed of many infrared active absorption processes. Stretching of C – O (e.g. polysaccharides) or C – N (e.g. amines), the C – O stretch of phenolic OH (e.g. lignins), the C – C stretch of aliphatic molecules and the out of phase ring stretching of aromatic compounds (e.g. cellulose) all contribute IR absorption bands in this region of the spectra. But several important inorganic groups also absorb in this region of the infrared; Si – O of silicates, Al_2OH, Al_2O_3, CO_3^{2-} and Fe_2OH all have bands (Table 4.2). The remaining wavelengths are most likely a combination of bands from silicates and metal oxides, although out of plane bending of C – H and CH_2 wagging bands can potentially contribute to this signal. The two small peaks at 3670 and 3630 on the left hand side of the O – H stretching band are indicative of kaolinite clays.

PCA of the spectral data set produced 10 components after varimax rotation. The decomposition of the variance for each component is shown in Table 4.3. As the first 5 components cumulatively contain 95% of the variance in the original data and further components contributed less than 2% of additional variance each, this was set as the cut off point to carry the new variables, the site scores data, thru to the next stage of analysis. The regions of the spectra loading on each component are shown in Figure 4.6. These plots show which regions of the spectra determined each sites factor score for that component. In turn the factor score represents a new variable that shows how the sites are located with respect to each other as described by the IR spectral data.

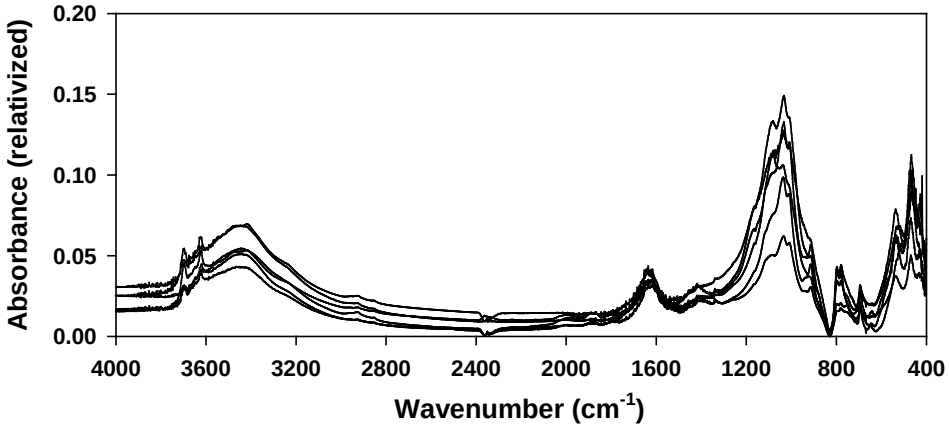

Figure 4.5 Example FTIR spectra taken from dried stream sediment. Most information is contained in the fingerprint region (400 – 2000 cm^{-1}). A comparison of this figure to those in Figure 4.6 shows that the main features are represented; the broad peak at 3400 cm^{-1}, the peaks at 1600 cm^{-1} and 1400 cm^{-1}. Peaks from 1200-400 cm^{-1} appear across factors.

Table 4.3 Variance explained by the first 10 principal components extracted from the FT-IR spectra of sediments pressed into KBr pellets.

Component	% Variance	% Cumulative
1	43.3	43.3
2	36.6	79.9
3	8.8	88.7
4	4.7	93.4
5	2.1	95.5
6	1.6	97.1
7	1.0	98.2
8	0.5	98.7
9	0.3	99.0
10	0.3	99.2

The first spectral component (Factor 1) shows a broad 'absorbance' (i.e. high loading) in the fingerprint region of the IR spectrum from 1600 – 700 cm^{-1}. At higher wavelengths (from 3600 – 2800 cm^{-1}) this component loads highly in a negative direction in the same region of the spectrum as the hydroxyl stretch. In the fingerprint region, there are peaks at 450 cm^{-1}, 780 cm^{-1}, 880 cm^{-1} and 970 cm^{-1}. From Table 4.2 these could suggest Silicates, aromatic CH (Lignin), ring stretching (Cellulose) or aluminates. The broad region centered on 1200 cm^{-1} (1600 – 1000) could be a combination of bands from the C=O stretch of carboxyl compounds and the C – O stretch of aliphatic molecules and alcohols but, Silicates also absorb in this region (see Table 4.2). Apart from some indeterminate signals in the fingerprint region at 530 cm^{-1} (possibly Ca or Mg oxides), 900 cm^{-1} &

1000 cm^{-1} (similar to assignments above), Factor 2 loads highest between 3000 – 1600 which may represent 'baseline adjustment' as spectra did not align perfectly after (see Figure 4.5). Factor 3 has a single large peak from ~1750 – 1550 cm^{-1} with a shoulder at 1450 – 1350 cm^{-1}. This broad peak is most likely a combination of bands arising from molecules containing the C=O group (carboxylic acids, ketones, amides) and possibly the C=C stretch of aromatics. Factor 4 has a prominent peak at 830 cm^{-1} suggestive of aromatics, but overlapping with CO_3^{2-} and a smaller band from 700 – 650 cm^{-1}, possibly mineral in nature. The last component, Factor 5, consists mostly of a single broad peak at 1440 which could be a combination of the C – O stretch of carboxyl compounds (e.g. humic material) with aliphatic C – H but this peak also could be due to CO_3^{2-}.

A further spectral component is shown in Figure 4.7. Although not used in later analysis this component shows the unambiguous signal of gaseous CO_2 trapped in the KBr pellets during pressing. Although a small nuisance signal, the near flat baseline of this factor demonstrates that the PCA method can extract "pure" signals when they are uncorrelated with, as this signal must be, other infrared active groups. Also shown in Figure 4.7 in the enlargement to the right of the CO_2 signal is the next factor. The signal for this factor can be seen in the baseline of Factor 9 demonstrating how, at its best, PCA can extract successive features.

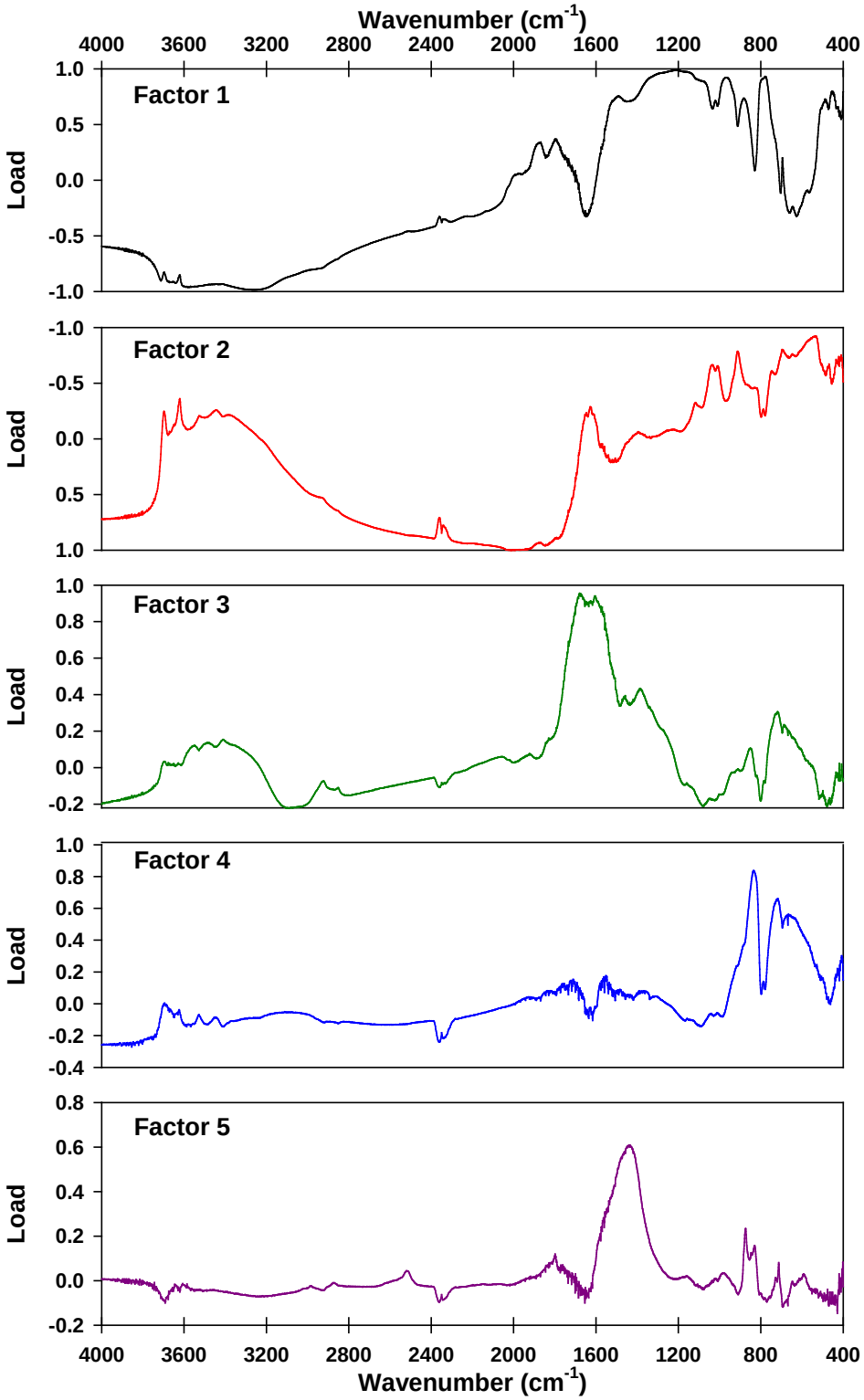

Figure 4.6 FT-IR loading plots for the first 5 principal components showing extracted infrared spectral components. Loads show which portion of the original variables most influence the extracted component.

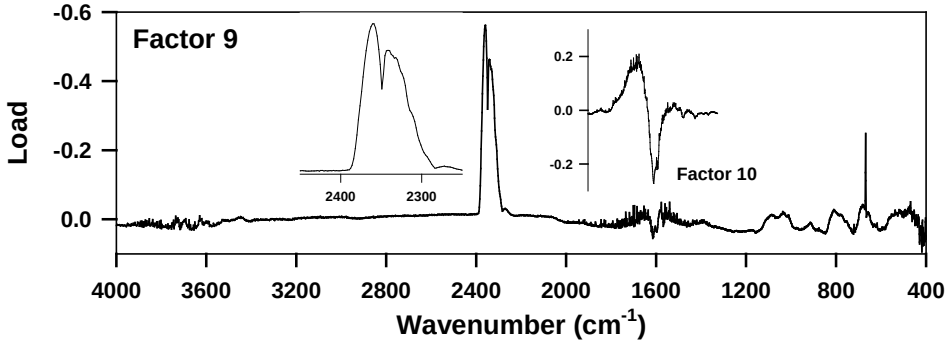

Figure 4.7 FT-IR loading plot showing an unambiguous assignment. Principal component 9 is gaseous CO_2. Enlargement to the left clearly shows the characteristic rotational signature of CO_2. Enlargement to the right is from the next principal component, the signal for which is visible in the baseline of this factor.

Interpretation of the EEM spectra and loading plots.

The three dimensional nature of the EEM spectra is illustrated in Figure 4.8. Instead of being located along a one dimensional wavelength continuum, spectral components are instead located at the intersection of an exciting wavelength and an emission wavelength. Figure 4.8 illustrates this principle using the fluorescence signal from 4 known materials (A – D, top panel) and from 4 representative sediment samples (E – H, bottom panel).

Figure 4.8 (Over) Example EEM spectra from hexane extracts. Top panel shows A - Tree fern fronds (*Dicksonia* sp.), B - Eucalyptus leaves (*Eucalyptus oblique*), C - Waterlogged Twig and Leaf Debris from Olinda Ck and D from dried Spirulina. Bottom panel shows sediment samples from E - Brushy Ck, F - Perrins Ck, G - Olinda Ck and H - Lyrebird Ck. A-D were not used in the PCA. All samples were dried before extraction with hexane.

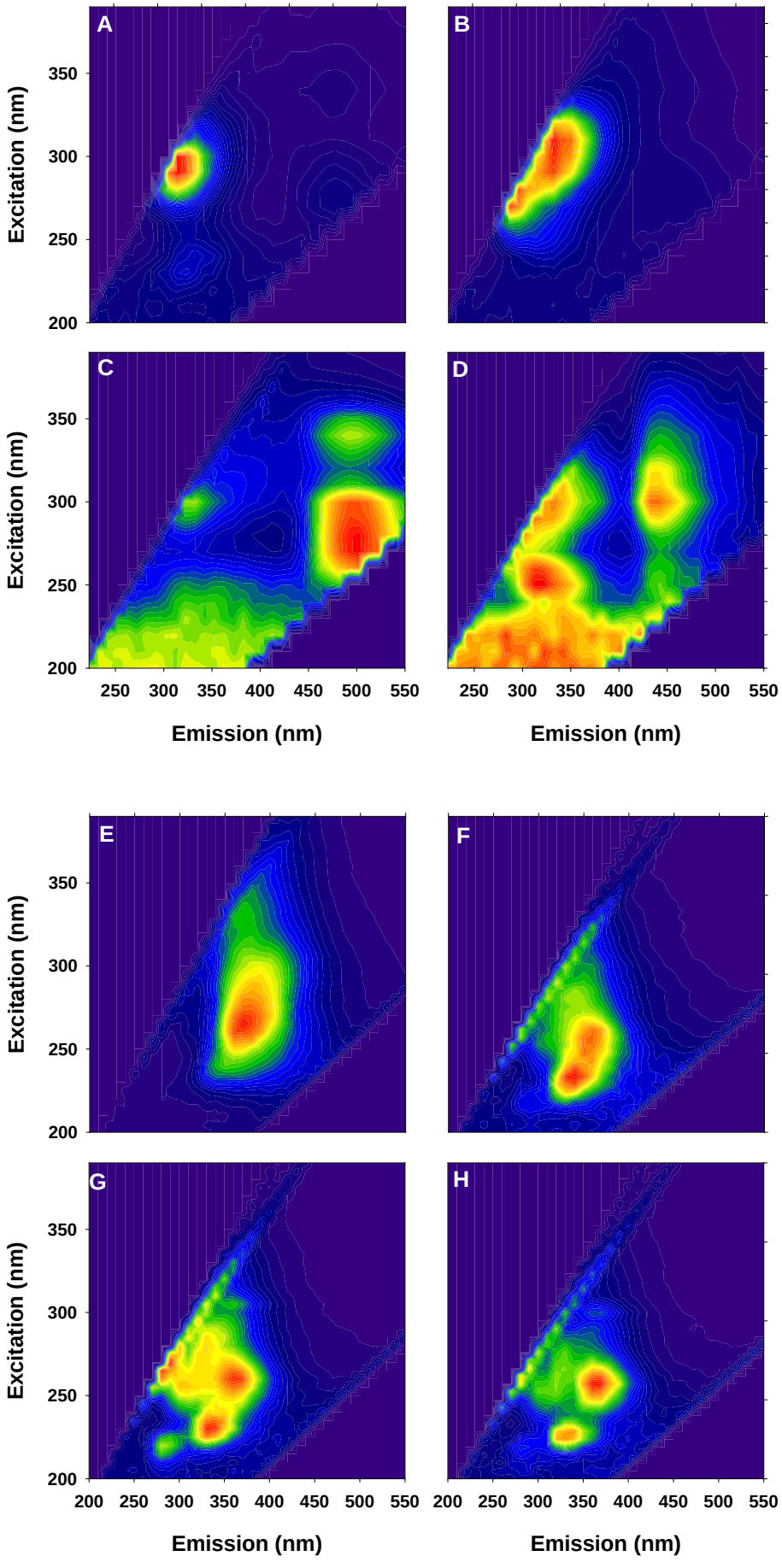
A
B
C
D
E
F
G
H
Excitation (nm)
Emission (nm)
200
250
300
350
400
450
500
550

Table 4.4 Variance explained by the first 10 principal components extracted from the EEM fluorescence spectra of hexane extracted sediments.

Component	% Variance	% Cumulative
1	68.8	68.8
2	13.7	82.4
3	6.5	89.0
4	5.3	94.3
5	0.7	95.0
6	0.7	95.7
7	0.9	96.6
8	0.4	97.0
9	0.9	97.9
10	0.2	98.1

The EEM loading plots show several clearly separated fluorescence signals (Figure 4.9). The bright red region of Factor 1 shows a strong fluorescence signal that encompasses Regions I and II, part of Region III and part of Region IV of Figure 4.3. The remainder of this factor has a strong weighting to regions of the EEM where there is no, or low fluorescence (see Figure 4.8 E – H). This factor explained 68.8 % of the variance in the data (Table 4.4) and appears to separate sites on the basis of "protein fluorescence" and microbial by products of proteins containing typtophan (Figure 4.9). Factor 2 (13.7% Variance) shows a broad region of fluorescence with a small peak at λ_{Ex} = 250 nm: λ_{Em} = 370 nm. This small peak is very close to the measured peak for tryptophan. Factor 3 (6.5% Variance) consists of a fluorescence band that extends from λ_{Ex} = 220 - 330 nm: λ_{Em} = 350 nm. Again, this region covers the tryptophan peak and extends into the region containing microbial by-products containing tryptophan. Factor 4 (5.3% Variance) consists of two broad peaks, one centered on λ_{Ex} = 270 nm: λ_{Em} = 500 nm and the other at λ_{Ex} = 350 nm: λ_{Em} = 500 nm. These two peaks are perhaps best interpreted as signals from fulvic and humic acids (Figure 4.3). Factor 5 (0.7%), the smallest of the retained components again shows a fluorescence peak near the tryptophan region.

Relating carbon to community structure.

The sites are clearly separated into 2 – 3 groups (Figure 4.10) that correspond to the land use gradient established in previous work (Hatt et al. 2004, Taylor et al. 2004, Harbott and Grace 2005) and in Chapter 3. The more urbanized sites cluster closely

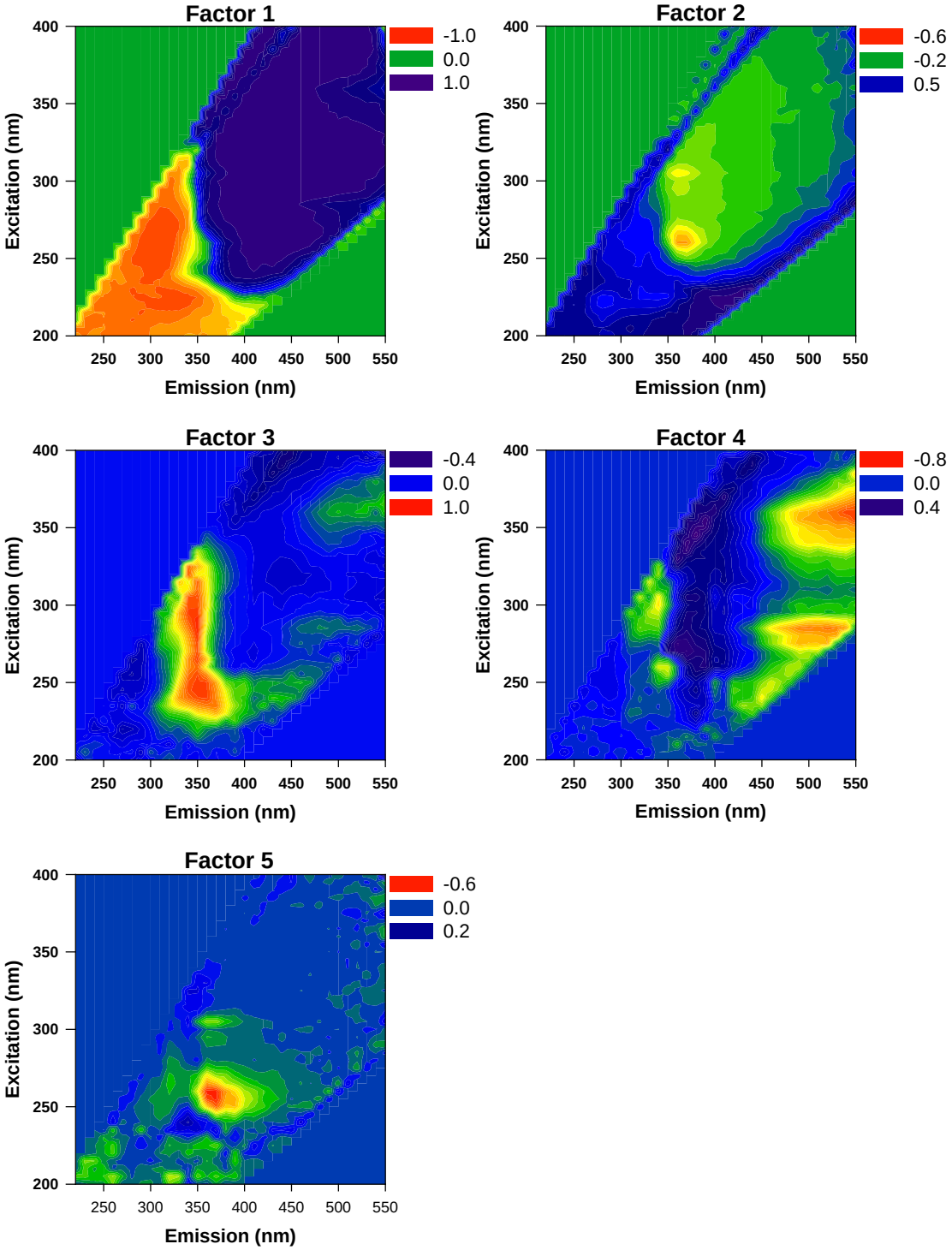

Figure 4.9 EEM loading plots for the first 5 principal components showing the extracted fluorescence spectral components. Loads show which portion of the original variables most influence the extracted component. Legend for each plot shows High (red) - Low (light blue/green) -loading: sign is relative. Dark blue is the highest load of the opposite sign to the red signal.

to the right of Axis one in Figure 4.10. Two carbon factors correlated strongly with this Axis; EEM Factor 1 and IR Factor 1. EEM Factor 1 was interpreted as indicating the presence or absence of protein compounds and the position of the sites (Figure 4.10) and the sign of the loading (Figure 4.9) of this component suggest that the non urban sites had a larger sediment protein signal than the urban sites. The IR Factor is harder to interpret. Although IR spectra are predominantly used for the identification of functional groups in organic molecules, inorganic components also contribute peaks to the spectra (Table 4.2). While the first EEM factor seems clear, the first IR factor could either be a description of the carbon, or a description of the mineralogy, or a combination of both. The high negative loading at ~3600 – 3200 was tentatively assigned to the hydroxyl group (a dominant functional group in cellulose) and this locates the non urban sites to the left on Axis 1 (Figure 4.10). This factor appears to separate sites based on silicate content and cellulose/organic content, a conclusion backed up by the carbon content, which was ~2% for the urban sites (Sc, Br & Fe) (Table 3.6, Chapter 3) compared to the ~10 – 15% for the non urban (Ly & Ol) and intermediate sites (Sa, Pe, Do & Da) . The urban sites also had a greater percentage of coarse sandy particles which would contribute a silicate signal to the IR spectra. The greater protein fluorescence signal may be accounted for by higher microbial biomass in the non urban sediments. Both the higher carbon content and the finer particle size fractions (i.e. greater surface area) are factors permitting colonization by greater cell numbers. Put together, Axis 1 (Figure 4.10) separates sites according to significant correlations with sediment protein content (EEM Factor 1), and a combination of silica mineralogy and organic matter content (IR Factor1).

The second CCA axis is composed of 3 factors. IR Factors 2 and 3 tend to separate the non urban sites (■ Fig 4.10) from the intermediate sites (■). IR Factor 2 was interpreted as being mainly due to differences in the baseline signal of samples (Figure 4.6) whereas IR Factor 3 appears to be predominantly due to absorption at 1750 – 1550 cm^{-1} (C=O) a signal suggestive of a combination the carboxyl group of cellulose and the amide bonds of protein. The shoulder signal at 1450 – 1350 cm^{-1} could be due to the C=C stretch of aromatics and might suggest either the polyphenyl propanoid subunits of lignin (Adl 2003) or the C – O bond of cellulose. Another molecule that has an IR absorbance in this region is *N*-Acetyl-D-glucosamine, found in chitin, a component of fungi and the cell walls of gram –ve bacteria (Moat et al. 2002, Adl 2003). EEM Factor 3 had a correlation of ~0.6 in the opposite direction to the two IR factors. This factor was interpreted as a

combination of protein fluorescence and the microbial by-products of tryptophan. These factors have the combined effect of separating the non urban sites from the intermediate sites on Axis 2. The remaining factors had lower or no correlations with the community data suggesting that those carbon signals were common to all sites and therefore applied no selective pressure on the bacterial community.

The results of the CCA analysis using the 10 retained components (5 IR components, 5 EEM components) are shown in Table 4.5 and Figures 4.10 A and B. The Monte Carlo statistic generated by PC-Ord for Axis 1 strongly suggests a significant relationship between the denitrifying bacterial community and the variables used to describe the carbon composition on this axis. High loadings were observed for IR Factor 1 and EEM Factor 1; lower loadings for IR Factor 4, IR Factor 5 and EEM Factor 3 were found. Axis 1 accounted for 16% of the variance in the community data and the CCA suggests that three axes describe 33.5% of the community data. While there is no test for the significance of the strength of the relationships in Axis 2, separation of the community data on this axis is principally due to IR Factors 2 and 3 and EEM Factor 3.

Table 4.5 Decomposition of the variance explained & correlation of species data with each canonical axis as determined by CCA of EEM and FTIR variables. The species and carbon data of axis 1 are significantly correlated.

Total variance ('inertia') in the species data: 3.8880	Axis 1	Axis 2	Axis 3
Eigenvalues	0.625	0.374	0.224
Mean	0.370	0.248	0.196
Min.	0.239	0.186	0.132
Max.	0.543	0.370	0.266
p**	0.002		
Variance in species data			
% of variance explained	16.1	9.6	5.8
Cumulative % of variance explained	16.1	25.7	31.5
Pearson Correlation* Spp-Envt	0.984	0.952	0.872
Mean	0.840	0.862	0.865
Min.	0.698	0.679	0.739
Max.	0.963	0.965	0.979
p**	0.002		

* Correlation between sample scores for an axis derived from the species data and the sample scores that are a linear combination of the environmental variables. Set to "0" if the axis is not canonical.
**p is not reported for axes 2 and 3 as using a simple randomization test for these axes may bias the p values.

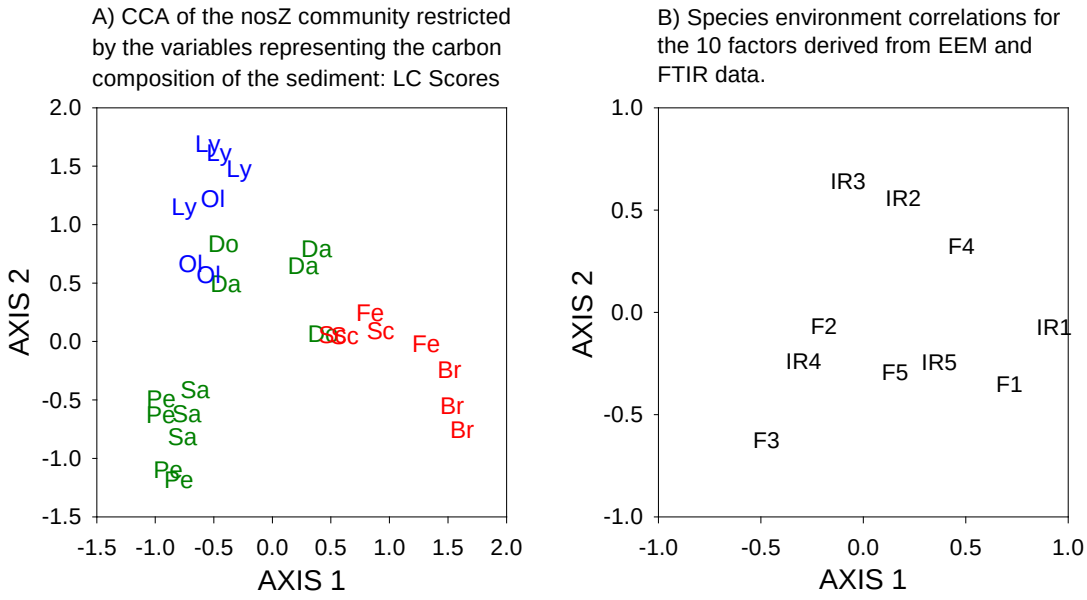

Figure 4.10 Restricted ordination of the *nos*Z bacterial community using variables that qualitatively described the carbon present in sediment samples. Axis 1 (left graph) has significant species environment correlations (Table 4.5) and forms a noticeable gradient from less urban to more urban (■ Urban, ■ Intermediate & ■ Non Urban). The right graph shows the strength of the correlations for each of the variables used. F 1-5 refers to EEM factors and IR 1-5 refers to infrared factors (refer to Figures 4.6 and 4.9).

4.4 Discussion.

Sediment carbon composition and the denitrifying community structure along a gradient of catchment urbanization.

Research into the effect of carbon concentration (as DOC) on denitrification is common, but fewer studies explicitly consider the effect of carbon type or bioavailability. For example, particulate organic carbon (POC) was a significant source of carbon supporting denitrification in agricultural streams when nitrate was not limiting (Arango et al. 2007) and experiments with leaf leachate and glucose showed that the bioavailability of carbon was a critical determining factor for the removal of nitrate in streams (Sobczak et al. 2003). While increased removal of nitrate via denitrification is not always a function of DOC concentration (Bernhardt and Likens 2002), factors affecting the accumulation and processing of carbon are strong regulators of nitrogen dynamics in streams (Groffman et al. 2005).

Microbial ecology predicts that carbon supply should be related to microbial abundance, and that different species should have preferences for different carbon substrates and as a consequence, the structure of bacterial populations should have some relationship to changes in the source of carbon being metabolized. The data presented here support a correlation between the heterotrophic denitrifying population and differences in the type of carbon present in the sediment. Since small temperate headwater streams usually receive most carbon allocthonously, the ratio of autotrophic production to respiration is low and heterotrophs are the dominate life forms (Cummins 1974, Cole and Caraco 2001). In the process of metabolizing allocthonous carbon, heterotrophs do two things; carbon is respired as CO_2 and new forms of carbon are produced and released in the cycle of cell growth and death (del Giorgio and Cole 1998). The carbon composition is altered by bacterial metabolism as the source carbon on which the heterotophs are feeding is degraded and the carbon is incorporated into cellular biomass.

Cellular uptake and incorporation leads to the production of proteins, lipids, chitin, polysaccharides and peptidoglycan (gram negative bacteria) the relative amount and type of which depends on the source species (Moritz et al. 2009). For example although both fungi and bacteria have cellular structures composed of glucosamine, only bacteria contain muramic acid. The ratio of these two can be used to indicate the relative contribution of fungi and bacteria to the soil or sediment carbon pool (Moat et al. 2002, Moritz et al. 2009). Gram positive bacteria have an additional cell wall structure rich in lipo-polysaccharide (Madigan et al. 2000, Moat et al. 2002). The sediment carbon signal is not just a signal from the allocthonous material that has fallen or been transported into the stream. Instead the carbon signal from the sediment is a composite of the allocthonous carbon substrate and the attached biofilm of microorganisms. In headwater streams where woody debris and leaves (i.e. cellulose, lignin) form a major component of the carbon, the population of fungal cells will be higher than in streams where cellulose and lignin are lower sources of carbon as fungi have a unique ability to degrade the complex structural macromolecules found in plants. It is possible that the separation on Axis 2 of Figure 4.10 is in part due to differences not only in the amount of cellulose but also the fungal biomass of the sediment.

The intermediately urbanized sites also receive carbon inputs directly from the urbanized catchment that may contain more easily metabolisable carbon and this might explain why

these sites and the urban sites tend to be located in the same direction on Axis 2. In a parallel study, Harbott and Grace (2005) measured the enzymatic activity in many of the same streams. In urbanized streams they found that the activity of the enzymes leucine amino peptidase and esterase was higher than in non urban streams where N-acetyl glucosaminidase and xylosidase activites (a marker for cellulose degradation) were, relatively, more important (Harbott and Grace 2005, Harbott et al. 2005). Esterase is a general indicator of enzyme activity while amino peptidase is a general indicator of protein decomposition (Harbott and Grace 2005). The increased peptidase activity could possibly result from decomposition of benthic algal material which was found to be higher in the urbanized streams (Taylor et al. 2004, Catford et al. 2007) while the increased esterase activity might be a signal of the slightly higher DOC concentrations of the urbanized streams (Hatt et al. 2004, Harbott and Grace 2005).

These observations and those from a previous study (Harbott and Grace 2005, Harbott et al. 2005) can perhaps be reconciled as follows. In the non urban streams where the greater carbon input is from trees, microorganisms release extra cellular enzymes that attack components of cellulose and lignin. Secondary to this, as fungi are the main decomposers of wood, fungal biomass increases: when fungal cells die bacteria release N-acetyl glucosaminidase to decompose the chitin present in fungal cells. In urban streams the input of cellulose is either lower or rapidly flushed from the system due to the flashy hydrology of urban streams (Walsh et al. 2005b). As a consequence these enzymes are less important in urban streams. Protein in urban streams can derive from in stream algal biomass, as contaminants from the surrounding catchment and from bacterial breakdown products resulting from secondary production. The increased DOC content and carbon inputs from road runoff containing a diverse mixture of substrates contributes to the generally increased non specific esterase activity.

A review of the spectroscopic methods used.

Both the FTIR and EEM spectra produced principal components that were interpretable and showed some significant correlation with the denitrifying community structure. Although FTIR has the potential to produce a spectral fingerprint with a large amount of information this is also a potential weakness. The procedure adopted here (modified from literature) produced complex spectra with overlapping carbon and mineralogical components. The IR component of the first Axis of the CCA (Figure 4.10) could only

tentatively be interpreted as a separation of sites based on mineral properties and organic content because of this. Later spectral components (Figure 4.6) were simpler and open to a more meaningful interpretation as they generally contained one or a few spectral features. The best example of this was the pure extraction of the gaseous CO_2 signal shown in Figure 4.7. Increased replication may help solve this problem. When this experiment was performed the number of samples analyzed was limited for logistical reasons, by the cost of the T-RFLP analysis. As a result the observed spectra may not have been derived from sufficient samples to fully separate individual covering regions of the spectra: producing instead 'general' principle components. The subsequent decreasing costs of DNA analysis, improvements in DNA techniques and the ability to use high throughput methods would greatly improve the analysis. However, although the first principle component always contains the most variance in the data, this may not be the information in the spectra that has the most ecological significance. Other researchers have used PCA to remove 'noise' (as the first PC) from spectral signals[1]; not that this is the case here. However, if sufficient spectra are recorded and if the mineralogical signal is much larger than the carbon signal, a result dependant on the amount of carbon in the sample, then the first 1 – 2 PCs will contain the majority of the mineral information and should be interpreted as such.

Of the two methods, EEM is simpler to perform though equally time consuming. The manipulation of the EEM data is more laborious however and the use of macros in excel is recommended if this technique were to be adopted as a routine analysis. Similar to the FTIR data the first extracted PC appeared to be general in nature, separating samples based on presence or absence of protein fluorescence. Later PCs produced spectra highlighting specific areas where separate fluorescence peaks appeared. While relating these PCs to specific carbon signals is still a somewhat subjective exercise, there is no subjectivity in the site score data used with the denitrifier community data. Unlike the FTIR spectra the EEM spectra have been derived solely from the carbon extracted from the sediments. Hexane was adopted as the solvent for several reasons. It is known that fluorescence signals in water are affected by several factors:

[1] for example see Hasegawa et al.. 2000, Hasegawa 2001.

1. fluorophores fluoresce at different wavelengths or not at all depending on pH.
2. oxygen in water can quench fluorescence.
3. metal ions that alter or quench fluorescence may be present.
4. the water Raman line appears in the spectra.

Hexane extraction avoided all of these potential problems at the possible expense of biasing the measured carbon signal towards non polar molecules. Given that many of the molecules thought to influence bacterial communities (see above) are large (humic material) and or non polar (lipids) this is probably not an issue. An equally valid argument is that water extracts bias the results the other way.

4.5 Conclusion.

Chapter 3 concluded that the denitrifying community was influenced by urban effects, that carbon had a role in structuring the denitrifying community, but that there were confounding structuring processes not directly related to urbanization. This chapter showed that the denitrifying community is directly correlated with some components of the organic carbon found in the sediments of streams in catchments urbanized to different degrees. The data shows that changes in carbon sources and dynamics have been influenced by urbanization and that this has had an effect on the bacterial community. This conclusion compliments previous research conducted in the same set of catchments that suggested, indirectly, that carbon was a structuring agent with a direct qualitative measure of the carbon and a direct measure of bacterial community structure.

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CHAPTER 5

CHANGES IN MICROBIAL COMMUNITY STRUCTURE IN RESPONSE TO DIFFERENT CARBON SUBSTRATE AND HEAVY METAL TREATMENTS.

"*A community adapted to elevated ambient levels of a particular pollutant is expected, compared to a non-exposed community, to display an increased tolerance to that pollutant*" (Soldo and Behra 2000).

OVERVIEW.

In this chapter changes in the community structure of both the denitrifying (i.e. *nosZ*) community and the 'total' (i.e. 16s rRNA gene) bacterial community were measured in response to the manipulation of sediments sampled from an urban and non urban stream. The experiment was predicated on the idea that the bacterial community from urban sediment would react to an 'urban' carbon source more readily than sediment derived from a non urban catchment (and vice versa). Additionally, in light of the results of previous chapters (i.e. Chapters 2 & 3), it was thought that the urban sample would be more resistant to the toxic effects of a heavy metal (zinc). The sediment community structure was determined before and after the incubation.

5.1 INTRODUCTION.

Chapters 2 and 3 demonstrated clear differences between the sediment denitrifying community structure observed in streams exposed to different degrees of urban influences. The second paper in Chapter 2 showed statistically significant differences between two sites at either end of the spectrum of streams examined and speculated that among the possible community structuring mechanisms, the type of carbon input into each stream might be significant. Chapter 3 outlined many of the factors found in previous studies that are associated with the process of urbanization. Among these are noticeable differences in the retention of organic matter, particularly large woody debris, and increased metal loadings in urban environments. Although the results of Chapter 3 indicated a weak relationship between sediment bacterial community structure and heavy

metal concentration, a number of other studies (reviewed below) have indicated that heavy metals can be important in structuring microbial communities.

Chapter 4 demonstrated a clear relationship between carbon type and denitrifier community structure. However, although a clear correlation existed, whether a change of carbon type might cause a change in community composition could not be determined. This Chapter and Chapter 6 (following) use two sites, non urban and urban, in an experimental manipulation to test the effects of a single addition of zinc in a series of microcosms treated with one of two types of carbon, chosen to represent the likely carbon sources found in urban and non urban streams. A factorial design was incorporated to test responses to zinc additions and the type of carbon supplied to either of the sediment types. Chapter 5 outlines how the community structure responds to the manipulations while Chapter 6 describes the physiological responses of the communities.

Carbon sources as drivers of community structure.

Many previous studies have investigated the importance of carbon sources to the bacterial community and the effects on community structure (Bott and Kaplan 1985b, Amon and Benner 1996, Preston-Mafham et al. 2002, Eiler et al. 2003, Romaní et al. 2004, Gao et al. 2005, Lennon and Pfaff 2005, Nakatsu et al. 2005, Wawrik et al. 2005, Docherty et al. 2006, Edmonds et al. 2008). The early study by Bott and Kaplan (1985) examined four diverse stream sites with different sources of dissolved organic carbon. Their modeling of the importance of different carbon substrates suggested that amino acids and carbohydrates had the greatest effect on the microbial activity, and that qualitative differences in carbon affected activity more than bacterial biomass as many bacteria can simultaneously use more than one carbon source. The reactivity of carbon substrates has also been examined by Amon and Benner (1996). Across the range of sites that they studied, Amon and Benner observed that the reactive pool of high molecular weight (HMW) DOC was larger than that of low molecular weight (LMW) DOC. In a generally recognized model (Wetzel 2001) the authors outline how the general flow of carbon is from larger "*diagenetically young or fresh material*" to smaller less reactive or refractory molecules. The uptake rate of organic molecules is related to a compounds lability, with preferential uptake of the more labile molecules (Romaní et al. 2004). Eiler et al. (2003) used samples from a humic lake treated with different levels of aged DOC solution to establish a concentration gradient to determine if concentration had a structuring effect on

the bacterial community. They found that an asymptotic relationship existed between the concentration of DOC and measures of community dissimilarity, with the greatest differences found between treatments at the lower DOC concentrations and only with minor changes in the community composition at higher DOC concentrations. Gao et al. (2005) used canonical correspondence to show that different communities of bacteria were present at sites with different concentrations of DOC. β- and γ-Proteobacteria dominated sites with high DOC and nitrate/nitrate concentrations, while α-Proteobacteria were more abundant in environments with low DOC and nitrate/nitrite concentrations. Sites with high DOC concentrations also had high sediment benthic organic matter and vice versa (Gao et al. 2005).

Using T-RFLP, Wawrik et al. (2005) observed that in three of the four soil and sediment samples treated with different carbon sources, the bacterial communities given the same substrates were more similar at the end of the treatment. They also found, as is commonly accepted, that dissimilar carbon sources selected for different communities; though the degree to which this selection overrode other selection mechanisms depended in part on the carbon source used (Wawrik et al. 2005). Microbial communities in samples from a garden soil, sandy soil and clay still showed a tendency to cluster together independently of the carbon treatment. Nakatsu et al. (2005) found that single carbon sources tended to select for a few types able to exploit that carbon source efficiently and Docherty et al. (2006) conclude that "*that microbial communities are selected by the DOM sources to which they are exposed*…"

To increase the total pool of available carbon, microorganisms release extracelluar enzymes into periplasmic space of free into the environment (Chróst 1992, Findlay et al. 1997, Findlay and Sinsabaugh 1999, Wetzel 2001, Harbott and Grace 2005). Findlay et al. (1997), through measurement of extracellular enzyme activities, demonstrated that microbial communities respond quickly (~ 24 hrs) to changes in the supply of carbon. The study by Harbott et al. (2005) utilized a subset of the same streams described through out this thesis. In their study, Harbott et al. showed that urban sites could be distinguished from less impacted sites on the basis of extracellular enzyme activities, and by extension, the availability of carbon for heterotrophic metabolism. In particular, there was a significant difference in the activity of Leucine amino peptidase, with the more

urban sites positively correlated with increased activity of this enzyme (Soldo and Behra 2000). This suggests that proteinaceous matter made up a greater proportion of the DOC available to heterotrophs in these urban streams.

Zinc and its effect on bacterial communities.

Recent studies have shown that zinc from a diverse range of materials is a major contributor to the total metal load in urban streams (Roger et al. 1998, Davis et al. 2001). Although zinc is an important biological metal (Blencowe and Morby 2003, Morel and Price 2003), results from Chapter 2 comparing sediment communities from the urban and non urban sediments suggested that heavy metals, particularly zinc, associated with the urban site had some correlation with changes in the community structure of denitrifiers. Other studies have found that heavy metals, including zinc, have a structuring effect on the microbial community (Kelly et al. 1999, Soldo and Behra 2000, Moffett et al. 2003, Duarte et al. 2004, Mertens et al. 2006, Sverdrup et al. 2006, Duarte et al. 2008). In a long term test of the exposure of zinc on microbial communities in soil, Kelly et al. (1999) found that, although after 420 days there were no differences in the culturable populations, other properties (i.e. metabolic profiles) were still affected. In an investigation of the effects of exposure to heavy metals (principally copper), Soldo and Behra (2000) started with the premise that a "*community adapted to elevated ambient levels of a particular pollutant is expected, compared to a non-exposed community, to display an increased tolerance to that pollutant*". Changes in the community composition of photosynthetic bacteria were accompanied by increased tolerance to heavy metals as measured by photosynthetic activity (Sverdrup et al. 2006). Exposure to 400 mg kg^{-1} zinc has been found to decrease the diversity of bacteria in an agricultural soil (Moffett et al. 2003). Similarily, Duarte (Duarte et al. 2004, Duarte et al. 2008) found that zinc and copper altered the structure of fungal and bacterial communities responsible for the decomposition of leaves. Bacterial biomass was reduced by all metal treatments investigated (Duarte et al. 2008). Mertens (Mertens et al. 2006) found that the ammonia-oxidizing community extracted from a long term contaminated field site was measurably different to a control site and could be related to the zinc concentration. However, recently Sverdrup (Sverdrup et al. 2006) has shown that many of the documented effects of heavy metal toxicity on bacterial communities are moderated to some extent by the bio complexity of the environment or sample being examined.

Hypotheses.

In light of the above observations and the tentative conclusions of Chapters 2 & 3 the following hypotheses are considered:

1. That urban and non urban sites have different community structures.
2. That the addition of protein will make the non urban bacterial community structure similar to the urban community structure (and vice versa for leaf litter leachate)
3. That the urban bacterial community structure will be unaffected by the zinc treatment but that the non urban bacterial community structure will become more like that of the urban sample.

5.2 METHODS.

5.2.1 Site descriptions.

Sediment was collected from Lyrebird Ck and Gardiners Ck. Lyrebird Ck was chosen to represent the least urban end of the collection of environmental gradients arising from the process of urbanization (see Chapter 3 for a more detailed description), whereas Gardiner Ck represents a fully urbanized system. Locations and detailed descriptions for these sites can be found in Chapter 3, Table 3.3.

5.2.2 Experimental design and analysis.

A factorial design was selected to compare the 2 sites (urban and non urban) with two levels of zinc treatment and three levels of carbon treatment (Table 5.1) This design resulted in a total of 48 treatment bottles, with 4 replicates for each treatment. Twelve additional bottles were included to provide a series of controls. These serum bottles were purged of headspace gas as described below. Four serum bottles were prepared with sediment from the non urban site and 4 from the urban site, each treated solely with 90 mL of the stream water collected from the urban and non urban sites respectively. A further 4 bottles that contained 90 mL of purged de-ionised water with no sediment were used to serve as blanks and to check for potential leakage of atmospheric gases. Sediments were sampled after the incubation and treated to extract community DNA for

subsequent T-RFLP analysis (see below). Headspace gas samples were taken throughout the incubation to measure the physiological responses of the various treatments; these results are discussed in Chapter 6. Although it was logistically possible to sample all four replicates for gas analysis financial constraints limited the replication for T-RFLP analysis to three of the four replicates.

Table 5.1 Experiment treatment matrix for comparing the effect of metal (Zn) and carbon (leaf/protein) treatments on the community structure of urban and non urban sediments. Numbers show replicates.

Site	Zinc	Carbon	Community Structure (T-RFLP)	Headspace Gas Analysis
	Yes	None	3	4
Lyrebird	Yes	Leaf	3	4
(non urban)	Yes	Protein	3	4
	No	None	3	4
Lyrebird	No	Leaf	3	4
(non urban)	No	Protein	3	4
	Yes	None	3	4
Gardiner	Yes	Leaf	3	4
(urban)	Yes	Protein	3	4
	No	None	3	4
Gardiner	No	Leaf	3	4
(urban)	No	Protein	3	4

The community structure data from both the *nosZ* and the 16s rRNA gene T-RFLP fingerprints (see below) were treated as described in Chapters 2 and 3. Similarity matrices calculated from the Bray-Curtis coefficient on presence-absence transformed T-RFLP fingerprints using PRIMER were used to produce NMDS ordinations. Differences between individual treatments were tested using a 1-way analysis of similarity (ANOSIM) and a 2-way crossed ANOSIM was used to explore interaction terms (Clarke

and Warwick, 2001). For the 16s rRNA gene fingerprints, statistical analysis was performed after truncating the data set by removing fragments smaller than 30 bp. For the nosZ fingerprints two comparisons were made, one between a data set that included peaks from 21 bp and the other from a data set treated similarly to the 16s rRNA gene fingerprints (i.e. incorporating peaks 30 bp and longer). The reason for this post hoc comparison of TRF populations will be explained in the discussion

Multivariate dispersion.

Multivariate Dispersion was calculated using the MVDisp routine of PRIMER. Dispersion is a procedure for comparing the rank similarities across the samples included in the analysis. A number close to 1 indicates 'average' dispersion – meaning that the relative distance between members within the groups under comparison is similar. Similar values for the dispersion index do not necessarily mean either that the same 'species' or the same number of species were present between comparisons. A number lower than 1 indicates a lower (on average) dispersion or community diversity for those samples being compared while a number greater than 1 suggests a greater diversity and therefore dispersion.

5.2.3 Sample collection and preparation.

A composite sediment sample was collected from the top 10 - 20 centimeters at two locations from both Lyrebird (28/7/04) and Gardiners Ck (29/7/04) and kept refrigerated in a 4 L plastic container until returned to the laboratory.

The following day, the composite sediment sample from Lyrebird Ck was passed through a coarse 1 cm^2 sieve to remove large debris and mixed in a plastic bucket. Approximately 50 g of sediment was then sub-sampled into pre weighed and labeled 125 mL serum bottles. Following the experimental design shown in Table 5.1, each zinc treatment was injected with 1 mL of a $ZnCl_2$ solution (see below) giving an approximate final concentration spike of 200 mg kg^{-1}, which is comparable to the levels detected at the urban site (Brushy Ck) of Chapter 2 (Table 2.0). This was then followed by 90 mL of either the leaf leachate solution, protein solution or blank solution (see below). The serum bottles were then capped.

The sediment from the urban site was treated in exactly the same manner the next day, preserving the 1 day difference in collection times.

After all samples and replicates had been processed, a 1 mL sample of water was extracted for pH measurement using a Horiba pH meter[1] and 1 mL of 100 mg $L^{-1}NO_3$-N injected to maintain nitrate levels and avoid substrate depletion. Serum bottles were inverted and allowed to settle again following the injection of nitrate to ensure mixing. Gas sampling (Chapter 6), pH measurement and nutrient injection was repeated every 4 days (with 2 exceptions; one 5 day interval and one 3 day interval) for 28 days.

5.2.4 Reagents and solutions.

Zinc solution.

A 10gL^{-1} zinc solution was made by dissolving $ZnCl_2$ in deionised water. The final pH of this solution was ~ 2.1.

Carbon sources.

Leaf Leachate. Woody material and leafy debris from the stream channel was collected at the non urban (Lyrebird Ck) site. This material was placed in a 1L Schott bottle and autoclaved at 120 ^oC for 20 minutes. After cooling, an aliquot of this solution was diluted 1:10 with 2 mL of a 1g-N L^{-1} nitrate (KNO_3, Merck, AnalR) stock solution and 80 μL of a 1g-P L^{-1} phosphate (KH_2PO_4, Merck, AnalR) stock solution to give final concentrations of 1 mg-N L^{-1} and 40 μg-P L^{-1} respectively in a total volume of 2 L. This solution was stored at 4 ^oC until used. This solution was analysed for TOC[2] after the experiment and found to have 36.4 (± 1.4) mg C L^{-1}.

Protein Solution. A source of carbon rich in protein was made by diluting 680 mg of Bacto Casitone (pancreatic digest of casein, Difco), 2 mL of the 1g-N L^{-1} nitrate stock solution and 80 μL of the 1g-P L^{-1} phosphate stock solutions to 2 L in deionised water.

[1] Model Twin pH B-211: ±0.1 pH unit repeatability calibrated before use with the supplied pH 4 and pH 7 bufffers.
[2] TOC analysis was performed at the NATA accredited analytical laboratory located at the MDFRC by Mr J. Pengelly

The final concentrations of nitrate and phosphorus were the same as the leaf leachate. Later analysis of TOC showed that this solution contained 133.7 (± 4.1) mg C L-1.

Control Carbon Solution. A control solution with no carbon was prepared using deionised water with nitrate and phosphate added to give the same initial concentrations as the above carbon solutions

Prior to distributing aliquots of the above solutions for the various treatments (Table 5.1), the above solutions were purged of dissolved gases by bubbling helium (UHP, BOC) through a ceramic diffuser for approximately 20 minutes. Specific details regarding the purging procedure to reduce the background levels of metabolic gases in the sediments, supernatant and headspace are described in Chapter 6.

5.2.5 Extraction and amplification of sediment DNA for community structure determinations.

Sediment material was retained at the beginning of the experiment and extracted from the serum bottles at the end of the experiment.

5.2.5.1 Denitrifying community structure as determined by *nosZ* T-RFLP fingerprints.

Methods for the extraction and determination of the sedimentary DNA and bacterial community structure via T-RFLP have been described in Chapters 2 and 3.

5.2.5.2 'Total' bacterial community structure as determined by T-RFLP of 16s rRNA gene.

DNA amplification.

After extraction of the DNA, the 'total' bacterial community structure was determined by amplification of an approximately 1465 base pair fragment of the 16s rRNA gene in a final volume of 50 µL, using the HotStar*Taq*™ DNA polymerase kit and master mix described by the manufacturer (Qiagen, Clifton Hill, Australia), with the exception that 2.5 units of Taq polymerase were used per reaction.

The forward primer 27f (5'-AGAGTTTGATCCTGGCTCAG-3') and reverse primer 1492r (5'-ACGGCTACCTTGTTACGATT-3') (Weisburg et al. 1991) were used for the polymerase chain reaction (PCR). For T-RFLP analysis, the forward primer was labeled at the 5' terminus with 6-carboxyfluorescein.

PCR was carried out on a BioRad iCycler thermal cycler (Regents Park, Australia) with the following conditions: 95 °C for 15 min to activate the HotStar*Taq*, denaturation at 94 °C for 15 s, 35 cycles starting with primer annealing at 48 °C for 15 s, chain extension at 72 °C for 1 min. The final extension on the last cycle was 72 °C for 6 min. DNA extracted from *Pseudomonas denitrificans* (NCTC 10688) was used as the positive control and amplicon size was confirmed by agarose gel electrophoresis. Autoclaved de-ionised water was used as the negative control. Amplified samples were purified using the MoBio UltraClean™ PCR clean-up kit following the manufacturer's instructions (MoBio Laboratories, Solena, CA, USA).

T-RFLP analysis.

Purified amplicons from PCRs with the forward-labeled primer were digested with *Hin6I* (Geneworks, Adelaide, Australia) for 3h at 37 °C. The 25 µL digestion reactions contained between 80 -100 ng DNA, 2.5 µL of incubation buffer[1] (prepared from the provided 10x stock solution: included with the *Hin6I* enzyme), 3 µL of endonuclease (3 U) and autoclaved Milli Q water. Post-digestion restriction fragments were precipitated in 3 volumes of 100% ethanol at -20 °C overnight. The precipitate was washed in 70% ethanol to remove excess salts and dried for final analysis. Dried pellets were resuspended in 10 µL water. A 2.5 µL volume was added to 0.3 µL Genescan Rox size standard (Applied Biosystems, Foster City, CA, USA), followed by 0.4 µL dextran Blue (50 mg ml^{-1} in 25 mmol L^{-1} EDTA, pH = 8), 1.8 µL formamide then denatured at 95 °C for 3 min. Final separation was carried out with a 377 DNA sequencer (Applied Biosystems)[2].

[1] The provided incubation buffer is 'proprietary': the ingredients are not stated.
[2] This analysis was performed at WEHI by AGRF; reagents and standards were prepared or obtained by this laboratory.

Nomenclature.

The following labels are used to identify two kinds of control samples not included in the main manipulation matrix shown in Table 5.1. These two controls are in addition to the de-ionized water blank used to check for gas leakage in the experimental units.

Zero Treatment Control refers to sediments that were incubated in serum bottles (4 from each site) with stream water collected at the time of sampling without being supplemented with nitrate or being subject to any of the treatments. This control was used to account for the changes in the community structure as a result of time isolated in the serum bottles.

Time Control refers to sub samples taken from each of the sediments and analyzed to determine the community composition as it existed prior to the manipulations (i.e. at time zero).

5.3 RESULTS.

5.3.1 pH changes.

Addition of the zinc solution did not appreciably change the pH between treatments (Table 5.2). The pH was initially lowered slightly (0.3 - 0.6 units) by addition of the zinc solution. By the end of the experiment although a statistically significant difference is still evident in the urban treatment, the difference between treatments was small (0.2 – 0.4 units) and the pH of the solution was near neutral (i.e. pH 7). Throughout the experiment the difference in pH due to zinc addition was less for the non urban treatment.

5.3.2 Structural response of the microbial communities represented by nosZ and 16s rRNA gene T-RFLP fingerprints from urban and non urban sediment samples.

Before presenting the main effects apparent in the data a short digression noting the effects of the data treatment adopted for this work will be made. In the process of calculating the relative multivariate dispersion from the *nosZ* data, a slight departure was made from the method employed in Chapters 2 and 3 where data routinely excluded from the analysis was included. When this data were then used to derive an MDS and the

ANOSIM test, significant differences were noted in the displayed community structure and statistical tests (Table 5.6). Including these 'shorter' (21 – 30 b.p. in length) terminal restriction fragments (T-RFs) altered the significance of one of the tests dramatically which then altered the interpretation of the results.

Table 5.2 Change in mean pH* over time for Urban and Non Urban samples treated with (n = 12) and without (n = 16) zinc. Underlined values are statistically significant (Students t; $\alpha = 0.05$).

Site	Urban				Non urban			
	without zinc		with zinc		without zinc		with zinc	
Day	pH	s.d.	pH	s.d.	pH	s.d.	pH	s.d.
0	7.11	0.12	6.53	0.11	6.83	0.06	6.52	0.04
4	7.20	0.12	6.73	0.15	6.87	0.10	6.59	0.03
9	7.33	0.13	7.02	0.14	7.00	0.08	6.83	0.07
13	7.48	0.18	7.42	0.26	7.29	0.18	7.07	0.08
17	7.29	0.25	6.98	0.20	6.86	0.12	6.76	0.09
21	7.17	0.15	6.95	0.27	6.71	0.07	6.61	0.09
25	7.11	0.16	6.88	0.19	6.71	0.08	6.61	0.09
28	7.31	0.17	7.04	0.20	7.27	0.06	7.15	0.09

* pH averages calculated using recorded pH values, not using means of $[H^+]$ and converting to the pH scale.

The bacterial communities represented by both *nosZ* and 16s rRNA gene T-RFLP fingerprints are different at each site (Figures 5.3, 5.4 and Tables 5.6 & 5.7). The difference was more pronounced (R = 0.872, p = 0.01) for the nosZ community structure than the 16s rRNA gene community structure (R = 0.560, p = 0.01). If the treatment blanks and the zero time treatments are included, these comparisons indicate a slightly lower, but still significant difference between the two sites (R = 0.843, p = 0.01 and R = 0.228, p = 0.01 respectively) for both genes. This observation is consistent with results from previous chapters and shows that the different community structures recorded at the end of the incubation were not solely due to selection arising from the experimental procedures.

5.3.2.1 The effect of experimental manipulations on the *nosZ* T-RFLP fingerprints.

Comparison with controls.

Comparison of the manipulated samples and the *Time Controls* (Experiment Effects in Table 5.6) reveals that the community isolated from the field (*Time Controls*) representing the community at t = 0, was marginally different from the manipulated sediments (R = 0.136, p = 5.5), but was not significantly different from those samples incubated for the duration of the experiment with stream water and here called *Zero Treatment Controls* (R = 0.004, p = 36.4). There was no difference between the compendium of manipulated sediments and the *Zero Treatment Controls* (R = 0.069, p = 15.9). This suggests that the communities from these treatments, though changing in time, changed in the same general direction, but with further changes imparted by the treatments (also see Multivariate Dispersion Results). Considering both sets of sediments together and excluding the *Zero Treatment Controls* and the *Time Control* sediments from the comparison, only the zinc treatment was jointly significant (R = 0.278, p = 0.1) for the case including all T-RFs. This effect was removed when routine exclusion of short (i.e. less than 30 bp) T-RFs was performed.

Effect of terminal restriction fragment selection.

The effect of inclusion or exclusion of the T-RFs noted above can be seen clearly in Figure 5.3 A & B. In Figure 5.3 A, both sites are clearly separated and the zinc samples (symbols with a white cross) show some tendency to gather together within sites, and tend towards the middle of the MDS plot. Figure 5.3 B shows clear separation of sites and a more coherent separation of the zinc treatments within and between sites, namely all zinc treatments (with one notable exception) are towards the top of the ordination. Table 5.6 shows the results of the ANOSIM tests for the two groups of terminal restriction fragments. The global R and significance levels for each of the tests is of the same order of magnitude for both sets of terminal restriction fragments except for those tests where zinc was a factor. The separation seen in Figure 5.3 B where shorter T-RFs were included is accompanied by a change from non significant to significant ANOSIM results where zinc was a factor both globally and for each site separately. Other tests remain largely unchanged.

Site effects.

It is clear from Table 5.6 and Figures 5.3 A & B that 'Site' was the single most important factor determining the separation of the community fingerprints derived from the *nosZ* gene.

Urban Samples. Considering the urban *nosZ* T-RFLP community structure alone, the results in Table 5.6 show that there is some support for the effect of carbon as a driver of community structural change, but that most of this was due to the effect of the protein carbon (R = 0.223, p = 3.9) and the effect, though significant, is only moderate. The evidence from this experiment is that the zinc treatment was a significant modifier (R = 0.363, p = 3.9) of the urban stream sediment community structure when all of the T-RF data was included. The effect was even stronger when the affects of carbon were accounted for (R = 0.475, p = 0.5). However, when the shorter T-RFs were excluded from the analysis, the ANOSIM test was not significant (R = 0.057, p = 17).

Non Urban Samples. The non urban *nosZ* sediment community responded strongly (Table 5.6) to the Zinc treatment (R = 0.435, p = 0.01) when considering all T-RFs but this effect was negligible (R = 0.088, p = 4.5), though still significant at the 95% level when only considering fragments greater than 30 b.p. The carbon treatments had no effect on the community structure.

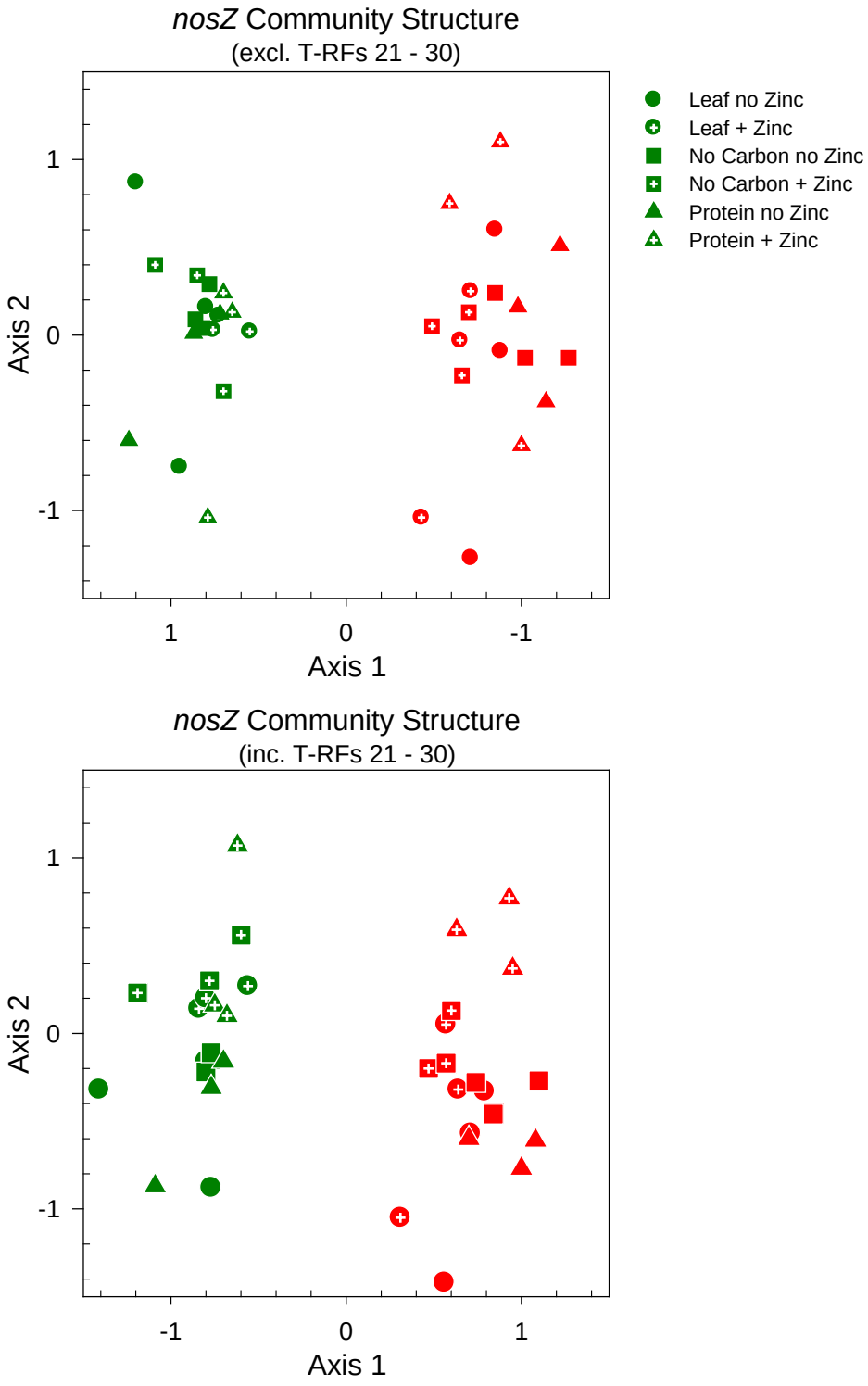

Figure 5.3 Community structure after incubation with different carbon sources with or without zinc. A: NMDS community ordination of *nosZ* T-RFLP fingerprints for sediments from both Urban (Red) and Non Urban (Green) sites – excluding T-RFs smaller than 30 bp. B: As for A, but including T-RFs smaller than 30 bp. Legend shows treatments for the non urban case. NB: Axis 1 reversed in B for ease of comparison.

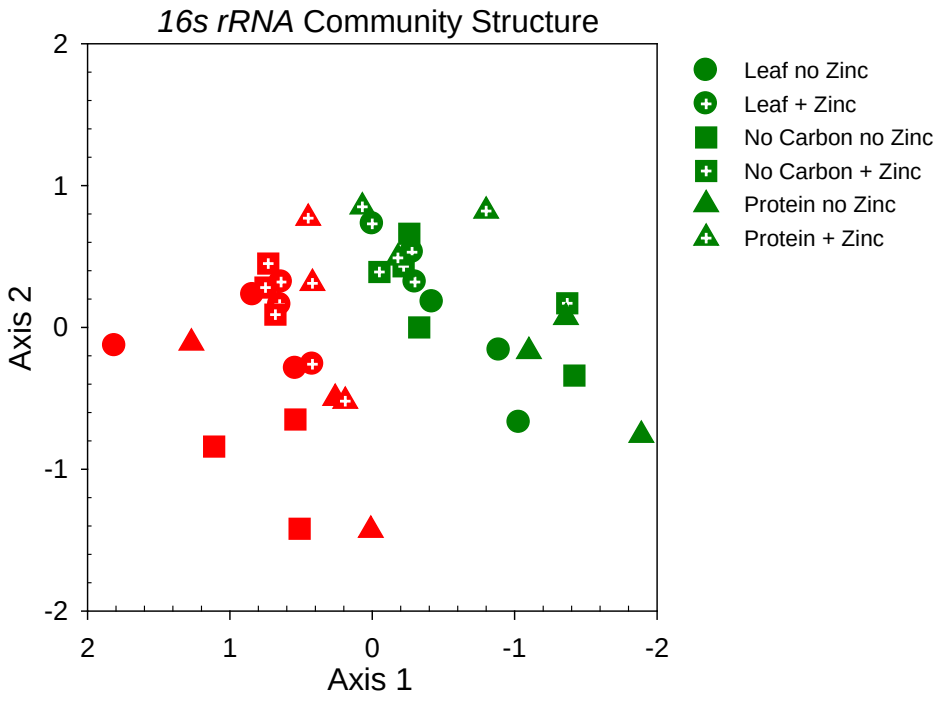

Figure 5.4 16s rRNA Community structure after incubation with different carbon sources with or without the addition of zinc for sediments from both Urban (Red) and Non Urban (Green) sites – excluding T-RFs smaller than 30 bp. Legend shows treatments for the non urban case. Red symbols are the urban cases.

5.3.2.2 The effect of experimental manipulations on the 16s rRNA T-RFLP fingerprints.

Comparison with controls.

The experimental treatments were significantly different (Table 5.7) from the *Time Control* (R = 0.956, p = 0.01). There was virtually no difference between the *Time Controls* and the *Zero Treatment Controls* (R = 0.142, p = 4.1). Although the largest difference was between the *Time Controls* and the manipulated sediments, there was also a significant difference between the *Zero Treatment Controls* and the manipulated sediments (R = 0.511, p = 0.01). These results suggest that although the communities changed during the experiment, the greatest change was for the manipulated sediments whereas the sediment that was treated only with stream water (*Zero Treatment Controls*) was similar to the *Time Control* (i.e. t = 0). This result is similar to that noted for the *nosZ* community structure above. Zinc was jointly significant for all treated samples (R = 0.241, p = 0.01), but carbon was not (R = 0, p = 43.5).

Effect of terminal restriction fragment selection.

The 16s rRNA gene electropherograms had few restriction fragments below 25 bp and inclusion of these terminal restriction fragments (20 -30 bp) in the analysis, although increasing the separation (R) and significance of the ANOSIM results (data not shown), made no overall difference to the interpretation of the patterns that emerged.

Site effects.

Although 'Site' was a significant determinant of community structure both when control samples (i.e. *Zero Treatment Control* and *Time Control*) were included in the analysis (R = 0.228, p = 0.01) and when these samples were excluded (R = 0.560, p = 0.01), the effect was about half that observed for the *nosZ* results. Comparing only those samples that received the zinc treatment showed that 'Site' was still the major determinant of community structure (R = 0.652, p = 0.004).

Urban Samples. No carbon treatment had either a large or significant effect on determining the structure of the overall microbial community (16s rRNA genes) in the sediment from the urban site (Table 5.7). The Zinc treatment however, had a significant, although moderate effect (R = 0.259, p = 0.20).

Non Urban Samples. Carbon was not a significant modifier of the overall bacterial community structure in sediment from the non-urban site (Table 5.7). However, the zinc treatment caused a significant change in the overall bacterial community structure (R = 0.357, p = 0.30).

Table 5.6 ANOSIM Test (10,000 permutations) for treatment differences of the *nosZ* T-RFLP derived community structure. The data has been manipulated in two ways; T-RF fingerprints including all T-RFs and T-RF fingerprints excluding T-RFs less than 30 bp (right most columns).[1]

Site/Treatment	Factor/Interaction	Comment	T-RF 20 -500 bp		T-RF 30 - 500 bp	
			Global R	Significance (%)	Global R	Significance (%)
All treatments	Site	Including *Zero Treatment Control* and *Time Control*	0.843	0.01	0.885	0.01
	Experiment Effect		0.096	6.3	0.106	5.3
		Time Control - *Zero Treatment Control*	0.004	36.4	0.015	30.2
		Time Control - Manipulated Sediments	0.136	5.5	0.143	5.4
		Zero Treatment Control - Manipulated Sediments	0.069	15.9	0.081	13.4
Manipulated samples: all sites combined	Site		0.872	0.01	0.92	0.01
	Carbon		-0.047	88	-0.036	79.9
	Zinc		0.278	0.1	0.008	30.3
Urban	Carbon		0.088	12.5	0.117	4.9
		No Carbon - Protein	0.223	3.9	0.255	0.9
	Zinc		0.363	0.01	0.057	17
	Carbon*Zinc	Carbon averaged across Zinc groups	0.152	13	0.175	8.2
		Zinc averaged across Carbon groups	0.475	0.5	0.173	12.1
Non Urban	Carbon		-0.134	99.7	-0.09	97.6
	Zinc		0.435	0.01	0.088	4.5
	Carbon*Zinc	Carbon averaged across Zinc groups	-0.171	98.3	-0.117	93.2
		Zinc averaged across Carbon groups	0.370	0.1	0.068	22.5

[1] The number of permutations for each treatment is shown in Table 5.7.

Table 5.7 ANOSIM Test (10,000 permutations) for treatment differences of the overall community structure - excluding fragments shorter than 30 bp. Determined from 16s rRNA gene T-RFLP fingerprints.

Site	Factor/Interaction	Comment	Global R	Significance (%)	Possible permutations
All treatments	Site	Including *Zero Treatment Control* and *Time Control*	0.228	0.01	Large
	Experiment Effect		0.691	0.01	Large
		Time Control - *Zero Treatment Control*	0.142	4.1	6435
		Time Control - manipulated sediments	0.956	0.01	177232627
		Zero Treatment Control - manipulated sediments	0.511	0.01	177232627
Manipulated samples: all sites combined	Site	All treatments	0.560	0.01	Large
		Zinc treatment only	0.652	0.004	24310
	Carbon		0	43.5	Large
	Zinc		0.241	0.01	Large
Urban	Carbon		-0.012	51.5	2858856
	Zinc		0.259	0.20	24310
	Carbon*Zinc	Carbon averaged across Zinc groups	0.066	28.9	78400
		Zinc averaged across Carbon groups	0.315	2.90	1000
Non Urban	Carbon		0.063	20.3	2858856
	Zinc		0.357	0.30	24310
	Carbon*Zinc	Carbon averaged across Zinc groups	0.047	33.9	78400
		Zinc averaged across Carbon groups	0.370	2.70	1000

Table 5.8 Relative multivariate dispersion for *nosZ* and 16s rRNA T-RFLP community structure data sets.

Site/Treatment	Factor/Interaction		Multivariate Dispersion		
			nosZ T-RF	*nosZ* T-RF	*16s r*RNA T-RF
			21 -500 bp	30 - 500 bp	30 - 500 bp
All treatments	Site	Non Urban	0.734	0.725	0.976
		Urban	1.266	1.275	1.024
	Experiment Effect	*Time Controls*	1.007	1.012	0.990
		Zero Treatment			
		Controls	1.128	1.140	1.444
		Manipulated			
		Sediments	0.994	0.993	0.981
Manipulated samples:					1.013
all sites combined	Site	Non Urban	0.833	0.831	
		Urban	1.167	1.169	0.987
	Carbon	None	0.834	0.848	1.036
		Leaf	1.058	1.042	0.829
		Protein	1.108	1.110	1.134
	Zinc	No	1.062	1.082	0.706
		Yes	0.938	0.918	1.294
Urban	Carbon	None	0.494	0.491	1.080
		Leaf	1.217	1.174	0.751
		Protein	1.288	1.335	1.170
	Zinc	No	0.906	0.931	1.371
		Yes	1.094	1.069	0.629
Non Urban	Carbon	None	0.778	0.777	0.972
		Leaf	1.094	1.088	0.778
		Protein	1.128	1.135	1.249
	Zinc	No	0.993	1.033	1.202
		Yes	1.007	0.967	0.798

5.3.2.3 Community variability within treatments – relative multivariate dispersion.

Table 5.8 shows the relative multivariate dispersion calculated from *nosZ* T-RFs for cases including and excluding fragments < 30bp and also for the 16s rRNA T-RF data.

Terminal restriction fragment diversity of the *nosZ* communities between treatments.
The relative multivariate dispersion calculated from the *nosZ* data suggests that in all cases the non urban site had a lower diversity than the urban site. This is clearest for the case where all treatments (including blanks and references) were included (Table 5.8). It is also noteworthy that there was little difference between the relative multivariate dispersion for the cases including or excluding the T-RFs < 30 bp across all treatments. This indicates that even though these two groups were defined by different species yielding a significant ANOSIM result, the overall diversity and multivariate distance within groups were similar.

The effect of carbon addition both across sites, and within each site separately, was a lowering of the relative dispersion for the 'no carbon' treatment – or conversely, to increase the number of unique T-RFs in both of the other two treatments (leaf & protein). This effect was consistent across all sites for *nosZ* and was largest for the urban site ('none' = 0.494 compared to 'leaf' = 1.217 & 'protein' = 1.288). The zinc treatment had little effect on the relative multivariate dispersion for each treatment suggesting that although different T-RFs (and by inference species) were present, the T-RF diversity of the two groups was similar.

Diversity of the 16s rRNA communities between treatments.
There was little evidence for large differences between sites based on a comparison of the multivariate dispersion from the 16s rRNA gene community data. Both sites have a near average 'diversity'. The *Zero Treatment Control* group had the greatest dispersion (1.444) of all treatments.

Both across sites and within sites, the 'Leaf' carbon treatment had a lower relative dispersion than other carbon treatments. Comparing the carbon treatments, the

dispersions were lower for the sites treated separately ('Urban – Leaf' = 0.751, 'Non Urban – Leaf' = 0.778) than when combined ('Leaf' = 0.829).

Zinc produced an interesting comparison. For the combined data (i.e. both sites), the no zinc treatment had a lower dispersion (0.706) than the zinc treatment (1.294). However, when considered separately, both the urban and non urban sites had higher dispersions for the no zinc treatment (1.371 & 1.202 respectively) than for the zinc treatment (0.629 & 0.798 respectively).

5.4 DISCUSSION.

Effect of manipulations on community structure.

T-RFLP analysis of the 16s rRNA gene community demonstrates that the bacterial community structure as a whole was different at urban and non urban sites. This site specific difference in bacterial community structure has already been demonstrated with the denitrifying community, as determined by the *nosZ* T-RFLP fingerprints. Another way of viewing this is that the *nosZ* community, being a broad subset of the 16s rRNA community, also reflects changes in the bacterial community as a whole. The greater separation of the sites in the NMDS ordinations (Figure 5.3; Table 5.5 & 5.6) derived from the *nosZ* community structure (ANOSIM R = 0.920) compared to the 16s rRNA gene community structure (ANOSIM R = 0.560) may reflect this. The implication being that because the total diversity of the denitrifying community (as measured by *nosZ* T-RFLP) must be lower than that of the 16s rRNA community, there are fewer species able to exploit the available environmental niches. It may also reflect the fact that the *nosZ* fingerprints are a coarser measure of the total number of denitrifying bacteria present in the sediments. However, it must be remembered that each fragment in a T-RFLP trace may represent more than one 'species' and that bacteria considered as separate 'species' with 16s rRNA gene comparisons may share a common lineage when compared with *nosZ*, as this gene may have been acquired for example via horizontal gene transfer between species with or from widely divergent 16s rRNA gene lineages – but able to exploit similar environmental niches. We must also remember that most bacterial species have multiple non identical copies (v. Wintzingerode et al. 1997, Weider et al. 2005, Case et al. 2007) of ribosomal coding sequences in their genomes which may also contribute to

the apparent greater diversity and also perhaps to the greater number of apparent shared 'species'.

The effect of different carbon additions.

Only one significant change in the community structure was observed as a result of the carbon treatment. For the urban sediment, addition of protein induced a moderate change (R = 0.223) in the *nosZ* community. A similar change was not observed when the two sediments were compared via 16s rRNA gene T-RFs. The only conclusion that can be drawn from this is that the protein treatment selected for a subset of denitrifying bacteria from the pool of organisms available from the urban sediment, but that these community differences were potentially obscured in the greater diversity represented by the 16s rRNA gene fingerprints. In other words, the potential exists for organisms with divergent ecological niches to be present in all (carbon) treatments that may be closely related thru a common 16s rRNA gene lineage. If this is the case then shared T-RFs contributing to both treated and non treated samples would diminish the observed magnitude of community changes. The phylogenetically diverse subset of organisms possessing the *nosZ* gene, may be contributing T-RFs in a 'coarser' (in the sense that there were fewer T-RFs) but more discriminating way for this particular treatment. This would depend on when and how an organism acquired the denitrifying trait in its evolution.

Organic carbon is a driving force that determines microbial communities (Madigan et al. 2000, Gao et al. 2005, Docherty et al. 2006) and single carbon treatments have been shown to have a strong effect on community composition (Preston-Mafham et al. 2002, Nakatsu et al. 2005, Wawrik et al. 2005). In many cases the carbon treatment is of relatively 'simple' molecules (Preston-Mafham et al. 2002); glucose and xylene in the case of Nakaktsu et al (2005). Using these single substrates and single soil samples the experimenter limits the study to essentially one of selective cultivation from among the diverse organisms present; those organisms that can utilize the substrate fastest dominate.

Two treatments, with addition of complex carbon sources were carried out to simulate more natural carbon inputs of the kind that occur in streams. The leaf leachate and protein solutions used contain a more complex suite of macromolecules than single substrate manipulations. This suite of macromolecules contain within them a greater

range of readily degradable loci on which the extra cellular enzymes released by bacteria can act. The Bacto Casitone (protein solution) has a greater number of compounds amenable to attack by peptidases, whereas the leaf leachate will have more molecules amenable to attack by various glucosidases. As each of these complex starting substrates are degraded, smaller less easily degraded (more refractory) molecules will also be produced (Amon and Benner 1996, Wetzel 2001). Harbott et al. (Harbott and Grace 2005, Harbott et al. 2005) have previously shown that enzyme activities in these streams are different, presumably in response to the different carbon inputs. As heterotrophic bacteria are capable of producing a range of extracellular enzymes (Chróst 1992, Findlay and Sinsabaugh 1999) these activity measures do not necessarily imply changes in species composition.

It was expected that the carbon enrichment experiment, would show that similar carbon treatments lead to similar community structure. This was not observed. The results show conclusively that the carbon treatments employed here did not produce similar communities from the two sampled sediments. The small change in community structure observed in the urban sample is probably an example of the selective effect described above. Warwrik et al. (2005) found that; "*…analysis of TRFLP data indicated that enrichment on structurally similar carbon sources selected for similar bacterial communities*." But they added, "*… communities first enriched on glucose or benzoate and subsequently transferred into medium containing an alternate carbon source retained a distinct community signature induced by the carbon source used in the primary enrichment*." In a more recent study similar in nature to the one conducted here, Langenheder et al. (2006) found that for short term (i.e. less than 1 month) incubations, clearly different bacterial communities from divergent habitats "*did not become more similar to each other upon growth under identical conditions*". Their conclusion was that while both the source community and environmental factors were both important factors determining microbial community structure, the source community was the dominant factor. They speculate that only in long term cultures on single substrates would the environmental pressure "*provoke the growth of very rare cryptic species*", thus creating a measurable change in community composition (Langenheder et al. 2006).

Although single substrate carbon treatments have been shown to induce some changes in community structure, where a diverse community of microorganisms has been conditioned as described above, the first carbon treatment has a significant bearing on the subsequent community structure. In the streams examined here, the prior carbon history is one of exposure to a complex suite of molecules. Thus although the community structures at the two sites are different, each population has among it sufficient diversity of organisms capable as a community to degrade and assimilate both of the chosen carbon sources. This suggests that within the range of normal fluctuations of carbon supply, natural populations of sediment bacteria may be relatively stable. High concentrations of simple single carbon treatments may be useful in isolating novel strains, but these treatments may only represent extreme situations normally only found at contaminated sites. In the context of the experiment described in this chapter, these observations suggest that either the 'prior exposure' (e.g. Warwrik et al. 2005) to the carbon sources derived from the catchment, and present in the sediments has imposed a dominant selective effect that is not fully overcome by subsequent carbon treatment; or that longer incubations are required to observe changes in the microbial community structure and that the preexisting community structure has a dominant selective pressure (e.g. Langenheder et al. 2006).

The effect of zinc addition.

Unlike carbon, zinc addition produced clear changes in the overall bacterial community structure when including both sites in the comparison and when each site was considered separately. This was contrary to expectations. It was hypothesized that the urban site should remain largely unaffected by the zinc treatment. The magnitude of the change relative to the pre-existing community structure at each site was slightly greater at the non urban site ($R = 0.357$, $p = 0.3$) than the urban site ($R = 0.259$, $p = 0.2$). The comparison between only those samples that received the zinc treatment demonstrated that "Site" remained the major determinant of community structure and that contrary to the hypotheses; the two communities did not converge.

From the *nosZ* T-RFLP results presented in Table 5.6 it is tempting to conclude that the effect of zinc on the bacterial community structure was greater for the non urban sediment ($R = 0.435$) than for the urban sediment ($R = 0.363$). However, given that the reverse

situation was found when using the two-way crossed ANOSIM averaging the zinc treatment effect across the carbon groups, and considering that these clear divisions are only manifest when T-RFs smaller than 30 bp were included in this analysis, the safest conclusion is that both communities changed to approximately the same extent: this includes the possibility that the *nosZ* community structure did not change (Table 5.6).

In many studies, and as standard procedure in the MDFRC laboratory, short T-RFs are frequently excluded from the analysis. The unstated argument for this appears to be concern about the reliability of short peaks. Reviewing some of the major papers in the development of T-RFLP as a technique to determine bacterial community structure, none of the author's state reasons for the almost arbitrary[1] lower size limit cut off for T-RFs to include in the statistical analysis (Liu et al. 1997, Marsh 1999, Osborn et al. 2000). The spread of lower sizes reported in the above papers for the shorter T-RFs ranges from sub 30 bp (Marsh 1999) through 37 bp (Liu et al. 1997) to 44 bp (Osborn et al. 2000). Where bias in the technique has been examined, although the reproducibility of T-RF size has been an issue, few authors have specifically investigated whether short T-RFs are inherently unreliable (Kaplan and Kitts 2003, Lueders and Friedrich 2003, Frey et al. 2006, Sipos et al. 2007).

The paper by Kaplan and Kitts (2003) specifically addresses measured T-RF size as an issue. The main finding of this study was that using the ABI 310 Genetic Analyzer, drift in the measurement technique (due to various factors) places a limit of ± 2 bp on the accuracy of measured T-RFs when compared to their 'true' length as determined by sequences retrieved from GENBANK. The authors state that other (presumably more recent) analyzers may have limits closer to ± 1 bp (Kaplan and Kitts 2003). This requirement for absolute accuracy in the determination of T-RF lengths is primarily aimed at those applications where researchers attempt to link peaks in the T-RFLP fingerprint to specific 'taxa' (Kent et al. 2003, Shyu et al. 2007, Thies 2007). These concerns were especially valid in the past when gel based systems were used to derive the fingerprint, but this concern may not be as valid with recent developments in sequencing technology using labeled size standards in laser based capillary electrophoresis systems.

[1] The practical lower limit is set by the ability of the sequencer to accurately resolve peaks in this range. The usable lower limit is set by the length of the labelled primer (usually ~ 20 bp), which must not include recognition sites for the restriction enzymes employed used to generate T-RFs.

For the case presented in this chapter the main requirement is that the error between samples is consistent.

The strong effect that the inclusion of these shorter peaks had on the *nosZ* data when interpreting the effect of the zinc treatment needs to be considered. Firstly, if these short peaks are unreliable in the sense that they are essentially random noise, then this should have had no effect on the ANOSIM test. A randomization test on random data should, by definition, produce a global R = 0 for this part of the data, and the final test result should be almost exactly the same as the test performed using only the longer peaks. The results for the 16s rRNA gene data (Table 5.7), where both sedimentary communities also changed to a similar extent, tend to support this conclusion.

Secondly, if there was a systematic error in this portion of the data set (i.e. the short peaks) then it was surprisingly consistent, being only associated with the zinc treatment (See Appendix 2 Table A5.1). Inclusion of the shorter peaks in the ANOSIM test did not alter the interpretation of the carbon treatment. It seems unlikely that significant quantities of zinc where carried through to the PCR and Restriction Enzyme digestion stages of the analysis. Even if some zinc were present, an explanation of the mechanism by which Zn might produce such a systematic error is required.

In future, there are two ways that this phenomenon can be examined[1]. If future researchers feel that the T-RFs produced from this region of the DNA strand are unreliable as they are 'too short', a primer site further upstream would need to be located and changes to the nosZ community due to Zn examined again using primer sets targeting this site; if one can be found. Alternatively, and perhaps more realistically, the reverse strand should also be selected for labeling as this would then place this region among the 'long' fragments in the derived fingerprints. However, if sites for the chosen restriction enzyme are located before this region of amplified DNA, the determination of variation in community structure (if real) due to this portion of the DNA may not be possible. A partial solution to this problem may be found by using the more recent primer sets proposed by Henry (2006).

[1] At the time of writing, reanalysis of the original DNA is no longer possible. The original samples were degraded when the -90^{o} C storage freezer lost power.

Community diversity.

Although some differences in the multivariate dispersion were found, diversity indices have been found to be poor indicators of community change when applied to bacterial communities (Hartmann and Widmer 2006), especially when comparing long term treatment effects (Gillan et al. 2005). In this study, carbon treatments at both the urban and non urban sites increased the multivariate dispersions for the *nosZ* community, but this effect was not as pronounced for the whole bacterial (i.e. 16s rRNA) community. The zinc treatment had no effect on the dispersion of the nosZ treatment but a noticeable effect on the 16s rRNA gene data. Although this particular index (Multivariate Dispersion) might be useful as a descriptive means of exploring differences between samples its use in studies of this nature may be limited. The values calculated using the MVDisp function are dependant on the primer and restriction enzyme combination used making comparisons between studies using different combinations impossible. Multivariate Dispersion has been employed in studies of macroinvertebrates (Clarke and Gorley 2001, Clarke and Warwick 2001), where the concept of the 'species' has a more precise meaning than in the case explored here. Terminal restriction fragments are a proxy indicator of species where each peak can represent multiple actual species of microorganism.

The amenity of diversity indices is further complicated by the fact that multiple copies of the 16s rRNA gene are found in most microbial genomes (v. Wintzingerode et al. 1997, Weider et al. 2005, Case et al. 2007). Phillipot and Hallin note (2005) "*for the denitrification genes, all known genomes are thought to carry only one copy*" with the exception of the *narG* gene. Thus it is possible that indices like Multivariate Dispersion either overestimate the diversity of organisms or otherwise cloud the results when using fragments from the 16s rRNA gene. At this stage it would appear that most of the denitrifying genes do not suffer from this effect. This may explain the more 'clear cut' differences between treatments noted in Table 5.8 using the nosZ data as compared to the 16s rRNA gene data; remembering that there is no actual statistical test applied to these descriptive results.

5.5 CONCLUSION.

Three hypotheses were proposed at the beginning of this chapter. Analysis of both *nosZ* and 16s rRNA gene TR-F fingerprints conclusively demonstrates that the two sites had different communities both before and after the manipulations thus confirming the first hypothesis. However, the chosen carbon treatments had only a minor effect on the community structure and then only when sites were considered separately. The hypothesis that similar carbon treatments would lead to similar community structures was found to be false under the conditions considered here. The prior carbon history and other factors considered in Chapter 3 are implicated as strong structuring mechanisms that were not altered by either the type or concentration of carbon treatments employed in this study. Finally, although the zinc treatment had the strongest effect on the community structure, with the zinc treated 16s rRNA communities appearing most similar, they were still measurably distinct communities at the end of the one month incubation period. The third hypothesis is also therefore false: zinc treatment alone was not sufficient to produce similar community structures from sediment sourced from two distinctly different streams, at least not under the conditions employed, and the time from adopted here.

The *nosZ* community results from the zinc treatment suggest that there may be a region in the amplified portion of this gene worth further investigation as a marker for heavy metal exposure. This speculation is based on the strong influence that inclusion/exclusion of a subset of the terminal restriction data had on the interpretation of the ANOSIM results.

Although commonly used in macro ecology, the use of diversity indices should be used with caution in fingerprint studies like this one – especially where multiple copies of non identical genes may be present.

In the following chapter, the effect that these manipulations had on the metabolic function of the sediment samples, as indicated by the measurement of N_2, CO_2 and CH_4 concentrations in the headspace of the microcosms will be explored.

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CHAPTER 6

METABOLIC RESPONSES OF SEDIMENT MICROBIAL COMMUNITIES TO CARBON SUBSTRATE AND HEAVY METAL TREATMENTS.

OVERVIEW.

In this chapter microbial activity was measured in response to the manipulation of sediments sampled from an urban and non urban stream. The experiment was predicated on the idea that the metabolic response of the bacterial community from urban sediment would react to an 'urban carbon source' more readily than sediment derived from a non urban catchment (and vice versa). Additionally, in light of the results from previous Chapters 2 & 3, it was thought that the urban sample would be more resistant to the toxic effects of a heavy metal (zinc). By incorporating measures of both metabolic function (gas generation rates) and community structure, the experimental design could provide some insight into any linkages between these important ecological concepts (structure and function) using two bacterial communities from the extremes of the urban gradient described in earlier chapters. The purpose of the gas determinations was not to make inferences about 'realistic' or 'natural' rates (i.e. on an area basis) in response to treatments; but rather as a way of gauging potential responses to treatments. Importantly, since all experimental units were handled in the same way, the gas production measurements can be compared across the experimental units.

6.1 INTRODUCTION.

Denitrification rates and community structure.

Frequently driven by concerns over the increased anthropogenic inputs of nitrogen in to the environment (Vitousek et al. 1997, Vitousek et al. 2002), studies of denitrification rates from many environments now exist (Seitzinger 1988, Barton et al. 1999, Martin et al. 1999, Simek et al. 2000, Watts and Seitzinger 2000, Zehr and Ward 2002, Dalal et al. 2003, Reay et al. 2003, Groffman et al. 2005, Piña-Ochoa and Álvarez-Cobelas 2006, Mulholland et al. 2008). While some studies have explored the merits of different measurement techniques (Payne 1991, Nielsen et al. 1997, Risgaard-Petersen et al. 1998,

Tomaszek and Czerwieniec 2000, McCutchan et al. 2003), others the factors thought responsible for the observed processes (Hedin et al. 1998, Stevens et al. 1998, Ettema et al. 1999, Luo et al. 1999, Hill et al. 2000a, Pinay et al. 2000, Bernhardt and Likens 2002, Simek et al. 2002, Stief et al. 2002, Sobczak et al. 2003, Öquist et al. 2004, Inwood et al. 2005, Pett-Ridge and Firestone 2005, Inwood et al. 2007), and yet others factors that structure the communities (Braker et al. 2001, Wolsing and Priemé 2004, Castro-González et al. 2005, Gao et al. 2005, Pett-Ridge and Firestone 2005, Hunter et al. 2006, Santoro et al. 2006), few studies have incorporated measurements of metabolic activity with determinations of the community structure (Cavigelli and Robertson 2001, Holtan-Hartwig et al. 2002, Araya et al. 2003, Rich et al. 2003, Rich and Myrold 2004, Enwall et al. 2005, Chénier et al. 2006).

In a review of the research into the links between denitrifier diversity and denitrification, Phillippot and Hallin (2005) observed with some surprise that;

> *"… almost none of the existing models to predict denitrification, nitrogen loss and nitrous oxide emissions in soils take into account the main players in this process — the actual denitrifying bacteria. This implies that either the diversity and density of this functional group are assumed to be the same, whatever the environment, and therefore can be modelled as a constant, or denitrifier diversity and density are unimportant for predicting gaseous nitrogen emission. However, denitrifier populations differ in physiological properties, such as their affinities for electron acceptors and donors or the relative reaction rates of the reductive steps*."

Cavigelli and Robertson (2001) used isolates from two fields of similar soil profile but different disturbance regime. One soil was a conventionally tilled agricultural soil while the other had never been tilled. Fatty acid profiles demonstrated that the bacterial communities were distinct. Isolation and cultivation suggested 27 denitrifying taxa were present with only 12 shared between the two sites. While community composition differed, diversity (as measured by the Shannon-Wiener index) did not. Although individual isolates clearly demonstrated different tolerances to oxygen levels, community level denitrification rates based on measurements from isolates were estimated to be

similar (Cavigelli and Robertson 2001). Thus although the potential exists for dentrifier community composition to alter *in situ* denitrification rates when isolates are grown under identical conditions (i.e. defined media), factors other than species composition may ultimately determine observed rates in the field (Cavigelli and Robertson 2001).

Rich and Myrold (2004) found that denitrifier community composition was different between stream sediment, riparian vegetation and nearby agricultural land, although the riparian and agricultural systems were more similar than the stream sediment (Rich and Myrold 2004). The distinct separation of riparian soil and stream sediment denitrifier communities has also been reported by Perryman (Perryman et al. 2008). There were also clear differences in the denitrification rates expressed on a per mass basis. But despite these clear differences in community structure and denitrification rates, Rich and Myrold (2004) conclude "*[b]ecause nosZ distribution and activities yielded different relationships among habitats, denitrifying community composition and functioning appeared uncoupled across this agro ecosystem.*" This conclusion was reached because the two sites that appeared most similar from a community structure point of view (i.e. riparian and agricultural soil), had dissimilar denitrification rates (Rich and Myrold 2004). It should be noted that in an earlier two site study Rich et al (2003) had come to the opposite conclusion. The studies of Cavigelli and Robertson (2001) and Rich and Myrold (2004) emphasize that this lack of agreement could have been due to one of several factors. The isolates of Cavigelli and Robertson (2001) may not have represented the *in situ* species and amplification of the *nosZ* gene does not measure expression of the gene and production of the actual *nosZ* enzyme (Rich and Myrold 2004). Also missing are measures of the interactions between organisms including plant roots (Cavigelli and Robertson 2001).

Inwood et al (2005, 2007) studied nine headwater streams each classified as either forested, agricultural or urban to explore the relationships between key environmental variables (e.g. [NO_3^-], [DOC]) and denitrification rates. Among these streams the availability of nitrate was the key environmental predictor of denitrification rates (Inwood et al. 2005) with DOC concentrations, oxygen levels and temperature proving to be of secondary importance. As the agricultural and urban streams had consistently higher levels of nitrate (and nitrogen species), these streams also had consistently higher

denitrification rates. Interestingly, Inwood et al. found that the proportion of nitrogen removed by denitrification was greater in the forested catchments, despite these lower rates (Inwood et al. 2005). Sediment core experiments revealed further details regarding the relative nutrient limitation at these sites with urban streams frequently limited by [NO_3^-], forested streams either nitrate limited or co limited by [DOC] and agricultural streams limited by [DOC] or co limited by [NO_3^-] (Inwood et al. 2007). In their study Inwood et al. were unable to comment on the qualitative effects of the carbon sources in these streams as carbon limitation was removed by the addition of readily available dextrose (Inwood et al. 2007).

Carbon sources and denitrification.

A number of studies have investigated the importance of carbon sources (electron acceptors) to the bacterial community and the effects on community structure (Bott and Kaplan 1985, Preston-Mafham et al. 2002, Eiler et al. 2003, Romaní et al. 2004, Gao et al. 2005, Lennon and Pfaff 2005, Nakatsu et al. 2005, Wawrik et al. 2005, Docherty et al. 2006, Edmonds et al. 2008). Sediments from the River Wiske (England) were used to explore how carbon sources can affect denitrification rates (Garcia-Ruiz et al. 1998). For their work Garica-Ruiz et al. (1998) used microcosm experiments prepared from the top 5 cm of extracted cores and the material incubated in sealed jars containing 20 mg L^{-1} nitrate and 100 mg L^{-1} of various carbon substrates. Using the simple substrates methanol, ethanol, glucose and acetate no increase in denitrification rate was observed relative to the control, but the ratio of nitrogen gases produced favoured N_2 for the substrates tested (Garcia-Ruiz et al. 1998)[1].

Addition of simple carbon sources can increase denitrification rates in agricultural soils (Frank and Groffman 1998, Luo et al. 1999) and wastewater treatment systems (Constantin and Fick 1997). For example, Constantin and Fick (1997) used additions of either ethanol or acetate to increase the denitrification rate in wastewaters with high nitrate concentrations. While complete dentrification was achieved with both substrates, acetate was found to be a more efficient substrate as it was more directly assimilated compared to ethanol.

[1] Garcia-Ruiz et al used the acetylene block method. Net N_2O production was determined from the difference between microcosms treated with and without C_2H_2. Denitrification rate here refers to the potential denitrification rate given the limitations of this method (see Discussion).

Responses of denitrifying communities to carbon additions have not always been predictable. From their extensive monitoring of piezometers along Smith Creek (Michigan), Hedin et al (1998) reasoned that only a narrow band of the riparian-hyporheic interface was responsible for the majority of dentrification. However, not all nitrate that reached this zone was consumed by denitrifiers. Denitrification rates could be increased by the addition of simple organic substrates (acetate, succinate, and propionate) and the authors suggest that management strategies that increased the provision of oxidisable carbon to this zone should be effective if nitrate removal was a goal (Hedin et al. 1998). However this increase in dentrification rate is not always observed. Bernhardt and Likens (2002) observed that although addition of labile carbon increased the uptake rate of nitrate, a corresponding increase in denitrification was not observed. Instead, the major response was increased bacterial growth, increased respiration and increased assimilation of nitrate (Bernhardt and Likens 2002).

Zinc and its effects on bacterial populations.

Results from Chapter 2 comparing sediment communities from the urban and non urban sediments suggested that heavy metals, particularly zinc, associated with the urban site had some correlation with changes in the community structure of denitrifiers. Results from Chapter 5 also suggest that additions of zinc had a strong effect on community structure even if not quite the effect that was originally hypothesized.

The addition of zinc to soil has been shown to dramatically affect both the structure and function of the soil microbial community (Kelly et al. 1999). Kelly et al. (1999) found that the addition of zinc (at a level of 6000 mg kg^{-1}) decreased the dehydrogenase activity of the soil by 95% after 15 days. As dehydrogenase has been shown to correlate with soil respiratory activity, suppression of this enzymatic pathway should have a strong effect on the processing of carbon. A similar conclusion was reached by Duarte et al. (2004) where the addition of Zn significantly decreased the decomposition rate of colonized leaf discs.

Treatment with multiple heavy metals (Cd, Cu and Zn) has been shown to decrease the rate of denitrification (Holtan-Hartwig et al. 2002). A single dose of metals (0.32 Cd; 80 Cu; 120 Zn: mg kg^{-1}) was sufficient to inhibit the nitrous oxide reduction rate although

nitrous oxide production i.e. nitrite reduction, was less affected (Holtan-Hartwig et al. 2002). The application of zinc alone (200 & 2000 mg kg^{-1}) has also been found to inhibit urease and nitrate reductase in soils (Hemida et al. 1997). Despite its essential role in cellular metabolism (Blencowe and Morby 2003) at elevated levels zinc can also affect cellular function by modulating turnover rates for those proteins susceptible to protease-dependent proteolysis (Kim et al. 2004). Thus, in addition to disrupting carbon processing (above), it is possible for one heavy metal to significantly perturb the nitrogen cycle by both inhibiting the release of ammonia from organic substrates, and preventing the reduction of nitrate to nitrite (the first step in denitrification).

Resistance to and recovery from heavy metal treatments is variable. Kelly et al. (1999) found that the dehydrogenase activity was still suppressed 420 days after treatment even though differences between the treatment and control community structures had become insignificant. The recovery of denitrifying enzyme activities in response to metal treatments depended on the enzyme (Holtan-Hartwig et al. 2002). While partial recovery of nitrite reductase activity was observed after 8 days and full recovery in 2 months, nitrous oxide reductase activity was still below that of the controls (Holtan-Hartwig et al. 2002).

Carbon composition and heavy metals are likely to be two factors that differ in streams from urban and non-urban catchments and this part of my work was designed to examine whether responses to further additions of different carbon sources and heavy metal (in this case zinc) would lead to different responses from the sediment microbes. Overall respiration of different pathways was used to measure microbial activity, and was done by measuring N_2, CO_2 and CH_4 production in the headspace of mesocosms. Results for these three gases were used to test the following hypotheses which mirror those of Chapter 5.

<u>Hypotheses.</u>

1. That gas generation rates are different between urban and non urban sites.
2. That gas generation rates from the urban samples will be less affected by the addition of zinc.

3. That the addition of carbon substrates will change the gas generation rates and that these changes will be characteristic to each site.

6.2 METHODS.

6.2.1 Site descriptions.

Sediment was collected from Lyrebird Ck and Gardiners Ck. Lyrebird Ck was chosen to represent the least urban end of the hypothesized urbanization gradient, whereas Gardiners Ck represents a highly urbanized stream. Locations and detailed descriptions for these sampling sites can be found in Chapter 3 Table 3.3.

6.2.2 Experimental design and analysis.

A factorial design was selected to compare the 2 sites (urban and non urban) with two levels of zinc treatment and three levels of carbon treatment: Table 6.1 showing the experimental design is reproduced from the previous chapter (where it appears as Table 5.1). In this chapter 4 replicates for the gas analysis were used. Further details on the experimental design can be found in the previous chapter.

Comparisons between treatments were made using Analysis of Variance (ANOVA) and the Student's t-test. The t-test was used to determine whether the gas production rates in the controls were different from sediments that received no zinc or carbon in the factorial design. The full effects of the experiment were then investigated using ANOVA. The ANOVA analysis was undertaken in two steps; first a global comparison was made for the effect of site (Urban - Non Urban) for all gas generation rates. The second step of the analysis investigated the effects of the two treatments (carbon and zinc) for each of the sediment samples separately.

Table 6.1 Experiment treatment matrix for comparing the effect of metal (Zn) and carbon (leaf/protein) treatments on the community structure of urban and non urban sediments.

Site	Zinc	Carbon	Gas Replicates	T-RFLP Replicates
	Yes	None	4	3
Lyrebird	Yes	Leaf	4	3
(Non Urban)	Yes	Protein	4	3
	No	None	4	3
Lyrebird	No	Leaf	4	3
(Non Urban)	No	Protein	4	3
	Yes	None	4	3
Gardiner	Yes	Leaf	4	3
(Urban)	Yes	Protein	4	3
	No	None	4	3
Gardiner	No	Leaf	4	3
(Urban)	No	Protein	4	3

6.2.3 Sample collection and preparation.

Descriptions of the sample collection, aggregation and pretreatment can be found in Chapter 5.

Once all sediment samples had been distributed (approximately 50g per serum bottle), and the appropriate treatments made (Table 6.1), the serum bottles were sealed with butyl rubber septa. The headspace of each bottle was purged by inserting two needles through the septa and connecting one needle to the Ultra High Purity (UHP) Helium (BOC Gases) supply. The headspace was flushed with UHP He for 1 minute, the two needles removed and the serum bottle shaken vigorously for ~ 10 seconds. Needles were then reinserted and the sparging procedure repeated 3 times. The serum bottles were then placed in a constant temperature room (20 ^{o}C) in the dark.

The sediment from the urban site was treated in exactly the same manner the next day, preserving the 1 day difference in collection times.

6.2.4 Reagents and solutions.

Zinc solution.

A 10g L^{-1}zinc solution was made by dissolving $ZnCl_2$ in deionised water. The final pH of this solution was 2.13.

Carbon sources.

Leaf Leachate. Woody material and leafy debris from the stream channel was collected at the non urban (Lyrebird Ck) site. This material was placed in a 1 L Schott bottle and autoclaved at 120 ^oC for 20 minutes. After cooling, an aliquot of this solution was diluted 1:10 to give a final volume of 2 L and Nitrate (KNO_3 AnalR, BDH) and Phosphorus (KH_2PO_4 AnalR, BDH) were added to give a final concentration of 1 mg-N $L^{-1}$and 40 μg-P L^{-1} respectively. This solution was stored at 4 ^oC until used. The TOC was 36.4 ± 1.4 mg C L^{-1}.

Protein Solution. A source of carbon rich in protein was made by diluting 680mg of Bacto Casitone (pancreatic digest of casein, Difco), 2 mL of 1g-N L^{-1} nitrate solution and 80 μL of 1g-P L^{-1} stock solutions to 2 L in deionised water. The final concentrations of nitrate and phosphorus were the same as the leaf leachate. Later analysis of TOC showed that this solution contained 133.7 ± 4.1 mg C L^{-1}.

Control Carbon Solution. A control solution with no carbon was prepared using deionised water with nitrate and phosphorus added to give the same initial concentrations as the above carbon solutions

Prior to distribution, the above solutions were pre purged of dissolved gasses by bubbling helium through a ceramic diffuser for approximately 20 minutes.

6.2.5 Head space gas sampling and analysis.

For the duration of the experiment, the concentrations of N_2, CH_4 and CO_2 were measured in the headspace of the experimental units. Oxygen levels were also monitored to detect potential contamination from the atmosphere.

Sampling procedure.

To avoid the introduction of atmospheric gases (especially N_2), a 5 mL syringe was filled and emptied 3 times with UHP He via an off take from the UHP He bottle. Each serum bottle was placed next to the sample introduction port of the GC. Two mL of headspace gas was then extracted from each serum bottle and again while gently expelling gas, the needle was quickly inserted into the sample injection loop and the final ~ 1mL of sample gas injected into the 250 μL injection loop of the GC.

After all samples and replicates had been processed, a 1 mL sample of water was extracted for pH measurement using a Horiba pH meter[1] and 1 mL of 100 mg L^{-1} NO_3-N injected to maintain nitrate levels and avoid substrate depletion. Serum bottles were inverted and allowed to settle again following the injection of nitrate to ensure mixing. Gas sampling, pH measurement and nutrient injection was repeated every 4 days (with 2 exceptions; one 5 day interval and one 3 day interval) for 28 days.

Gas analysis.

Gas analysis was performed using a Varian Star 3400 gas chromatograph fitted with a 250 μL gas sampling valve, thermal conductivity detector (120 ^oC), Alltech CTR-1 column (35 ^oC) and UHP He as carrier gas at 30 mL min^{-1}. The instrument was calibrated using a one point mixed gas standard (Scotty II, Mix234; Alltech 19792). Corrections were made to the gas concentrations to account for dilutions resulting from the multiple sampling described above and the gas generation rates were determined by fitting curves to the data using SigmaPlot (Systat Software). Estimates of metabolic activity were made using linear fits to the data (see Results and Discussion for further details) and comparisons between treatments made using Analysis of Variance (ANOVA). Rates, expressed as partial pressures, were normalized to the dry mass of sediment remaining in the serum bottle after all liquids were removed and the sediment dried at 110 ^oC at the end of the experiment.

[1] Model Twin pH B-211: ±0.1 pH unit repeatability calibrated before use with the supplied pH 4 and pH 7 bufffers.

Nomenclature.

The following labels are used to identify two kinds of control samples not included in the main manipulation matrix shown in Table 6.1. These two controls are in addition to the de ionized water blank used to check for gas leakage in the experimental units.

Zero Treatment Control refers to sediments that were incubated in serum bottles (4 from each site) with stream water collected at the time of sampling without being supplemented with nitrate or being subject to any of the treatments. This control was used to account for the changes in the community structure as a result of time isolated in the serum bottles.

Time Control refers to sub samples taken from each of the sediments and analyzed to determine the community composition as it existed prior to the manipulations (i.e. at time zero).

6.3 RESULTS.

6.3.1 pH changes.

Addition of the zinc solution did not appreciably change the pH between treatments (Table 6.2). See the previous chapter for further details.

Table 6.2 Change in pH* over time for Urban and Non Urban samples treated with (n = 12) and without (n = 16) zinc. Underlined values are statistically significant (students t; $\alpha = 0.05$)

Site	Urban				Non urban			
	without zinc		with zinc		without zinc		with zinc	
Day	pH	s.d.	pH	s.d.	pH	s.d.	pH	s.d.
0	7.11	0.12	6.53	0.11	6.83	0.06	6.52	0.04
4	7.20	0.12	6.73	0.15	6.87	0.10	6.59	0.03
9	7.33	0.13	7.02	0.14	7.00	0.08	6.83	0.07
13	7.48	0.18	7.42	0.26	7.29	0.18	7.07	0.08
17	7.29	0.25	6.98	0.20	6.86	0.12	6.76	0.09
21	7.17	0.15	6.95	0.27	6.71	0.07	6.61	0.09
25	7.11	0.16	6.88	0.19	6.71	0.08	6.61	0.09
28	7.31	0.17	7.04	0.20	7.27	0.06	7.15	0.09

* pH averages calculated using recorded pH values, not using means of $[H^+]$ and converting to the pH scale.

6.3.2 Measured headspace partial pressures and calculated rates of denitrification, methanogenesis and carbon dioxide production.

Figures 6.1, 6.2 and 6.3 are examples of the measured headspace concentrations for Nitrogen (N_2), Methane (CH_4) and Carbon Dioxide (CO_2). Each graph shows four replicate time series concentrations; gaps indicate accidentally missed sampling times for individual replicates. Gas generation rates calculated from these curves are shown in Table 6.4. These data were used to test for differences between treatments as shown in Table 6.5 and Figures 6.4 and 6.5. Figure 6.1 A & B show a steady increase in the concentration of N_2; one replicate in Fig 6.1 B is offset slightly due to incomplete flushing of the background N_2. These two graphs (A & B) are examples of the complete denitrification observed from sediments from the urban treatments. In contrast, Figures 6.1 C – F show the results from several of the non urban treatments. Individual replicates show either an increase, decrease or no change in the headspace concentration of N_2. The mean rate determined from these traces results in a near zero estimate of gas production with accompanying high variance.

Figures 6.2 A – D show the strong methane production from the non urban sediment samples relative to the much lower gas production rates observed in Figs 6.2 E – F. Also apparent in these traces is the inhibitory effect of the zinc addition. Figs 6.2 A & B show this effect for the non urban sediments treated with leaf leachate while Figs C & D show that the inhibitory effect was also present in samples treated with protein as a carbon source. Even though methane was produced at a much slower rate in the urban sediment samples, Figs E – F show that these samples also experienced an inhibitory effect due to the addition of zinc. Two other features are worth noting in these curves. First, each set of replicates starts from a near zero concentration demonstrating the effectiveness of the helium flushing procedure for this gas and second, replicates show close agreement to each other. Figures 6.3 A – F show the replicate headspace concentrations for CO_2. Figs A – D show the reduced CO_2 production in the urban samples relative to the non urban samples. Also notable is that the replicates, like those in Figure 6.2 show close agreement.

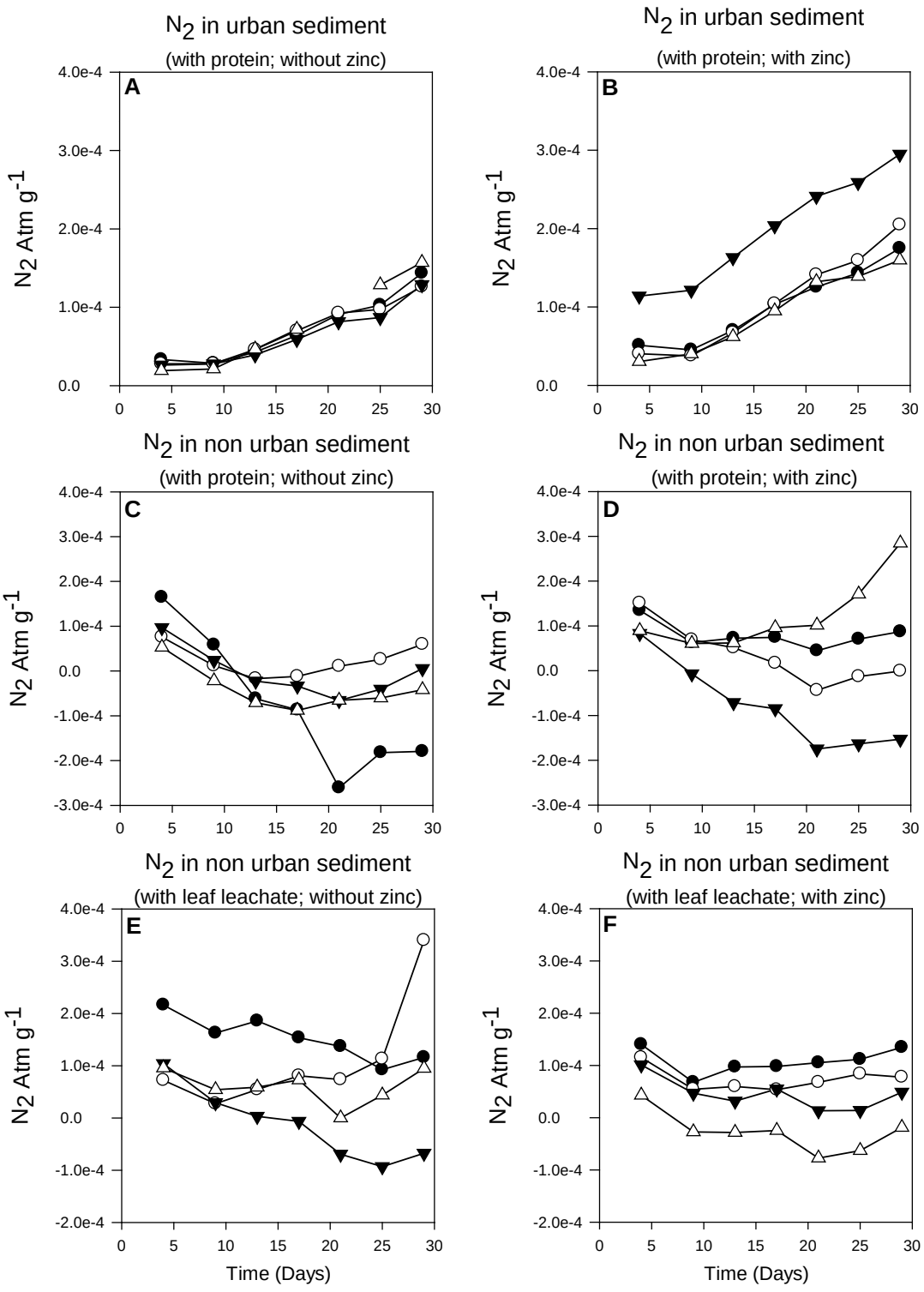

Figure 6.1 Replicate headspace nitrogen concentration time series plots from sediment samples from urban & non urban sites. Not all treatments are shown.

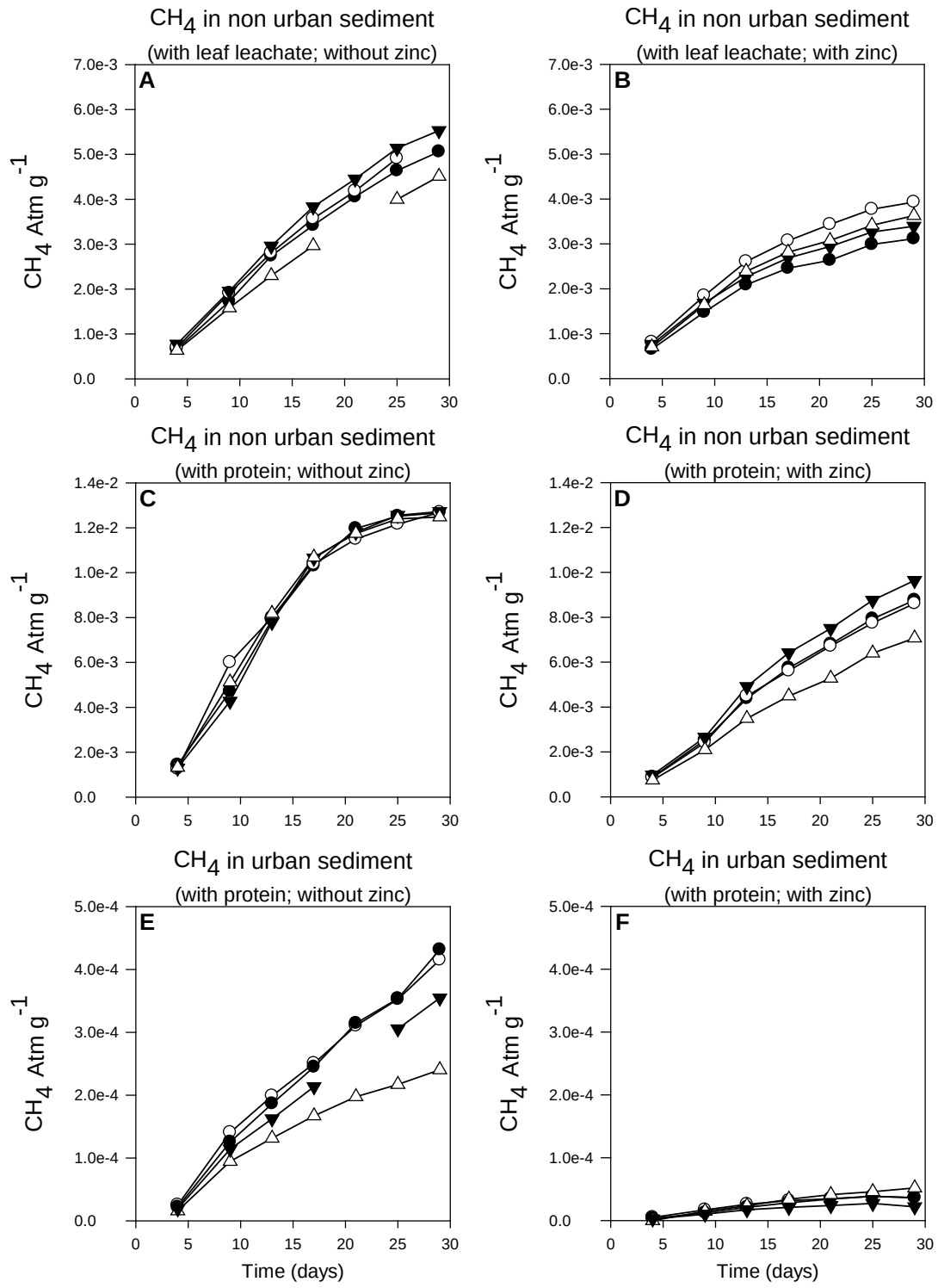

Figure 6.2 Replicate headspace methane concentration time series plots from sediment samples from urban & non urban sites. Not all treatments are shown.

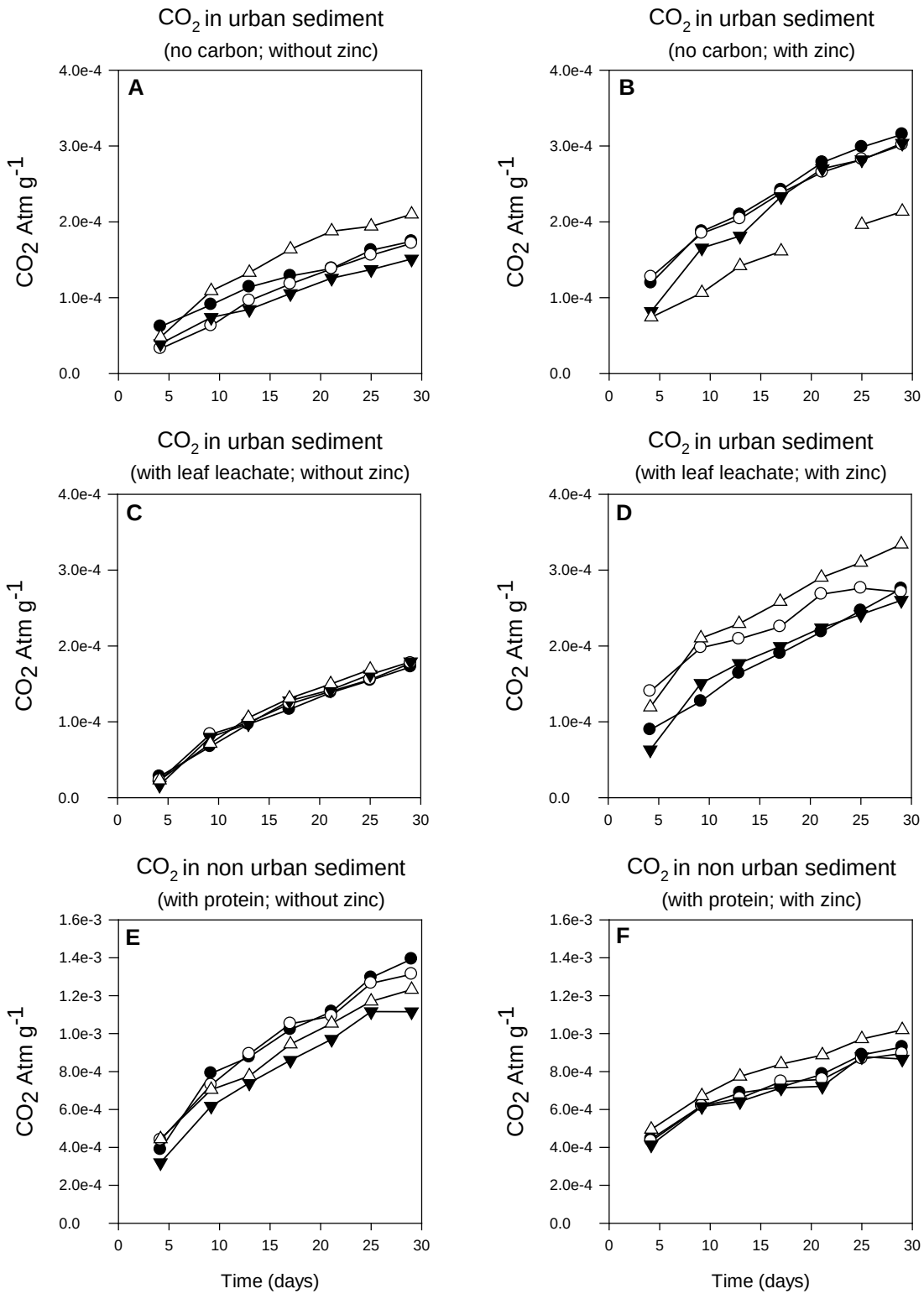

Figure 6.3 Replicate headspace CO_2 concentration time series plots from sediment samples from urban & non urban sites. Not all treatments are shown.

6.3.3 Response to nitrate addition.

The results of a Student's t-test (Table 6.3) suggest that process rates between the control samples used in the factorial design, and those treated with stream water (and no other treatment) were not significantly different. There was weak evidence for the CO_2 rate in the urban samples being lower in the control, and less convincing evidence for the CH_4 rate being higher in the control for the non urban sample. These observations are not statistically significant at $\alpha = 0.05$. This indicates that the addition of NO_3^- did not significantly affect the background gas generation rates of the controls.

Table 6.3 Students t-test of differences between samples not treated with Zn or Carbon (but treated with NO_3^{-1} injections), and samples treated with stream water and no additional treatment.

Site	Gas	N	t-value	p
Urban	N_2	8	0.548	0.603
	CH_4	8	0.699	0.525
	CO_2	8	-2.345	0.057
Non Urban	N_2	8	-0.843	0.448
	CH_4	8	2.205	0.087
	CO_2	8	-1.96	0.122

6.3.4 Comparison of treatment effects.

The effect of site.

The between site (all treatments) mean process rates were noticeably different (Figure 6.1 and Table 6.4). Figure 6.1 is a summary of the differences between sites that combines all treatments for each of the metabolic processes measured. Details of treatment differences within each site can be seen in Table 6.4 where the process rates for denitrification, methanogenesis and carbon dioxide production are shown for each treatment and the experimental controls. Of particular note are the increased rates of de-nitrification in the urban samples and the much larger rates of methanogenesis in the non urban samples. The variability in the combined denitrification rates (Figure 6.1) from the non urban site is large, and together with the observed low rates, suggests that the result is

near zero. Methane concentrations in the urban samples were initially very low and increased at a much lower rate than the non urban samples. Headspace CO_2 concentrations were also lower in the urban sediments. The clear differences in process rates between sites shown in Table 6.4 are reflected in the highly significant ($\alpha = 0.01$) ANOVA statistics shown in the top panel of Table 6.5.

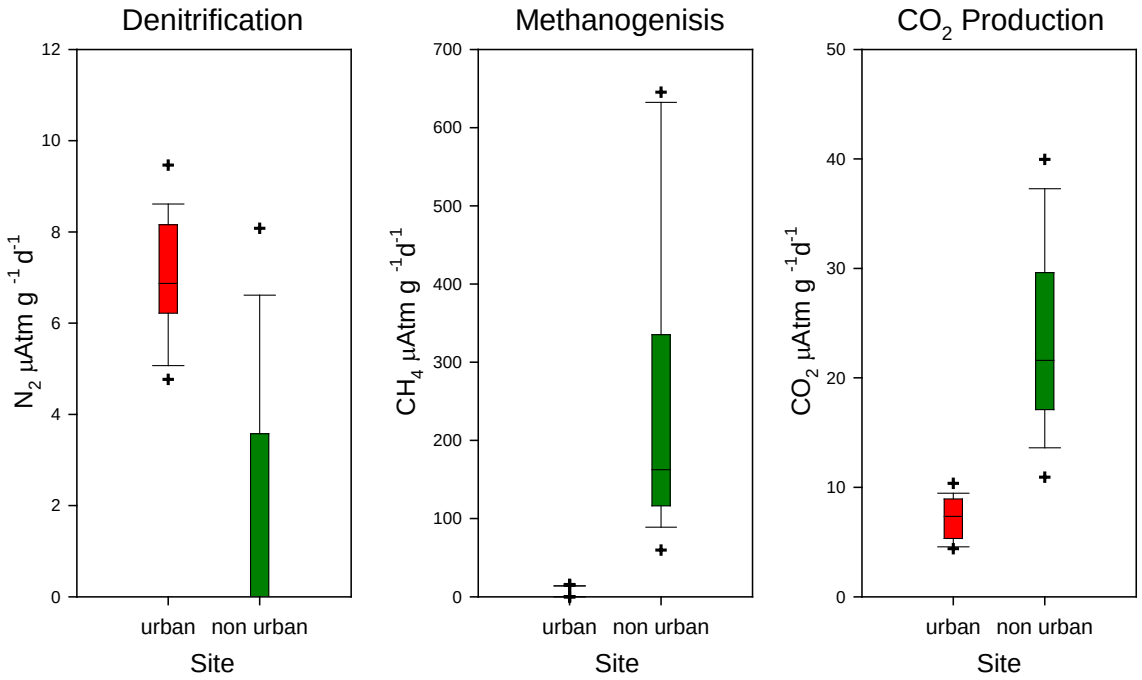

Figure 6.4 Box plots of overall site differences as determined from calculated gas production rates. The red boxes (■) are the urban samples and the green boxes (■) are the non urban samples. Boxes show 25th, 50th and 75th percentiles; whiskers show 5th and 95th percentiles; '+' indicates possible outliers.

<u>The effect of zinc.</u>

Inspection of Tables 6.4 and 6.5 and Figures 6.2 and 6.5 reveal some striking differences in the rate of methanogenesis between treatments with and without zinc at both sites. Denitrification and CO_2 generation, in comparison, generally showed a less consistent response. The gap in the data set for denitrification for the non urban sediment - protein treatment is a result of non linear process rates (see Discussion) for this treatment. For both sediments the addition of zinc had the effect of decreasing methanogenesis by half. However, addition of zinc to the urban sediment supplemented with protein as a carbon source appeared to completely suppress methanogenesis (Table 6.4). The zinc treatment also had a significant inhibitory effect on CO_2 generation for the non urban sediment (Table 6.5).

Table 6.4 Mean generation rates and standard deviations (n = 4) of the metabolic gases N_2, CH_4 and CO_2. All rates are in units of μ Atm g^{-1} d^{-1}.

Site	Treatments		N_2	stdev	CH_4	stdev	CO_2	stdev
	Carbon	Zinc						
Urban	None	-	7.4	1.9	1.5	2.4	6.0	1.6
Urban	None	+	6.8	1.3	0.60	0.84	8.6	1.6
Urban	Leaf	-	7.2	0.42	0.99	0.54	7.0	0.49
Urban	Leaf	+	7.7	1.1	0.34	0.26	8.2	1.2
Urban	Protein	-	5.5	0.96	13	3.2	8.9	0.87
Urban	Protein	+	7.3	1.2	1.4	0.49	4.7	0.27
Urban	Control	Control	-0.20	0.19	0.40	0.42	4.9	1.1
Non Urban	None	-	0.11	4.9	144	14	22	2.4
Non Urban	None	+	3.7	4.9	78	22	15	3.8
Non Urban	Leaf	-	-2.5	5.4	180	20	26	3.1
Non Urban	Leaf	+	0.69	1.7	109	12	14	1.6
Non Urban	Protein	-			630	24	38	2.5
Non Urban	Protein	+			347	53	19	2.4
Non Urban	Control	Control	-8.3	3.7	166	4.8	30	3.7

The response of bacterial communities to additions of different carbon sources.

Urban sediment. There was no significant difference between denitrification rates for any of the treatments (Table 6.5). Methanogenesis rates were significantly different between treatments. In contrast to this, CO_2 generation rates were not significantly different for either the carbon or zinc treatment, but were different for the combined effect of carbon*zinc (Table 6.5). This result appears to be driven by the strong suppression of both methanogenesis and CO_2 generation rates by the addition of zinc (see above).

Figure 6.5 (Over) Treatment differences between process rates (N_2 = denitrification, CH_4 = methanogenesis, CO_2 = carbon dioxide generation) showing the total or mixed effect of carbon and zinc treatments (left column) and the effect of carbon treatments with and without the zinc treatment (middle and right columns). The red circles (●) are the urban samples and the green circles (●) are the non urban samples. Error bars are one standard deviation.

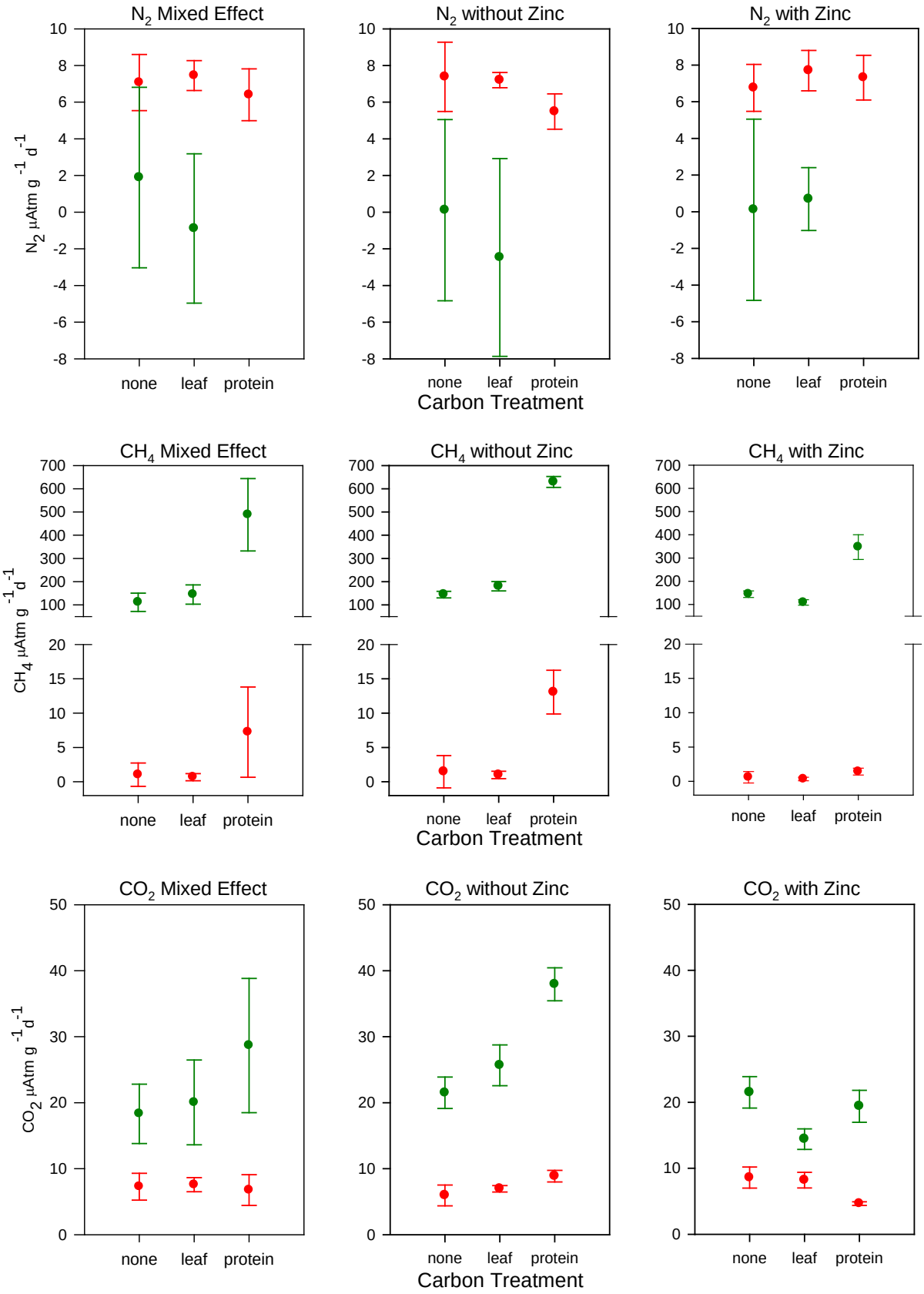
N2 Mixed Effect
N2 without Zinc
N2 with Zinc
N2 µAtm g-1 d-1
10
8
6
4
2
0
-2
-4
-6
-8
none
leaf
protein
Carbon Treatment
CH4 Mixed Effect
CH4 without Zinc
CH4 with Zinc
CH4 µAtm g-1 d-1
700
600
500
400
300
200
100
20
15
10
5
0
CO2 Mixed Effect
CO2 without Zinc
CO2 with Zinc
CO2 µAtm g-1 d-1
50
40
30
20
10
0

Table 6.5 Statistical comparison of denitrification (N_2), methanogenesis (CH_4) and carbon dioxide generation (CO_2) rates using ANOVA from urban and non urban sediments. Upper panel shows the result of a comparison between sites (including all treatment effects). Lower two panels indicate the significance of treatment effects for each sediment type separately. Results are for untransformed data, transformed data was also tested to remove some skewness in the raw data. See Appendix 3 Table A6.1& Figure A6.1.

Site	Factor or Interaction	Process	N	R^2	F-Ratio	η^2 (Effect size)[1]	p	Significance level
All	Site	N_2	40	0.531	42.96	N/A	9.91E-08	$\alpha = 0.01$
		CH_4	48	0.448	37.30	N/A	2.00E-07	$\alpha = 0.01$
		CO_2	48	0.615	73.36	N/A	2.33E-11	$\alpha = 0.01$
Urban	Carbon	N_2	24	0.315	1.501	0.114	2.50E-01	ns
	Zinc	N_2			1.278	0.048	2.73E-01	ns
	Carbon*Zinc	N_2			2.007	0.152	1.63E-01	ns
	Carbon	CH_4	24	0.906	38.49	0.402	3.15E-07	$\alpha = 0.01$
	Zinc	CH_4			40.82	0.213	5.13E-06	$\alpha = 0.01$
	Carbon*Zinc	CH_4			27.86	0.291	3.08E-06	$\alpha = 0.01$
	Carbon	CO_2	24	0.710	1.077	0.035	3.61E-01	ns
	Zinc	CO_2			0.053	0.001	8.20E-01	ns
	Carbon*Zinc	CO_2			20.90	0.674	2.03E-06	$\alpha = 0.01$
Non Urban	Model	N_2	16	0.240	1.265	N/A	3.30E-01	ns
	Carbon	CH_4	24	0.985	455.8	0.784	1.16E-11	$\alpha = 0.01$
	Zinc	CH_4			72.26	0.062	1.02E-07	$\alpha = 0.01$
	Carbon*Zinc	CH_4			80.76	0.139	1.02E-09	$\alpha = 0.01$
	Carbon	CO_2	24	0.919	33.32	0.299	8.91E-07	$\alpha = 0.01$
	Zinc	CO_2			49.51	0.222	1.45E-06	$\alpha = 0.01$
	Carbon*Zinc	CO_2			44.46	0.399	1.09E-07	$\alpha = 0.01$

1. η^2 calculated as $SS_{effect}/(SS_{total})$ is the effect size of the interaction term. As per Tabachnick and Fidell (2001). N/A means this test is not applicable for this case as the effect size is the same as R^2.

Non urban sediment. The non urban data is limited by the non linear response of the N_2 signal for some treatments (see 6.2.2 above). For this comparison, missing data points resulted in a non significant model using ANOVA. Besides this limitation, both methanogenesis and CO_2 generation rates were altered by the addition of carbon

substrates and the zinc treatment. Figure 6.4 shows that this result is driven by the suppression of gas generation rates by the zinc treatment. The protein carbon source acted as a readily used substrate – increasing both CO_2 and CH_4 generation rates (Table 6.4). The leaf leachate carbon source was a less effective treatment for increasing the production rates of both gases (Figure 6.4). The main treatment effects across both sediment types and treatments are the notable increases in CO_2 and CH_4 due to the protein carbon substrate and the suppressive effect of zinc. Although the urban and non urban sediments demonstrated differences in denitrification rate, neither of these sediments (considered individually) demonstrated any other treatment effects (Table 6.5).

For both sets of sediments, the individual treatment effects on denitrification rates were not significant, and because of the difficulties encountered with the non urban samples no further investigation can be made of these results. For the methanogenesis rates and to a lesser extent CO_2 generation rates, we can compare the calculated effect size (η^2) between sites (Table 6.4) for those results found to be significantly different by ANOVA. The effect size is the portion of variance due to that interaction term; effect sizes sum to the total explained variance (R^2). For the methanogenesis results, all treatments were found to have a significant effect on generation rates from sediments sampled from both sites. For the non urban sediment the carbon treatment had the single largest effect size (η^2 = 0.784) compared to the urban sediment (η^2 = 0.402). In both cases the zinc treatment, though significant, had the smallest effect size but the effect size for the urban sediment was greater than for the non urban sediment. For the CO_2 generation rates, the non urban samples were significantly affected by all treatments, but the combined effect of zinc and carbon had a larger η^2 than each of these treatments individually. A similar result was observed for the urban sediments, the individual effects of carbon and zinc were not significant but the combined effect (carbon*zinc) was significant (Table 6.5).

6.4 DISCUSSION.

Experimental methodology and effects.

The denitrification rate measurements reported here were potentially limited by the necessity of relying on the N_2 flux method[1]. The greater sensitivity of the Electron Capture Detector (ECD) compared to the Thermal Conductivity Detector (TCD) (Skoog et al. 1998) would have enabled more frequent sampling of the headspace gases using a smaller sub sample. The risk of contamination from atmospheric gases during sample acquisition would also have been reduced. However, these advantages need to be considered in relation to the disadvantages; inhibition of nitrification, poor penetration of C_2H_2 into the sediments and incomplete inhibition (Garcia-Ruiz et al. 1998, Watts and Seitzinger 2000). In addition Watts and Sietzinger (2000) have found that the acetylene block method may underestimate the actual denitrification rate by two orders of magnitude in the systems they studied. Although Watts and Seitzinger (2000), using the N_2 flux method, found that denitrification rates could potentially be measured after only 2 days[2], sampling in the case reported here was limited to a ~ 4 day interval to help ensure that adequate denitrification had occurred before measurement; especially since the repetitive sampling regime employed (see methods) and small microcosm volumes led to regular dilution of the headspace gases.

In order to minimize the major source of error from the N_2 flux method, namely detecting small changes in the partial pressure of N_2, the liquid phases and headspace of each bottle were rigorously flushed with ultra high purity (UHP) Helium gas to reduce the background concentration of N_2. This process, combined with the sample homogenization steps before the sediment was distributed to individual serum bottles, destroyed any overall physical structures the sediment cores may have retained. Any measurements derived from this experiment do not attempt to give *in situ* rates of denitrification, rather they give a potential rate that can be generated from the sediments. Since all treatments were handled the same way, the information derived

[1] The initial plan was to use the acetylene block method coupled with N_2O detection via an Electron Capture Detector potentially (ECD) allowing greater measurement frequency. However, the available ECD was damaged and the N_2 flux method was substituted.

[2] The volume of sediment used by Watts and Seitzinger (2000) was approximately 250cm^3; twice the total volume of the serum bottles used in this study.

Table 6.6 Cross comparison of denitrification rates with other studies after conversion to common units.

Description		Denitrification ($\mu g\ N\ kg^{-1}\ h^{-1}$)		Reference
		Mean	Stdev	
Forest Stream	Debris Dam	185	31	(Groffman et al. 2005)
	Pool	48	33	
	Riffle	15	9	
	Gravel Bar	8	6	
Suburban Stream Forested	Debris Dam	1604	76	(Groffman et al. 2005)
	Pool	36	30	
	Riffle	18	7	
	Gravel Bar	21	15	
Suburban Stream Unrestored	Pool	21	15	(Groffman et al. 2005)
	Riffle	8	7	
	Gravel Bar	16	15	
Gravel Sand Cores	Upflow	0.46	0.05	(Storey et al. 2004)
	Down flow	1.96	0.08	
Headwater Streams	Forested	30	ns*	(Inwood et al. 2005)
	Agricultural	200	ns*	
	Urban	690	ns*	
Lowland Stream	Gravel Bar	15	ns*	(Hlavácová et al. 2005)
Melbourne Streams	Urban	13	3	This study
	Non Urban	1	6	

* not stated.

from the experiments shows the relative change in rates in response to the treatments and whether these changes were reflected in the community structure. These caveats aside, the measured denitrification rates for the urban samples are comparable to similar sediments (Table 6.6). Sediments from urban or lowland streams that consisted of sand or gravel typically had lower denitrification rates than those found in pools or debris dams.

Effect of manipulations on process rates.

Evolution rates for all three measured gases were different between sites but comparable to other sediment studies (Table 6.7). The most obvious difference between sites was the much larger production of methane from the non urban samples (Table 6.4). Only one treatment from the urban site showed methane generation rates significantly greater than the control treatment; the samples treated with protein substrate (Table 6.4). Treatment of these samples with zinc reduced the generation rates to a level comparable to the control. This inhibitory effect was also noticeable in the non urban samples where the gas generation rates (Table 6.4) were almost halved from ~ 630 μ Atm g^{-1} d^{-1} to ~350 μ Atm g^{-1} d^{-1} for the protein treatment. The relative magnitude of this inhibitory effect was comparable for the other two carbon treatments for the non urban samples. Inhibition of methane production has been reported previously (Bhattacharya et al. 1996, Codina et al. 1998, Lin and Chen 1999, Zayed and Winter 2000). Both Zayed and Winter (2000), and Codina et al. (1998) found that Zn concentrations in excess of 50 mg/L reduced methanogenesis by ~ 50%, whereas Bhattacharya et al (1996) found that acetate utilization was inhibited when Zn was present at 20 mg L^{-1}. Lin and Chen (1999) also noted that the inhibitory effect (in their case for anaerobic sludge blankets) depended on the type of fatty acid being utilized as a carbon source. On a total volume basis (90 mL aqueous phase plus ~ 50 mL of sediment), the Zn added in this work had an approximate concentration of 70 mg L^{-1}. As percentages the reductions in the methanogenesis rates reported here as a result of Zn toxicity are comparable to those reported in the literature (above).

Table 6.7 Cross comparison of methanogenesis rates from various studies. Measurements are μg CH_4 kg^{-1} hr^{-1}. Figures in square braces [ ] are ranges, those in parenthesis ( ) are standard deviations.

Description	Methanogenesis (μg kg^{-1} hr^{-1})	Error	Reference
Salt Marsh Sediment	22	[3 – 41]	(Capone et al. 1983)
Salt Marsh Sediment	75 765	[36 – 99] [522 – 933]	(Jones and Paynter 1980)
Intertidal Sediments	4.8-340	5%	(Mountfort et al. 1980)
Peatlands	2 - 220	ns*	(Williams and Crawford 1984)
Tropical Rice Soils	174	ns*	(Mishra et al. 1999)
Urban (+Zn)	0.23	(0.16)	This Study
Urban (-Zn)	13	(6)	
Non Urban (+Zn)	54	(14)	
Non Urban (-Zn)	361	(31)	
Biogas Digester	~11,000	ns*	(Vieitez and Ghosh 1999)

* not stated.

There are two gaps in the rate data for denitrification from the non urban samples. As mentioned briefly in the results section, the rates for these samples were either not linear over time, some exhibiting an initial decline followed by an increase (Figure 6.1), or the response from replicates was inconsistent. Two options presented themselves for analysis. A linear fit to these results would result in an estimate from the replicates of an approximately zero rate with a very high error term undermining the robustness of comparisons made via ANOVA. Alternatively a non linear fit could be applied to some results, but then the results across replicates and treatments would be hard to compare directly. These samples were excluded from this part of the analysis for these reasons. A linear fit was applied to all remaining samples and treatments for ease of comparison. Although a non linear fit may have been suitable for some traces (Figure 6.2), in the case of the methane results this would only be needed to incorporate the last point in these traces. Although the urban treatments (with the exceptions described above) did not exhibit significant methanogenesis, they were the only samples to exhibit denitrification

rates significantly greater than zero. All urban samples that received nitrate exhibited denitrification (Table 6.4) and denitrification rates for these sediments were not affected by any of the treatments. This is somewhat contrary to the results of previous research in this area where (partial) inhibition of either nitrous oxide reductase or nitrate reductase has been reported (Hemida et al. 1997, Holtan-Hartwig et al. 2002).

There are two probable reasons for the lack of denitrification in the non urban sediment. Firstly, as has been covered in Chapters 2 and 3, one of the signature characteristics of the non urban sediment was the finer texture, with a greater mass fraction in the < 355 μm size class (Table 2, Chapter 2; Table 3.6, Chapter 3). The direct consequence of this is a correspondingly greater surface area available for colonization by bacteria, and therefore a greater biomass per unit mass of sediment. Assuming, as seems likely, that the established bacterial community from the non urban sediments was dominated by methanogenic and other fermentative organisms, addition of organic substrates may have gone directly to bacterial growth. Bernhardt and Likens (2002) concluded this after a whole stream addition of soluble DOC remarking that this was "*[c]ontrary to expectations*". They found that the "*…principal effect of DOC enrichment was to stimulate bacterial growth, leading to high respiration and a corresponding increase in the assimilative demand for nitrogen*." The greater surface area of the sediment coupled with the potential stimulation of bacterial growth and consequent high demand for nitrogen may have prevented denitrifying bacteria present in the sample becoming dominant. Where Bernhardt and Likens (2002) speculate that nitrate is being incorporated at the surface of the sediments, thus potentially limiting the rate of uptake, this limitation would not be expected in the experiment conducted here as the serum bottles were inverted every four days.

It may be possible to extend this line of thinking to account for the low rates of methane generation in the urban samples. The coarser sedimentary material from the urban site has a lower surface area and likely a lower bacterial biomass. It is also likely that the greater porosity of this source material allowed more aerobic heterotrophs in the initial bacterial community as oxygen ingress to the sediment is less restricted. Under these assumptions, the total removal of oxygen from the experimental unit and the regular addition of nitrate should have favored facultative and obligate anaerobes capable of utilizing nitrate as

electron acceptor. Another possibility might be the existence of consortia capable of anaerobically oxidizing CH_4 with nitrate as described by Raghoebarsing et al. (2006) and also Thauer and Shima (2006). Under this scenario, methane produced by fermentation of the added organic substrates is oxidized to CO_2, reducing the accumulation of CH_4 in the headspace. Raghoebarsing et al. (2006) isolated these slow growing organisms from a freshwater canal contaminated by agricultural runoff, an environment not dissimilar to that encountered in urban streams.

6.5 A Re-Appraisal of The Initial Hypotheses.

It is clear that the gas generation rates for the three gases studied here are different at both sites, thus confirming the first hypothesis. This finding is uncontroversial as it is well known that differences in the rates of denitrification and methanogenesis (for example) are controlled by site specific differences in the availability of substrates, among other factors, at multiple scales (Wahlen 1993, Ronald R et al. 1997, Jacobson 2000, Madigan et al. 2000, Pinay et al. 2000, Schimel 2004, Groffman et al. 2005, Piña-Ochoa and Álvarez-Cobelas 2006, Inwood et al. 2007). Confirmation of this hypothesis mirrors the finding in Chapter 5 that community structure at each site was unique, which implies that site specific factors that structure the bacterial community may also be a factor in determining the rates of denitrification, methanogenesis and carbon dioxide production.

The addition of zinc produced mixed effects, inconsistent with the second hypothesis. It was assumed that an environment that was regularly exposed to heavy metals (i.e. urban) would produce a bacterial community with some resistance to a heavy metal treatment. The zinc treatment clearly suppressed the generation rates of methane at both urban and non urban sites and carbon dioxide at the non urban site. If the second hypothesis were applied to methanogenesis alone then the conclusion must be that zinc treatment unilaterally suppresses methanogenesis; in fact the sensitivity of methanogenesis has been proposed as an assay for determining metal toxicity (Codina et al. 1998). The same conclusion can not be made for CO_2 generation rates, partly as the rates for the urban samples were significantly lower than those found in the non urban samples.
The situation with respect to dentrification is confounded by a lack of data, or data indicating no denitrification from the sediments collected from the non urban site. The

data from the urban site however, suggests that denitrification was not affected by the addition of zinc which is contrary to previous studies and tends to support the second hypothesis. However, until further investigation across more sites is attempted the safest conclusion for the second hypothesis may be that the effect of zinc on metabolic processes from urban and non urban sites depends on the metabolic process.

The third hypothesis considered the effect of carbon. The results suggest that carbon addition had no effect on the rates of denitrification for the urban site; for the non urban site the denitrification rate was no different to the control. Leaf leachate produced no change in the generation rates of either methane or carbon dioxide but the addition of the more labile protein substrate produced clear increases in methanogenesis. Thus although clear changes in gas generation rates were observed, in the form they are presented here they can not be said to be characteristic of each site.

6.6 Community Structure and Metabolic Function: A Synthesis of Results from Chapters 5 and 6 and Recommendations for Future Work.

Community differences, metabolic rates methodological issues.

Chapter 5 opened with the following quotation;

> "*A community adapted to elevated ambient levels of a particular pollutant is expected, compared to a non-exposed community, to display an increased tolerance to that pollutant*" (Soldo and Behra 2000).

The evidence relating the changes in community structure and denitrification rates to the zinc treatment are not clear cut. Chapter 5 discussed at some length the possibility of significant changes to the community structure in response to the zinc treatment and the probable source of confounding error in these determinations. The gas generation rates are also confounded by the fact that essentially little useful denitrification data was available for the non urban case. Considering only the urban case, two alternate conclusions could be made. Given that the community data from Chapter 5 suggested a significant structural change in the nosZ community in response to the zinc treatment, then the denitrification rate data indicates that such a change was accompanied by no

change in the denitrification rate. One conclusion from this is that the community structure changed rapidly (i.e. between sampling periods) to a more zinc tolerant community as there was no transitional period during which the denitrification rate decreased. An alternate conclusion being that the denitrifying population at the urban site has developed a protection mechanism to metal stress, thus maintaining the rates of denitrification, and that the changes in community structure occurred subsequent to this.

A third conclusion is possible. The observed rates may be the maximum possible rate and nitrate limitation has prevented increases in the headspace concentrations. This conclusion does not totally invalidate either of the above alternatives as the rates did not decrease. But if this conclusion is true, it would potentially mask differences between treatments within sediment groups as the rate of nitrate introduction[1] described in the methods puts an upper limit on the rate of denitrification.

Chapter 5 concluded that the community structures represented by both the *nosZ* and 16s rRNA gene systems were unique at the two sites and that carbon treatment had no measurable effect on the community structure. Thus, in response to the remarks of Phillipot and Hallin (Philippot and Hallin 2005) at the beginning of this chapter we can say that the community can not be modeled as a constant, and that the answers lie elsewhere. While, the results from this chapter show that the generation rates of three metabolically important gases are also different, it is worth recognizing that this comparison was made on a per gram sediment basis and not on some universal measure of biomass or metabolic activity. Measures of function expressed on a per mass or per area basis are usually made for estimating gross environmental process rates (Seitzinger 1988, Groffman et al. 2005, Mulholland et al. 2008). As the sediment itself is one of the factors that influences community structure (Chapter 3), what is missing in this and most other studies is a measure of cell biomass. With this, the extent to which changes in process rates are due to changes in community structure or changes in either the total or relative biomass of individual members of the bacterial community could be determined. Thus although we know that the bacterial community structure is qualitatively different at the two sites (Chapter 5) and that the absolute rates are different (this chapter), we need to know whether on a per active biomass basis the differences in rates are comparable. In

[1] Chosen to maintain a concentration near the median DIN concentration ~ 1mg/L (see Table 3.3).

other words, were measured denitrification rates controlled by the biomass and physiology of the taxa present, or by the physical and chemical properties of the sediment?

To understand the specific activity of the sediment organisms, some means of normalizing the community data and the metabolic data on a common basis needs to be adopted. This is problematic as DNA extraction efficiencies depend on both the method used and the sediment or soil being extracted (Steffan et al. 1988, Cullen and Hirsch 1998, Yeates et al. 1998). There are several approaches to this problem. Some authors (Kelly et al. 1999, Bossio et al. 2005) have used phospholipid fatty acid (PLFA) assays as a means of tracking both community composition and biomass. But the method requires careful extraction and derivitization of the fatty acids before analysis by gas chromatography; the taxonomic resolution is more limited than T-RFLP and the method relies on signature fatty acids which are often produced at different levels between different taxa (e.g. bacteria compared to fungi) and in response to stress (Hill et al. 2000b). Signature fatty acids are related to taxonomic groupings and not to functional traits like denitrification. Fluorescence *in situ* Hybridization (FISH) is another method used to study changes in community composition (Hill et al. 2000b, Araya et al. 2003, Gao et al. 2005). It can differentiate between live and dead cells, employs taxon specific probes and avoids PCR related biases, but it requires the use of expensive confocal microscopic equipment (Hill et al. 2000b).

Real Time PCR offers a means of quantifying the amount of DNA in a sample and has recently been applied to determining the number of *nosZ* copies relative to the number of 16s rRNA genes in soil samples (Henry et al. 2006, Philippot 2006). Using these ratios it might be possible to estimate the biomass of denitrifiers as a percent of the total biomass determined from epifluoresence using sensitive dyes like SYBR (Noble and Fuhrman 1998, Ponchel et al. 2003). However the method is still limited by the DNA extraction efficiencies from different soil samples. To over come this, a means of determining the extraction efficiency from different soil/sediment samples needs to be developed. For the purpose of T-RFLP analysis one approach might be the construction of a short sequence of double stranded DNA (ds-DNA) containing just the forward and reverse primer sequences on complementary strands. In the case of the *nosZ* primers used in this study,

such a construct would be about 40 bps long. As the primers do not contain the restriction enzyme recognition sequence, amplification and digestion of this artificial construct will produce a fragment 40 bps long in the final T-RFLP trace. To distinguish this fragment from any other potential T-RFs of the same length, the entire T-RFLP procedure should be conducted with differentially labeled forward and reverse primers. Only this artificial construct will posses both fluorescent labels, all other fragments having been cut by the restriction enzyme will either have the forward or reverse label[1]. This artificial ds-DNA could be used as an internal standard by adding a known (small) quantity to each of the sediments to be extracted, and then carried through the entire procedure. Real Time PCR can then be used to quantify the total number of copies of the gene and, knowing the number of copies added as the artificial construct, it should be possible to determine the relative proportions of the other fragments. Using forward and reverse primers that are both labeled will also increase the number of useable peaks in the community data set, potentially increasing the resolution of the method.

Reverse transcription RNA community analysis is another recently used method in the analysis of nitrogen cycle dynamics (Nogales et al. 2002, Burgmann et al. 2003). This method, if coupled to T-RFLP analysis, produces a fingerprint of those members of the bacterial community actively expressing the gene of interest. Coupled with the approach described above, these two methods could give a measure of both the number of potential denitrifiers present and potentially the proportion of those actually doing so. However, both of these methods are more complex than the relatively simple T-RFLP procedure employed through out this thesis, and would require extensive method development before routine application (if possible) could be attempted.

<u>Carbon additions and rates of methane and carbon dioxide production.</u>

Carbon addition as protein had a significant effect on methane production at both sites, but carbon dioxide rates were only affected at the non urban site; denitrification was unaffected. This finding is perhaps consistent with the theory proposed in Chapter 5 that prior carbon treatment has a major structuring effect on the bacterial community and that the concentrations of the added carbon substrates was insufficient to drive major changes.

[1] Excluding the unlikely case of a sequence that doesn't posses the restriction enzyme.

In addition, as has been shown elsewhere, bacteria are capable of expressing a myriad of extra-cellular enzymes to increase the pool of carbon available for cellular uptake (Findlay et al. 1997, Findlay and Sinsabaugh 2003, Harbott and Grace 2005, Harbott et al. 2005). The lack of community structure changes in response to the chosen carbon treatments while metabolic changes were recorded reinforces this conclusion. In the case of the non urban site the leaf leachate carbon treatment represents a source of (refractory) carbon almost identical to the carbon sources that this community was already exposed to. It is therefore unsurprising that no community structure changes occurred. The fact that this treatment did not stimulate additional rates of gas production may be due to the greater concentrations of similar carbon already present in these sediments. The addition of the protein carbon however, provided a ready supply of labile carbon which immediately stimulated both methane and carbon dioxide production rates, again, without changing the community structure. Unlike, the simple single carbon treatments employed in other studies (Preston-Mafham et al. 2002, Nakatsu et al. 2005, Wawrik et al. 2005) the addition of complex carbon sources may contain a sufficiently diverse source of carbon moieties that selection pressure on individual organisms able to express a range of organic carbon cleaving enzymes is minimized.

At the urban site methane production also increased in response to the protein treatment, but there was also evidence for a small shift in the denitrifying bacterial community structure for this treatment (Chapter 5.3.2.1, Table 5.6). Although previous studies have found links between dentrification, denitrifiers and methane, this has been attributed to denitrifiers consuming the intermediate products of methane oxidation (by methanotrophs) as carbon sources (Roy et al. 1994, Knowles 2005, Osaka et al. 2008). Under the conditions used for this experiment, the major source of electrons in the system is probably the added nitrate. It has recently been suggested that microbial consortia work in concert to couple the anaerobic oxidation of methane with denitrification (Raghoebarsing et al. 2006).

6.7 Conclusion.

The rates of gas generation were found to be different on a per mass basis confirming the first hypothesis. It was thought that the effect of zinc on metabolic processes would be less in samples from the urban site. However zinc treatment inhibited methane production

at both sites while denitrification was not affected in samples from the urban site; the only samples to show significant denitrification. The addition of carbon altered gas generation rates but the observed changes could not be considered characteristic of each site. These findings, together with those of the previous chapter, suggest that microbial community structure was resistant to change by both carbon and zinc additions. Bacterial communities probably responded by alterations in metabolic activity, by releasing different enzymes to process the carbon substrates and by changes in cell growth rate. As a result, the carbon substrates used here did not have a significant selective effect on members of the bacterial community. The combined results of Chapters 5 & 6 support the findings of previous chapters (Chapters 3 and 4) that although carbon type has a role in structuring the community, in these sites it was not the dominant factor structuring the microbial community.

While demonstrating that the community gas production rates were different on a per sediment dry mass basis, a greater understanding of the microbial community response would be achieved if routine methods were available to normalize these rates on a per biomass basis. Also, to get some idea of the relative contribution of individual ‘species’, a modification of the T-RFLP method was proposed that should account for the differences in extraction efficiencies of DNA from sediments.

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CHAPTER 7

FROM BIG THINGS LITTLE THINGS GROW

7.1 SYNTHESIS AND CONCLUSION.

Linking catchment scale processes to bacterial community structure.

"*Everything is everywhere – the environment selects*."[1]

Chapters 2 and 3 demonstrated that T-RFLP of the denitrifying community could distinguish sites affected by urbanization. Incorporating environmental variables into the analysis suggested that there was a significant relationship between the community structure and variables describing the geology and geography of the sites. While not describing all of the variation in the community data, this partial relationship suggests that there are measurable variables or factors at the catchment scale that affect the distribution of denitrifying bacteria at the micro scale. Chapter 3 proposed that both the source material and texture of the sediment were important structuring variables by influencing the surface chemical properties of the sediment and the delivery of nutrients by advection. Further, since alterations to catchment imperviousness and connection directly affect the hydrology of streams this can have a modifying affect on the texture and therefore the bacterial community.

As one factor controlling the rate at which nutrients and carbon substrates are delivered to bacteria in the hyporheic zone, sediment texture in part determines the transition from oxic to reducing conditions. This alters the population dynamic of the sediment, selecting for microorganisms that can respire with electron acceptors other than oxygen. If present in sufficient concentrations, nitrate can be used as an alternate electron acceptor (Figure 1.4) potentially leading to the production of nitrous oxide, but in anaerobic conditions generally leading to the removal of nitrate as nitrogen gas. Both of these relationships were demonstrated in Figure 3.4 where texture was correlated with measures of urbanization on Axis 1 and with nitrate on Axis 2. Recalling the two axis resource

[1] Baas Becking (1934)

gradient depicted in Figure 1.7, this could be interpreted as catchment scale processes interacting, affecting both the sediment texture and nutrient concentrations at the micro scale to produce a gradient of resource conditions along which different bacterial species are selected. However as many of the geographic variables are correlated, and texture is not the sole determinant of bacterial species distributions, the amount of variation explained by the selected variables tended to plateau. This is demonstrated in Tables 3.9 and 3.10, where although the inclusion of geographic variables describing the effects of urbanization marginally improved the ordination (compare Figure 3.3 A to Figure 3.4 A), the amount of variance explained by the first two axis increased by only 4%.

Carbon type is an important factor controlling the distribution of microbes; especially heterotrophs. Using spectral decomposition of both dried sediment FTIR and EEM spectra of hexane extracts, Chapter 4 demonstrated that the denitrifying community was significantly related to the carbon composition in the sediment. Interpretation of the spectra together with results from previous reports conducted in these streams also suggested that the process of urbanization has had some effect on the community structure by altering the sources, supply and residence time of carbon. Comparison of the ordinations in Figure 3.4 and Figure 4.10 show that sites group in a nearly identical way. That these ordinations were constructed from two sets of independent data (elemental and geographic versus carbon composition), is evidence for a stable and consistent ecological gradient. While this research demonstrates that an unambiguous gradient exists, the degree to which the community structure has been directly shaped by catchment urbanization can not be stated, only that there is a correlation.

<u>Linking bacterial community structure to measures of community function.</u>

Based on initial measurements (Chapter 2) and evidence available in the literature, it was hypothesized that two significant or dominant controlling factors in the studied streams were heavy metal pollution and the composition of available carbon. By manipulating the heavy metal concentrations and carbon composition, Chapters 5 & 6 attempted to investigate the links between the community structure and function.

The conclusion reached was that these treatments had significant effects on some metabolic functions, with zinc having a strong inhibitory effect on methane production, but that the hypothesized structural changes did not completely eventuate: although there

was some evidence of change due to the zinc treatment. Of the two carbon treatments investigated, one stimulated methane production, but to a greater extent in the sediment from a non urban site. These results support the conclusions of previous chapters that carbon alone is not necessarily the most important structuring agent.

Directions for future studies.

Although the research in these chapters was 'limited' by the number of samples achieved by the T-RFLP analysis, the number of replicates is comparable to similar reports found in the literature (see Rich and Myrold 2004, Gleeson et al. 2006, Culman et al. 2008) . At the time the research was conducted T-RFLP, though 'cheap', was still a major project cost. This restriction was the initial impetus behind the data reduction techniques used extensively in Chapters 3 & 4. However, the successful reduction of data to useful or interpretable factors in Chapter 3, led to the adoption of this technique for the more complex task of producing objective qualitative descriptors of carbon type based on spectral data employed in Chapter 4.

Although daunting at first, the data reduction methods used offer an objective method of consistently producing qualitative descriptors from complicated data sets. Combined with ordination methods like NMDS and CCA, changes in the microbial community structure can be directly related to environmental variables and a measure of the strength and significance of these relations produced. This is a useful first step in exploring species environment relations when the significance of individual variables on species composition is unknown. This technique helped keep the number of variables low compared to the number of samples – a problem frequently encountered if a cursory examination of the literature is any guide.

A call for a common approach to examining bacterial communities using ordination techniques and consistent environmental factors.

> "*Microbial ecology is currently undergoing a revolution, with repercussions spreading throughout microbiology, ecology and ecosystem science. The rapid accumulation of molecular data is uncovering vast diversity, abundant uncultivated microbial groups and novel microbial functions. This accumulation*

> *of data requires the application of theory to provide organization, structure, mechanistic insight and, ultimately, predictive power that is of practical value, but the application of theory in microbial ecology is currently very limited*" (Prosser et al. 2007).

Bacterial community or diversity studies are frequently sequence rich and descriptor poor. The advent of new sequencing techniques (see for e.g. the 454 protocol, Rothberg and Leamon 2008) threatens to amplify this situation. Often the only description accompanying a sequence (or "*unknown environmental clone*") is a statement like 'sandy sediment'. While this statement may have meaning at the human scale, the same probably doesn't apply at a scale relevant to bacteria. Is it calcareous or silica sand? Are the particles a similar size or well graded[1]? The first question, among other things, will alter chemical properties like adsorption and pH while the second goes to the issue of hyporheic flow, sediment disturbance and the delivery of nutrients. Although the methods for determining bacterial community composition will change, some consistency in the measured environmental factors needs to be achieved if microbial ecological theory is to advance beyond 'mere' collections of sequences. Chapter 3 is an example of the difficulties faced in comparing data to the work of other researchers. Although the number of papers on bacterial communities is vast, the search is complicated by the fact that one must frequently compare sections of research to small portions of several published papers in order to comment on the effect of environmental variables.

A defining physical property of soils and sediments is particle size and composition. That these ecologically significant environmental factors are not regularly included in studies of soil and sediment microbial communities is telling. Despite the observations that over 90% of bacterial species are uncultured or uncultivable, the mindset behind many environmental studies using powerful molecular methods is reminiscent of the 'colony counting' methods of the past. Unless guidelines are agreed for the minimal set of common factors that must be included in bacterial community studies from different environments, comparison of results and meta analysis will be problematic, and accomplishing the objective in the above quote by Prosser et al. (2007) will be difficult.

[1] In order to be classified as well-graded particles must have a range of all representative particle sizes between the largest and the smallest. A well graded sediment packs more tightly.

In a similar vein, clustering based on sequences may not demonstrate meaningful *environmental* relationships; these relationships might exist up to the point that 'species' diverged[1]. For clustering based solely on 16s rDNA genes to have environmental validity, it should be possible for the organisms that cluster with *E. coli*, for example to be isolated on the same media. But this is *not* always the case; instead many of these organisms remain an "*unknown environmental clone*".

Ordination methods coupled with 'chemometric' analysis of environmental descriptors are a potentially more ecologically meaningful approach. In the research conducted here multiple variables describing the mineral (XRF) and carbon (FT-IR & EEM) composition of the sediment were reduced to interpretable factors with minimal loss of information. In future, with sufficient replication, once a factor is shown to be significant, the individual variables within that factor could be separately examined. Conversely, with sufficient replication the overlap of variance and interaction between factors can be explored (e.g. see Borcard et al. 1992, Okland 2003).

Other factors to be considered in future research.

That the variables examined in this research only accounted for ~30% (if all three generated CCA axes are included: see Tables 3.10 or 4.5) of the community variation means that there are other significant structuring variables not captured in this analysis. This is not surprising as many of the environmental factors explored were at a macro scale, and had indirect effects on the bacterial community structure. Although no one study can explore all relevant factors, important factors that perhaps should be explored in similar research are:

1. stream hydrology,
2. vegetation,
3. the effect of other functional bacterial groups (i.e. other than denitrifiers),
4. sediment nutrients and oxygen concentrations,
5. the possible effect of protists and natural viral populations.

[1] Very close sequence similarity does not explain, for example, the divergent ecologies of apes, monkeys and humans.

Stream hydrology was not directly included in this research; its effects were included indirectly in the texture, imperviousness and connection variables. What the best measures of stream hydrology are, and how to make comparisons with the sediment bacterial populations will need to be considered. For example, discharge alone is probably not useful as the same discharge produces different stream velocities in different stream morphologies. Velocity or bed shear force may be better variables to study. It may be possible to treat flow or bed shear data as a spectrum and use PCA to reduce this to a system of components: this may prove to be a useful approach. Determining whether samples come from an upwelling or down welling location should also be considered in future studies.

Though carbon type was included in this study the effect of vegetation type was not. Plants are known to have symbiotic relationships with many bacterial and fungal species and can release stimulatory or inhibitory compounds into the soil (Kourtev et al. 2002, Bais et al. 2006, Bremer et al. 2007). Whether plants and macrophytes adjacent to streams have a similar effect has not been explored. Adequate and relevant descriptors of stream side vegetation need to be adopted if meaningful ecological information is to be extracted[1].

Bacteria in the environment do not exist in isolation. The community structure - environment relationships explored here were only for those denitrifiers that posses the *nos*Z gene. In reality, bacteria are commonly found in symbiotic relationships as biofilms (Paerl and Pinckney 1996, Findlay and Sinsabaugh 2003, Romaní et al. 2004). The effect that other functional bacterial groups (e.g. nitrifiers, nitrogen fixers) have on the community structure of denitrifiers has not, as yet, been investigated. However, how this can be achieved is problematic, as the effect of shared variation due to environmental factors would have to be accounted for. If the equipment is available, this could perhaps be accomplished by using some variant of Fluorescent *in situ* Hybridization (FISH) (Amann et al. 1995, Araya et al. 2003) with probes for different functional groups, or by the specific inhibition of the metabolic function of the groups being investigated.

[1] Just as ‘sandy sediment’ is an information poor descriptor, so to perhaps is ‘temperate forest’

Although stream water nutrient concentrations were included in the analysis (Chapter 3), the effect of interstitial nutrient concentrations, especially nitrate, was not examined. Future research should investigate if nitrate concentrations affect the community structure or, since many denitrifiers are obligate anaerobes that use the denitrifying pathway only in the absence of oxygen, whether nitrate concentration only affects the community function. Oxygen was not measured in the stream sediments; this is a measurement that perhaps should be linked to the question of upwelling and down welling flows mentioned above.

Future research might also consider the effect that the protozoan and viral populations have on bacterial species. It is known that protozoan grazing influences soil, marine and freshwater microbial community structures (Sherr et al. 1992, Rønn et al. 2002) and that viral populations also have a significant structuring effect in the aquatic environment (Fuhrman 1999, Jardillier et al. 2005, Tucker and Pollard 2005).

7.2 Concluding Remarks.

This research explored the role of environmental factors at multiple scales in determining the structure and function of the denitrifying communities in streams variously impacted by the process of urbanization. Sediment composition, sediment texture and sediment carbon composition were all correlated with the community structure. Additionally, indices of the degree of urbanization of the streams were also correlated with the bacterial community structure (and the forgoing variables) suggesting that the process of urbanization has had an effect on the ecologically significant variables responsible. The variance explained by the examined variables accounted for 30% (at most) of the community variance indicating that important microbial community structuring variables remain to be discovered. Manipulating sediments with carbon and heavy metal (zinc) treatments demonstrated that the community structure was resistant to change but that metabolic function was affected. Treatment with zinc suppressed methanogenesis in sediments from urban and non urban streams but did not affect denitrification, which was only detected in the urban stream.

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APPENDIX 1.

TREEFLAP MACRO ROUTINE.

Instructions for use.

To use this macro.
1. Have this file open in the background.
2. Paste the AGRF data into an Excel worksheet in a new workbook, making sure that the data begin in column C. Column A should contain a sequence of column numbers and column B should contain a list of sample names (no two sample names should be the same).
3. Press Ctl-Shift-T.
4. A dialog box will appear asking:
    a. if you want the macro to return a table of peak heights or peak areas. The macro defaults to peak heights.
    b. how you want the size values rounded. The macro defaults to rounding to the nearest 1. Any positive number can be selected. A larger rounding number (say 10) will most likely result in truly different fragment sizes being rounded to the same number. A smaller rounding number (say 0.1) will most likely result in truly the same fragment sizes being rounded to different numbers.
5. Press OK.

The macro creates two new worksheets: 'Count crosstab' and 'Sum crosstab'. (Make sure that the workbook you are analysing doesn't already have worksheets of these names). Count crosstab should be used to check that the rounding has not lumped any two peaks as a single number (all values in the Count crosstab table should be blank or 1).
It retains the original data in a third sheet, but with it re-arranged into a database form with samples grouped in the third column.

Cell J1 on this third sheet contains the rounding factor. You can explore the effect of value of this factor by changing it and then opening one of the crosstab tables and pressing the run button (red exclamation mark) on the PivotTable dialog that should be visible. Pressing this after changing cell I1will re-format the crosstab table on that sheet.
To check the effect of the numbers to which you are rounding, add half the rounding factor to all peak sizes and re-run the pivot table. To do this type half the rounding factor into cell I3 (the cell below the rounding factor). Copy this and then select all data in column D (select D2 and press Ctl-shift-down) and paste-special-add. Then select the crosstab sheet of interest and press the red exlcamation mark in the PivotTable dialog box. (To undo the changes to the peak sizes, copy I3 again and paste-special-subtract.)

Code

```
Public heightorarea As String
 Public sround As Double

Sub TREEFLAP()
'
   crosstab_options_dialog
   ActiveSheet.Name = "Original data"
   Cells(1, 1).Select
   Selection.End(xlDown).Select
   norows = ActiveCell.Row - 1
   Cells(1, 1).Select
   Selection.End(xlToRight).Select
   noPeaks = (ActiveCell.Column - 2) / 3
   Columns(3).Insert Shift:=x1ToRight
   activerow = 2
   Range(Cells(activerow, 3), Cells(activerow + norows - 1, 3)).Value = 1
   For Count = 2 To noPeaks
   Range(Cells(activerow, 1), Cells(activerow + norows - 1, 2)).Copy
```

```
Cells(activerow + norows, 1).Select
ActiveSheet.Paste
Range(Cells(activerow, 7), Cells(activerow + norows - 1, noPeaks * 3 + 3)).Cut
Cells(activerow + norows, 4).Select
ActiveSheet.Paste
Range(Cells(activerow + norows, 3), Cells(activerow + 2 * norows - 1, 3)).Value = Count
activerow = activerow + norows
Next Count
Range(Cells(1, 4), Cells(1, noPeaks * 3 + 3)).Clear
Cells(1, 4).Value = "Size"
Cells(1, 5).Value = "Height"
Cells(1, 6).Value = "Area"
Cells(1, 1).Value = "Number"
Cells(1, 2).Value = "Name"
Cells(1, 3).Value = "Peak"
Range(Cells(1, 1), Cells(norows * noPeaks * 3 + 2, 6)).Select
   Selection.Sort Key1:=Range("D2"), Order1:=xlDescending, Header:=xlGuess, _
   OrderCustom:=1, MatchCase:=False, Orientation:=xlTopToBottom
Cells(1, 4).Select
Selection.End(xlDown).Select
firstblank = ActiveCell.Row + 1
Cells(1, 1).Select
Selection.End(xlDown).Select
lastblank = ActiveCell.Row
Range(Cells(firstblank, 1), Cells(lastblank, 6)).Clear
Range(Cells(1, 1), Cells(firstblank - 1, 6)).Select
Selection.Sort Key1:=Range("b2"), Key2:=Range("c2"), Order1:=xlDescending, Header:=xlGuess, _
OrderCustom:=1, MatchCase:=False, Orientation:=xlTopToBottom

Cells(2, 9).Value = sround
Cells(2, 7).Value = "=ROUND(D2/I$2,0)*I$2"
Range(Cells(2, 7), Cells(firstblank - 1, 7)).FillDown
Cells(1, 7).Value = "Size rounded..."
Cells(1, 9).Value = "to the nearest"
Set myRange = Worksheets("Original data").Range(Cells(1, 1), Cells(firstblank - 1, 7))
Sheets.Add
ActiveSheet.Name = "Count crosstab"
Sheets.Add
ActiveSheet.Name = "Sum crosstab"
Sheets("Original data").Select
myRange.Select
ActiveSheet.PivotTableWizard TableDestination:=Worksheets("Count crosstab").Cells(1, 1), _
  TableName:="CountTable", ColumnGrand:=False, RowGrand:=False
ActiveSheet.Cells(3, 1).Select
With ActiveSheet.PivotTables()
End With
ActiveSheet.PivotTables("CountTable").AddFields RowFields:="Name", _
   ColumnFields:="Size rounded..."
If heightorarea = "Peak Area" Then
With ActiveSheet.PivotTables("CountTable").PivotFields("Area")
   .Orientation = xlDataField
   .Caption = "Count of Area"
   .Function = xlCount
End With
Else
With ActiveSheet.PivotTables("CountTable").PivotFields("Height")
   .Orientation = xlDataField
   .Caption = "Count of Height"
   .Function = xlCount
End With
End If
Sheets("Original data").Select
```

```
    myRange.Select
    ActiveSheet.PivotTableWizard TableDestination:=Worksheets("Sum crosstab").Cells(1, 1), _
      TableName:="SumTable", ColumnGrand:=False, RowGrand:=False
    ActiveSheet.Cells(3, 1).Select
    With ActiveSheet.PivotTables()
    End With
    ActiveSheet.PivotTables("SumTable").AddFields RowFields:="Name", _
       ColumnFields:="Size rounded..."
    If heightorarea = "Peak Area" Then
    With ActiveSheet.PivotTables("SumTable").PivotFields("Area")
       .Orientation = xlDataField
       .Caption = "Sum of Area"
       .Function = xlSum
    End With
    ActiveSheet.PivotTables("SumTable").NullString = "0"
    Else
    With ActiveSheet.PivotTables("SumTable").PivotFields("Height")
       .Orientation = xlDataField
       .Caption = "Sum of Height"
       .Function = xlSum
    End With
    ActiveSheet.PivotTables("SumTable").NullString = "0"
    End If
End Sub
Private Sub crosstab_options_dialog()
    With UserForm1
       .heightareacombo.AddItem "Peak Height"
       .heightareacombo.AddItem "Peak Area"
       .sizeround.Text = "1"
       .Show
       ' ...
    End With
End Sub
```

ACKNOWLEDGMENT.

This code was modified by Dr Chris Walsh from an exisiting routine of his for use with T-RFLP data.

Citations for the following tables and figures can be found at the end of Chapter 3.

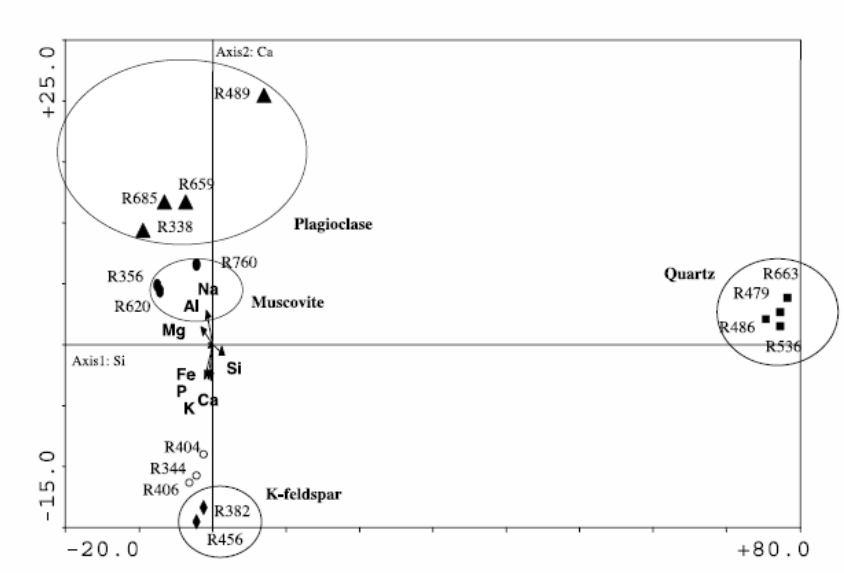

Figure 2. Canonical correspondence analysis (CCA) ordination diagram of fungal ARISA data, with chemical variables represented as *arrows* and ribotypes represented by *symbols*. Ribotypes are labeled according to size (bp), and have been coded and delineated according to mineral type on which they occur (●: muscovite; ▲: plagioclase; ◆: K-feldspar; ■: quartz; ○: granite). Axis 1 explains 15.1% of the ribotype–chemical variance, while Axis 2 explains a further 15.1% of the ribotype–chemical variance.

Figure A3.1 Reproduction of Figure 2 from Gleeson et al. (2005) showing the effect of mineral type and elemental composition on fungal community structure.

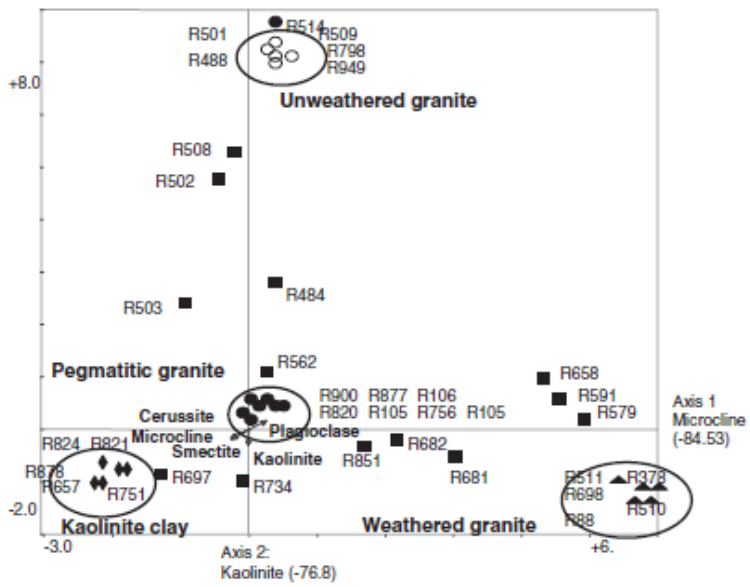

Fig. 2. Canonical correspondence analysis ordination diagram of bacterial ARISA ribotypes in relation to measured mineralogy. The 36 most abundant bacterial ribotypes from ARISA are shown as dots, with chemical variables represented as arrows. Ribotypes are labelled according to size (bp), and are coded as follows: PG (●), UG (○), WG (▲) and KC (◆).

Figure A3.2 Reproduction of Figure 2 from Gleeson et al. (2006) showing the effect of mineral type and weathering processes on fungal community structure.

Table A3.1 Reproduction of Table 1 from Hawkesworth & Kemp (2006).

Table 1 | Average and modelled compositions of the continental crust

	Average continental crust[3]	Average upper continental crust[3]	Upper crust magma composition*	Model new crust[19] (OIB-arc mix)	Residue[19]
Compositions (wt%)					
SiO_2	60.60	66.60	68.02		
TiO_2	0.72	0.64	0.48	0.89	0.95
Al_2O_3	15.90	15.40	15.41		
FeO_t	6.70	5.04	3.30		
MnO	0.10	0.10	0.07		
MgO	4.66	2.48	1.63		
CaO	6.40	3.59	3.62		
Na_2O	3.07	3.27	3.60		
K_2O	1.81	2.80	2.76	0.61	0.27
P_2O_5	0.13	0.15	0.14	0.15	0.16
Compositions (p.p.m.)					
Rb	49	82	80	10.9	0.1
Ba	456	628	568	212	156
Sr	320	320	301	347	354
Nb	8	12	10.3	4.8	3.9
Y	19	21	21	18.0	17.6
Zr	132	193	157	58.4	42.8
Hf	3.7	5.3	ND	1.6	ND
La	20	31	34	7.8	3.7
Ce	43	63	64	17.9	10.6
Nd	20	27	26	11.0	8.6
Sm	3.9	4.7	5.0	2.9	2.5
Eu	1.1	1.0	1.0	1.0	1.0
Gd	3.7	4	4.1	2.8	2.6
Tb	0.6	0.7	ND	0.5	ND
Dy	3.6	3.9	ND	3.0	ND
Er	2.1	2.3	ND	1.8	ND
Yb	1.9	2	1.9	1.7	1.7
Lu	0.3	0.31	ND	0.3	ND
Pb	11	17	12	2.7	1.3
Th	5.6	10.5	9.3	1.5	0.2
U	1.3	2.7	2.1	0.4	0.1
Ratios					
Eu/Eu*	0.93	0.72	0.70	1.06	1.18
ASI	0.85	1.03	0.99	ND	ND
Mg#	55.3	46.7	0.47	ND	ND

The composition of the average continental crust and the average upper continental crust[3], and the upper crust magma composition calculated from global granite data arrays at Eu/Eu* = 0.7 (ref. 19). Also presented is the proposed composition of model new continental crust (itself calculated as a mixture of 92% average arc and 8% OIB, Fig. 1); this has higher U and Th contents than the average lower crust[19], although these are broadly consistent with continental heat flow data[76]. The residue composition is that formed assuming that the *D* values for Rb and Th are ~0 during the differentiation of the continental crust, whereupon the upper crust magma composition reflects 14% melting (or 86% crystallization) of the newly added crust[19]. (ASI, alumina saturation index; Mg#, magnesium number; Eu/Eu*, europium anomaly, calculated as in ref. 17; ND, not determined.)

Table A3.2 Primary and Secondary Minerals commonly found in soils.[1]

Primary Minerals	Approximate Composition	Weatherability
Quartz	SiO_2	-
K-Feldspar	$KAlSi3O_8$	+
Ca, Na-plagioclase	$CaAl_2Si_2O_8$ to $NaAlSi_3O_8$	+ to (+)
Muscovite	$KAlSi_3O10(OH)_2$	+(+)
Amphibole	$Ca_2Al_2Mg_2Fe_3Si_3O_{10}(OH)_2$	+(+)
Biotite	$KAl(Mg,Fe)_3Si_3O_{10}(OH)_2$	++
Pyroxene	$Ca_2(Al,Fe)_4(Mg,Fe)_4Si_6O_{24}$	++
Apatite	$[3Ca_3(PO_4)_2].CaO$	++
Volcanic Glass	Variable	++
Calcite	$CaCO_3$	+++
Dolomite	$(Ca,Mg)CO_3$	+++
Gypsum	$CaSO_4.2H_2O$	+++

Secondary Minerals	Approximate composition	Type
Kaolinite	$Al_2Si_2O_5(OH)_4$	1:1 layer-silicate
Vermiculite	$(Al_{1.7}Mg_{0.3})Si_{3.6}Al_{0.4}O_{10}(OH)_2$	2:1 layer-silicate
Montmorillonite	$(Al_{1.7}Mg_{0.3})Si_{3.9}Al_{0.1}O_{10}(OH)_2$	2:1 layer-silicate
Chlorite	$(Mg_{2.6}Fe_{0.4})Si_{2.5}(Al,Fe)1.5O_{10}(OH)_2$	2:1:1 layer-silicate
Allophane	$(SiO_2)1\text{-}2Al_2O_5.2.5\text{-}3(H_2O)$	Pseudo crystalline, spherical
Imoglite	$SiO_2Al_2O_3.2.5H_2O$	Pseudo crystalline, strands
Hallyosite	$Al_2Si_2O_5(OH)_4.2H_2O$	Pseudo crystalline, tubular
Gibbsite	$Al(OH)_3$	Hydroxide
Goethite	FeOOH	Oxyhydroxide
Hematite	Fe_2O_3	Oxide
Ferrihydrite	$5Fe_2O_5.9H_2O$	Oxide

[1] Reproduced from Table 8-1, Montgomery, D. R. et al. in Jacobson et al. 2000, *Earth System Science*.

APPENDIX 2.

Table A5.1 Distribution of small (21 – 31 bp) T-RFs from the manipulation experiment. Red highlights T-RFs more generally associated with the zinc treatment, blue highlights T-RFs more generally associated with the non zinc treatment. Sediment samples (G1-4 & L1-4) collected in the field (Time Controls) and blanks (R1-4 and U1-4) appear to have a random association with these T-RFs. These samples were not treated with zinc.

Sample	T-RF									
	21	22	23	24	25	26	27	28	30	31
L1					14	4				
L2	2		7				2			
L3					13		3			
L4		4	3							
R5					4	2		1		
R6			2							
R7					4		3	1		
R8	2		6							
RLN1					5			1		
RLN2					14	9		6		5
RLN3					13			1		1
RLY1	5		18						3	
RLY2	23	7								
RLY3		15	23				2			
RNN1					3			2		3
RNN2					2			4		3
RNN3					2			4		
RNY1	7		13				3		4	
RNY2	26	25								
RNY3	2		18				2			
RPN1					14			2		3
RPN2					5					
RPN3					7	2		2		2
RPY1	18	8	17				5		3	
RPY2	2		24							
RPY3		5	7							

Sample	T-RF									
	21	22	23	24	25	26	27	28	30	31
G1					6					2
G2	3		9							
G3					15					
G4	1		6				1			
U5					27			3		
U6		5	10				2		1	
U7					23			2		
U8		5	7							
ULN1				10				2		
ULN2					13	14				
ULN3					13	2		4		
ULY1		18		7			3			
ULY2	16		6							
ULY3	26	11					2		2	
UNN1			2					2		2
UNN2					21	4		3		4
UNN3					2	7		2		
UNY1										
UNY2	6		1						2	
UNY3	16		1							
UPN1				18		13		7		6
UPN2					34	15				
UPN3					9	9		4		6
UPY1	25		1							
UPY2	16	6					1			
UPY3	12		20				4			

APPENDIX 3

Table A6.1 ANOVA using transformed variables for N_2 and CO_2 .CH_4 results not shown as no easy transform could be applied. Untransformed results were used in the analysis. Transforming variables had no effect on the overall result.

Site	Factor/Interaction	Gas	N	R^2	F-Ratio	η (Effect size)	p	significance level
		N_2	40	0.593	4.864	N/A	4.703E-04	$\alpha = 0.01$
All	Site	CH_4	48	0.951	600.8	N/A	1.56E-11	$\alpha = 0.01$
		CO_2	48	0.956	613.7	N/A	1.56E-11	$\alpha = 0.01$
	Carbon	N_2	24	0.225	0.785	0.07	4.71E-01	ns
Urban	Zinc	N_2			0.163	0.01	6.92E-01	ns
	Carbon*Zinc	N_2			1.744	0.15	2.03E-01	ns
	Carbon	CO_2	24	0. 749	2.159	0.06	1.44E-01	ns
Urban	Zinc	CO_2			0.296	0.004	5.93E-01	ns
	Carbon*Zinc	CO_2			24.56	0.68	7.17E-06	$\alpha = 0.01$
Non Urban	Model	N_2	16	0. 286	1.603	N/A	2.40E-01	ns
	Carbon	CO_2	24	0.861	16.915	0.26	7.34E-05	$\alpha = 0.01$
Non Urban	Zinc	CO_2			21.366	0.16	2.12E-04	$\alpha = 0.01$
	Carbon*Zinc	CO_2			28.25	0.44	2.803E-06	$\alpha = 0.01$

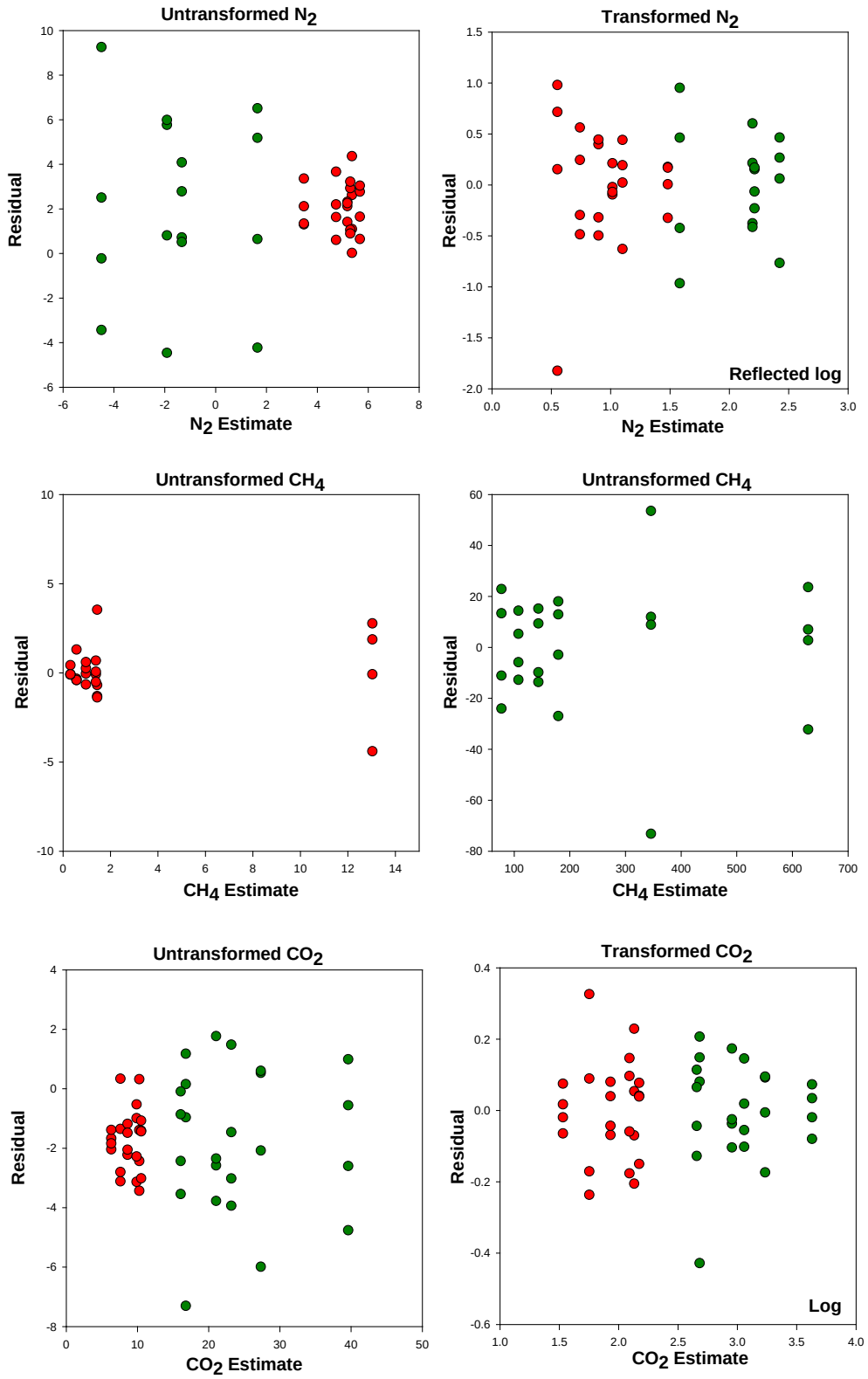

Figure A6.1 Residuals as a function of estimates plots for untransformed and transformed data used in the ANOVA of treatement effects on gas generation rates. Transforming variables decreased the residuals. Urban sites ●; Non Urban sites ●.