A comparative assessment of groundwater ecosystems under irrigated agriculture and pasture

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Declaration

I wish to acknowledge the following assistance in the research detailed in this report: A/Prof Grant Hose, my principal supervisor, for comments on earlier drafts of this thesis. All other research described herein is my own original work. This work has not previously been submitted for a degree or diploma in any university.

Signed: John Little

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Abstract

The impacts of intensive agriculture on underlying groundwater ecosystems are not yet fully understood, although agricultural practices are firmly implicated in the modification of stygofauna (invertebrate) and microbial populations in aquifers, primarily through changes in water quality associated with irrigated agriculture. The aim of this study was to determine how biological communities in groundwater vary with agricultural land-use, and along environmental gradients. Biological and environmental samples were collected from groundwater under irrigated agricultural landscapes, and pasture in the Condamine Catchment (QLD), and Gwydir Catchment (NSW). Bacterial populations were evaluated through the analysis of high-throughput 16S rDNA sequence data. The findings of this study did not support the hypothesis that stygofauna distributions vary with agricultural land-use. Molecular data did not support a relationship between microbial distributions and agricultural land-use. Microbial assemblages became more dissimilar with increasing geographic distance. Microbial and stygofauna distributions were poorly correlated with groundwater chemistry gradients. In summary, the influence of agriculture on stygofauna community structure is likely to be limited, since their distributions in the landscape are largely defined by physical limitations of the alluvial aquifer environment. In contrast, the influence of agricultural land-use on groundwater quality is likely to have a quantifiable bearing on microbial distributions, although evidence to support this hypothesis was not forthcoming from the environmental and molecular analysis here. To assess the hypothesis that microbial communities differ with agricultural land-use, further studies should use a metagenomic sequencing approach to estimate the relative abundance of microbes between sites.

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1 Introduction

1.1 Groundwater ecology overview

A significant portion of the total freshwater reserve of many continental landmasses is stored in subterranean groundwater systems (aquifers) (Boulton, Humphreys & Eberhard 2003). In semi-arid regions of eastern Australia, or indeed any continental location where the occurrence of rainfall is unpredictable or periodic, aquifers are often the primary source of water underpinning agricultural production, industrial development, and supplies of fresh water for stock and human consumption (Tomlinson & Boulton 2010). From both management and research perspectives, aquifers have traditionally been viewed in terms of hydrogeological attributes, whilst biological aspects of groundwater remained largely overlooked in comparison (Humphreys 2009).

Biological communities in aquifers consist of two broad groups of organisms: the invertebrates and microbes (microorganisms). Relatively little is understood of the biology and ecological requirements of obligate groundwater invertebrates in Australia, or how their trophic interactions with microbial populations relate to holistic ecosystem processes (Hancock, Boulton & Humphreys 2005). Microorganisms, i.e., prokaryotes (bacteria and archaea) (Campbell et al. 2009), are responsible for the majority of carbon and element cycling in groundwater (Wrighton et al. 2014). However, the taxonomic and functional breadth of microbial communities in aquifers remains largely uncharacterised (Griebler & Lueders 2009; Sirisena et al. 2013; Smith et al. 2012).

Relatively recent innovation in second generation DNA sequencing technologies, have provided micro-biologists with the tools to describe taxonomic and functional diversity at a level of resolution previously unattainable with preceding community profiling techniques (Bohmann et al. 2014). In the application of second generation genetic analyses in aquatic microbial ecology thus far, researchers have focused largely on the description of diversity in marine environments (Logares et al. 2014b; Pedros-Alio 2012), whilst the diversity of microbial assemblages in groundwater has yet to be adequately explored.

1.2 Groundwater ecosystems

1.2.1 Stygofauna description and habitat attributes

Groundwater fauna (stygofauna) are aquatic invertebrates which are categorized by the degree of affinity they exhibit towards groundwater. Facultative stygofauna which may inhabit groundwater on a temporary to permanent basis are termed ‘stygophiles’, whereas obligate groundwater residents are termed ‘stygobites’ (Humphreys 2008). Stygobites, which are largely represented by crustacean groups (e.g., copepods, amphipods, isopods and syncarids) exhibit specific adaptations to environmental conditions below ground, and can be differentiated from stygophiles on those criteria. The absence of eyes and pigmentation, and minute or attenuated body forms often equipped with numerous spines or extra-sensory setae, are diagnostic features of stygobitic fauna which differentiate them from facultative, or otherwise temporary groundwater invertebrates (Gibert et al. 1994; Stumpp & Hose 2013).

Alluvial groundwater systems underlie many of the major river systems and drainage basins (catchments) along the eastern seaboard of Australia (Humphreys 2008). In terms of geology and structure, an alluvial aquifer can be considered a largely permeable layer of loose regolith and saturated sediments associated with a river channel, which may extend laterally underground up to several kilometres, particularly under alluvial floodplains (Geoscience Australia 2015). Research initiated largely in the past decade has resulted in the discovery of relatively diverse (in comparison to western Australia) stygofaunal communities inhabiting alluvial aquifers in New South Wales (Hancock & Boulton 2008, 2009; Korbel, Lim & Hose 2013b), and Queensland (Cook et al. 2012; Schulz, Steward & Prior 2013).

1.2.2 Ecological conditions in alluvial groundwater

Aquifers provide relatively stable and predictable ecological conditions for aquatic organisms (Gibert et al. 1994). Structural longevity of the subterranean environment, and low variability in ambient physicochemical conditions, are characteristic of groundwater environments (Dumas 2002). Such conditions may be regarded as favourable for aquatic organisms in comparison to life in surface waters, or benthic sediments, where desiccation or flood induced disturbance may be relatively frequent, and environmental parameters subject to steep or rapid change (Gibert & Deharveng 2002; Hancock 2006).

Irrespective of the physical environmental benefits in a subterranean existence, biological conditions are considerably less favourable in groundwater in comparison to surface waters. In the absence of light there is no significant autotrophic production below ground, and thus the occurrence of organic carbon, nutrients, and dissolved oxygen, is dependent upon the downward percolation of terrestrial surface waters, or the mixing of surface and groundwater flows (Tomlinson & Boulton 2010). Alluvial groundwater with strong hydrological connectivity to surface waters need not necessarily be oxygen deficient, or bereft of organic material (Hancock & Boulton 2008); however, groundwater ecosystems are generally considered to be low energy, poorly oxygenated environments (Hancock et al. 2005). It is to such stable and yet relatively harsh biological conditions, that groundwater organisms are well adapted (Griebler & Lueders 2009; Malard & Hervant 1999).

1.2.3 Predictors of stygofaunal diversity in groundwater

Few studies to date have been able to demonstrate clear relationships between patterns of stygofauna distribution and environmental gradients. At the regional landscape scale, it appears that the occurrence of stygofauna can rarely be predicted by a particular set of physicochemical conditions, or variation within the range of any one water quality variable (Dole-Olivier et al. 2009; Schmidt et al. 2007).

Halse et al. (2014) found that variation in dissolved oxygen concentration, electrical conductivity, pH, and major ions: (Na, K, Ca, Mg, Mn, Cl, HCO_3/CO_3, and SO_4), appeared to have little influence on the occurrence of stygofauna in the Pilbara region, Western Australia. During their research, a total of 350 stygofaunal species were collected from 507 widely distributed groundwater monitoring wells, and it was noted that stygofauna and the hydrogeological environments in which they occur in the Pilbara are diverse (Halse et al. 2014). Given the high level of faunal diversity in their study, and the variety of habitats surveyed, it may have been difficult to identify parameters regarding the physicochemical preferences, or tolerance levels of stygofauna as a broad group. Despite poor correspondence between patterns of stygofauna distribution and pH in the above mentioned study, other authors suggest that pH is an important physical factor in groundwater, with fewer taxa collected below pH 5 (Hancock & Boulton 2008; Humphreys 2008).

The relationship between the occurrence of stygofauna and groundwater salinity gradients in eastern Australian alluvial aquifers is unclear, since the results of similar

studies can appear contradictory; for example, Hancock & Boulton (2008) noted that stygofauna were most likely to occur in groundwater with relatively low level Electrical Conductivity (EC <1500 µS cm^{-1}), in their investigation of four alluvial aquifers in Queensland and New South Wales. In comparable circumstances however, stygofauna were more recently collected in alluvial groundwater with high level EC, even approaching that of sea-water (36 300 - 54 800 µS cm^{-1}) (Schulz, Steward & Prior 2013).

Stygofauna are physiologically and behaviourally well adapted to the characteristic spatial heterogeneity and low levels of dissolved oxygen (DO) in alluvial groundwater (Malard & Hervant 1999); in an Australian context, stygofauna are perhaps typically associated with suboxic groundwater (DO < 1 mg/L) (Humphreys 2008). Therefore, whereas epigean freshwater species are often sensitive to or intolerant of low dissolved oxygen concentrations (Arthington & Pusey 2003), distributions of stygofauna need not necessarily reflect dissolved oxygen gradients (Tomlinson & Boulton 2010); thus, the concentration of dissolved oxygen is generally considered an unreliable determinant of faunal distributions (Halse et al. 2014; Reeves at al. 2007).

Environmental factors which appear likely to influence stygofauna distributions are largely hydrogeological in nature (Dole-Olivier et al. 2009; Korbel & Hose 2015). Particle size and porosity are important determinants of groundwater flow within alluvium, and consequently the supply of dissolved oxygen and organic material to aquifer ecosystems (Tomlinson & Boulton 2010). Porous alluvial media facilitates the circulation of groundwater and provides living space for stygofauna; in contrast, clay dominated sediments inhibit groundwater flows, and can reduce porosity to such a degree to preclude living space for stygofauna (Hancock, Boulton & Humphreys 2005); thus, it is perhaps unsurprising that the occurrence of stygofauna is most often associated with coarse grain alluvial sediments (Hancock & Boulton 2008; Korbel & Hose 2015).

1.2.4 Predictors of microbial diversity in groundwater

In alluvial groundwater, a strong relationship between microbial diversity and activity patterns can be expected with sediment mineralogy, organic content, and particle size (Griebler & Lueders 2009). At micro to macro scales, community dynamics are governed by species' responses to variation in physicochemical conditions, which are driven by the influx of organic compounds and nutrients from the terrestrial environment

(Hug et al. 2015; Smith et al. 2012). The majority of microorganisms in groundwater actively colonize sediments, exhibiting an 'attached' as opposed to 'free-living' life history strategy, which is likely because sediment surfaces are chemically diverse and offer more ecological niches than groundwater itself (Griebler & Lueders 2009). Interestingly, attached and free-living prokaryotes appear to segregate on the basis of resource affinity in aquifers, typically sharing less than a third overlap in community composition (Flynn et al. 2013; Hug et al. 2015).

Given the ease with which microorganisms disperse by virtue of their size and vast population numbers (Pedros-Alio 2012), and avenues of dispersal via surface to groundwater pathways (Schmidt et al. 2007), strictly endemic groundwater microorganisms are not expected (Griebler & Lueders 2009). However, given ecological conditions common to most aquifers, such as low carbon and nutrient concentrations, darkness (and thus the absence of photoautotrophs), or similarities in geology and temperature between adjacent aquifer systems, then characteristic or diagnostic communities might be expected (Sirisena et al. 2013; Smith et al. 2012).

1.3 Methods and applications in molecular microbial ecology

1.3.1 Microbial taxonomy

Orthologous genomic regions serve as 'molecular markers' enabling the analysis of phylogenetic relationships amongst organisms, and their taxonomic classification (Fahey et al. 2014). In terms of the classification of microorganisms, ribosomal DNA (rDNA) markers are considered to be sufficiently conserved (slow evolving), to be informative in the assessment of prokaryote and micro-eukaryote diversity (Logares et al. 2014a; Pedrós-Alió 2006). Specifically, gene 16S rDNA is used in the taxonomic assessment of bacteria and archaea (Pedrós-Alió 2006), and gene 18S rDNA for micro-eukaryotes (Logares et al. 2014b); it is suggested however, that broad resolution within the fungi is likely best achieved with the ribosomal ITS (internal transcribed spacer) marker (Schoch et al. 2012). Despite the utility of rDNA markers as a standard tool to classify microorganisms, some authors suggest that nucleotide sequence variation in gene 16S may be insufficient for species level delineation amongst the prokaryotes (Achtman & Wagner 2008; Wang et al. 2013).

1.3.2 High-throughput DNA sequencing (HTS) and metagenomics

Prior to the development of molecular profiling methods, isolation in pure culture was the only means available to assess microbial diversity; culturing techniques however, are unable to isolate the vast majority of microorganisms in the environment (Schloss & Handelsman 2008). In contrast, targeted high-throughput sequencing (HTS) of specific genes, and metagenomics: defined as the direct genetic analysis of genomes contained within an environmental sample (Thomas, Gilbert & Meyer 2012), have enabled the recovery of a much greater portion of diversity (Pedros-Alio 2012).

Polymerase chain reaction (PCR) based high-throughput sequencing and metagenomic approaches differ, primarily in that PCR based HTS recovers solely taxonomic information, whereas a metagenomic analysis has the advantage of retrieving taxonomic and information regarding potential function from the same microbial community (Logares et al. 2014a). Functional information is derived by screening for genes which code for enzymes with known biological functions. Thus, organisms active in the cycling of particular elements can be identified, and dominant biological processes defined as sets of genes (Smith et al. 2012; Wrighton et al. 2012).

1.3.3 HTS sample processing and analytical issues

The key question in any molecular assessment of microbial diversity is whether the phylogenetic information gained is representative of the environment from which the sample was taken (Thomas et al. 2012; Wang et al. 2013). The recovery of DNA which is proportionally representative of the focal community is essential for the integrity of subsequent analysis; however, DNA extraction methods have been shown to work more efficiently within certain taxonomic groups than in others, which may result in the underrepresentation, or absence of some taxa (Wang et al 2013). A combination of extraction protocols is advocated to optimise the recovery of community wide DNA from environmental samples (Delmont et al. 2011).

The success of PCR can be hindered by the non-specificity of ‘universal’ rDNA primers, which fail to anneal to primer binding sites on target genes (Wang et al. 2013). In microbial community analyses, rDNA primer mismatch may result in considerable underestimation of diversity, given that extraction products are likely to contain the genomic DNA of a large number of species and individuals, and thus a corresponding number of opportunities for primer binding failure (Hong et al. 2009; Wang et al. 2013).

In contrast, random sequencing errors and the formation of chimeric sequences (i.e., adjoined DNA fragments originating from different organisms), can result in the overestimation of diversity. Bias introduced by artificial sequences can be mitigated however, by filtering out chimeric sequences, discarding unique sequences which by their rarity are likely the result of sequencing error, and the clustering of sequences into Operational Taxonomic Units (OTUs) under a similarity threshold (e.g., ≥ 97 %), which is likely to incorporate erroneous sequences resulting from a small number of incorrectly assigned nucleotide bases into a correct OTU (Huse et al. 2010).

1.4 Microbial biogeography

1.4.1 Microbial species-abundance distributions

Biological communities are typically composed of a few abundant and many rare species (Logares et al. 2014b). This pattern is particularly prevalent in microbial communities, in which a subset of taxa predominate numerically, whilst the vast majority of taxa are extremely rare in comparison (Jones & Lennon 2010; Logares et al. 2014b). Analyses of HTS datasets reflect this species-abundance relationship in the marine biome (Pedrós-Alió 2012); for example, Logares at al. (2014b) found that although microeukaryote community composition varied between collection sites in dissimilar coastal waters, rare and abundant taxa occurred in consistently similar proportions across all samples. Similarly, a metagenomic analysis of adjacent (but unconnected) aquifer systems, found that a small fraction of microbes within each community were disproportionately abundant (Smith et al. 2012).

Within microbial communities, the many taxa present in relatively low abundance are collectively referred to as the ‘rare biosphere’ (Jones & Lennon 2010; Sogin et al. 2006). The rare biosphere can be viewed as a seed-bank, or reservoir of functional diversity in which species are either slow growing or dormant, most likely because current ecological conditions are unfavourable for their proliferation (Logares at al. 2014b; Pedrós-Alió 2012). Rarity however, need not necessarily imply inactivity or functional redundancy in aquatic communities (Jones & Lennon 2010; Logares at al. 2014b). Within the bacteria for example, functional specialists may remain relatively rare yet still contribute significantly to the cycling of specific elements; albeit given low biomass, their relative contribution to carbon turnover is likely to be less important (Pedrós-Alió 2012). Comparative analysis of the ratio of rRNA to rDNA in freshwater (Jones & Lennon 2010) and marine (Logares et al. 2014b) communities, revealed

considerable and unexpected metabolic activity in ‘rare’ bacterial and micro-eukaryote cohorts, respectively. It is likely that a fine line exists between microbial activity and dormancy depending on resource gradients (Pedrós-Alió 2012); thus at any given juncture, an evaluation of the metabolic status of rare and abundant sub-communities is inherently complex (Jones & Lennon 2010).

1.4.2 Microbial community assembly

Ecological theory predicts that biological communities generally become more dissimilar with increasing geographic distance, usually as a result of the combined effects of environmental factors and geographical separation; i.e., dispersal constraints (Nekola & White 1999). The degree to which dispersal constraints influence processes of microbial community assembly in freshwater aquatic ecosystems is uncertain (Gibbons et al. 2014); it is clear however, that environmental gradients drive and maintain community structure to a large extent (Freimann et al. 2014; Gibbons et al. 2014; Hug et al. 2015). Under stable geochemical conditions for example, such as those occurring in groundwater, community composition and even the abundance of many core (abundant) organisms, may persist largely unchanged for years, at least over distance of up to one metre (Hug et al. 2015).

Taxonomic affiliations may also persist across diverse environmental gradients. For example, Gibbons et al. (2014) found that a core group of organisms were consistently selected for in hyporheic sediments along a 134 kilometre river transect, irrespective of significant shifts in underlying (rare) diversity between sites. Dominance of the core group was attributed to the metabolic plasticity of group members. Functional plasticity, or the capacity of species to adjust their physiology and metabolism in response to environment cues (Comte & Del Giorgio 2011), extends the ecological niche of a given community, and contributes to community resistance and resilience (Freimann et al. 2014; Gibbons et al. 2014). Commensal relationships between microbial taxa are also expected to occur: that is, the activities of one organism increasing the bioavailability of a compound utilized by another (Flynn et al. 2013). Given commensalism, and the diverse metabolism of many microbial taxa (Mou et al. 2008), taxonomic affiliations might be expected to reoccur across space, perhaps irrespective of relatively minor changes along environmental gradients.

1.5 Anthropogenic disturbance and groundwater biota

1.5.1 Microbial distributions

Few studies have explored the relationship between microbial diversity in groundwater and environmental gradients at the landscape scale (Griebler & Lueders 2009). The major challenge in such research is to tease apart the relative influence of a typically diverse array of environmental factors, on the distributions of what are ecologically complex populations (Gibbons et al. 2014). Collectively, or in any given combination, it appears that local geochemical characteristics, land-use effects, and geographic distance (dispersal constraints), may contribute to spatial variation in aquatic microbial diversity to some extent (Gibbons et al. 2014; Sirisena et al. 2013; Smith et al. 2012).

Land-use related disturbance of aquatic ecosystems varies with the type and intensity of the gradients involved, and thus in degree of impact on microbial community structure. At one end of the disturbance spectrum for instance, exposure to high concentrations of metal elements, organic solvents, and nitric acid, can reduce microbial diversity in groundwater (Hemme et al. 2010); whereas at more moderate levels of disturbance (in estuarine sediments), sensitive taxa were replaced by more robust forms, at no net loss in biotic richness (Chariton et al. 2010). In association with land use, variation in redox potential was identified as a key driver of bacterial diversity in a geographically extensive survey of New Zealand groundwaters (Sirisena et al. 2013). This finding is consistent with the observation that shifts in bacterial diversity correspond to the switch from aerobic to anaerobic respiration in groundwater (Lee et al. 2010). Fluctuations in nutrient and organic carbon concentrations are also important determinants of microbial community structure (Smith et al. 2012). In a southern Australian landscape for example, dominant taxa in an ‘unconfined’ aquifer which had been subject to the input of dairy farm runoff, were those associated with the degradation of organic pollutants and wastewater (Smith et al. 2012); in contrast, dominant taxa within an adjacent ‘confined’ aquifer system, reflected the generally oligotrophic status of an aquatic environment bereft of terrestrial inputs (Smith et al. 2012). Interestingly, although hydrologically unconnected, the confined and unconfined aquifer metagenomes were more similar to each other in terms of taxonomy and metabolism, than to those of other sediment or freshwater environments; which suggests that conditions common to most groundwater systems (e.g., darkness and geological stability) may engender relatively similar communities (Smith et al. 2012).

1.5.2 Agricultural land-use

The impacts of intensive agriculture on underlying groundwater ecosystems are not yet fully understood (Smith et al. 2012), although agricultural practices are firmly implicated in the modification of biological communities in aquifers, primarily through changes in water quality associated with irrigated agriculture (Korbel et al. 2013a, 2013b). In landscapes dominated by agricultural production, the absence of natural vegetation coupled with farming practices which disturb the soil profile (tillage), engender greater hydrological connectivity between surface and groundwaters, than exists under rural landscapes on which natural groundcover is relatively intact (Foley, Silburn & Greve 2010). Elevated salinity and nutrient levels can be expected in groundwater replenished via precipitation and seepage through the soil profile (Gunawardena et al. 2011; Schmidt et al. 2007). Under irrigated agricultural land specifically, rainfall and irrigated water contribute collectively to deep drainage (i.e., the movement of water below the root zone of crops), and the concomitant leaching of nutrients (particularly fertilizer derived nitrates), salts and organic compounds, which have the potential to compromise groundwater quality on reaching the water-table (Foley, Silburn & Greve 2010; Gunawardena et al. 2011). Accordingly, Korbel et al. (2013a) found greater nitrate levels and organic carbon concentrations in groundwater under irrigated agriculture, in comparison to that under relatively undisturbed pasture in the Gwydir Valley, New South Wales.

Groundwater communities varied in composition and activity patterns between agricultural land-uses, and between catchments in rural New South Wales (Korbel et al. 2013a, 2013b). Differences in microbial assemblages between catchments were attributed to changes in groundwater chemistry following a period of irrigation activity (Korbel et al. 2013b). Microbial activity (as measured by loss in the tensile strength of cotton strips submerged in groundwater bores for 6 weeks), increased in response to a period of intense rainfall following protracted drought, and presumably the flushing of organic material through the soil profile and into groundwater (Korbel & Hose 2015). Stygofaunal abundance was greater under irrigated agricultural land in comparison to pasture (Korbel et al. 2013a), and correlated positively with microbial activity and dissolved organic carbon content (Korbel et al. 2013b). Given that many stygofauna consume biofilm (microbial film) to obtain energy (Hancock, Boulton & Humphreys 2005), their abundance might be expected to correlate positively with microbial activity.

1.6 Project Background

The focal taxa of this project are the stygofauna and microbial populations present in groundwaters of the Gwydir River Catchment, New South Wales, and the Condamine River Catchment, Queensland. A recent state government survey collected stygofauna from groundwater monitoring bores located in the Border Rivers region of the upper Condamine Catchment (Schulz, Steward & Prior 2013), and there is unpublished data from a connected survey in the central Condamine in 2009 (Shultz, C 2015, pers. comm.); in broad terms however, relatively little is known regarding the diversity and distributions of stygofauna in the Condamine Catchment (Hancock, PJ 2015, pers. comm.). In contrast, stygofauna diversity of the Gwydir Catchment is better characterized, although faunal distributions are highly variable over space and time (Korbel et al. 2013a). In terms of the microbial diversity present in aquifers of either project catchment, no taxonomic or distributional information could be sourced for this review.

Following rainfall and or irrigation, groundwater recharge through percolation (seepage) may occur in hours, days, or months; depending on soil properties and condition, and depth to the water-table (Silburn et al. 2004). The proportion of deep drainage that actually reaches groundwater in the project areas is not quantified, although detection of agrochemicals at irrigated sites, and elevated organic carbon concentrations following heavy rains, suggests a strong link between surface and groundwaters in the Gwydir Catchment (Korbel et al. 2103a, 2013b). In the Condamine Catchment, geophysical survey methods and deep coring (up to 20 metres) along transects running through natural vegetation and into irrigated paddocks, revealed evidence of significant long-term deep drainage under irrigation, whist soils under relatively natural vegetation were very dry in comparison (Foley, Silburn & Greve 2010).

1.7 Project aims

The primary aim of this study is to determine how biological communities in groundwater vary with agricultural land-use, and along environmental gradients. In regard to microorganisms specifically, bacterial populations are evaluated through the analysis of high-throughput 16S rDNA sequence data. Given the potential for differences in groundwater chemistry and organic content between irrigated and pasture landscapes (Korbel et al. 2013a), microorganisms might be expected to segregate on the basis of resource or chemical affinity between these two land-use

categories; that is, collectively, microbial communities under irrigation may be more similar to each other in composition than to communities under pasture, and visa versa.

Specific aims of microbial community analysis are to:

(1) Determine whether microbial community composition in groundwater differs significantly between irrigated and pasture land-uses

A taxonomic profile common to irrigated sites will imply that aspects of groundwater quality associated with irrigated agriculture influence community assembly. Alternatively, the absence of a clear taxonomic signal corresponding to land-use, may indicate that a more complex suite of environmental factors mediate microbial distributions, perhaps including, or irrespective of land-use related effects.

(2) Determine whether microbial community composition differs significantly over geographic distance

It is predicted that sites nearest in space will be most similar in taxonomic composition, as has been demonstrated in one study of microbial beta-diversity in benthic river sediments (Gibbons et al. 2014), and would be consistent with general patterns of macro-organism biogeography in ecological theory (Nekola & White 1999). Accordingly, it is expected that microbial assemblages will differ between catchments.

(3) Identify patterns of correspondence between microbial distributions and variation in groundwater chemistry

Specific aims of stygofauna community analysis are to:

(4) Determine whether stygofauna community structure differs significantly between irrigated and pasture land-uses

(5) Identify relationships between stygofauna community structure and variation in groundwater chemistry

2 Methods

2.1 The study area

The study areas are located in the Condamine Catchment, south-east Queensland, and the Gwydir Catchment, north-west New South Wales. Both catchments are located in the northern region of the Murray-Darling Basin, and are separated geographically by approximately 160 kilometres (Figure 2.1). The Condamine Catchment is approximately 19,190 km^2 in extent (Queensland Water Commission 2012), and the Gwydir Catchment approximately 26,500 km^2 in extent (Barrett 2009). The Condamine River is a headwater of the Darling River system and drains the catchment to the southwest (Queensland Water Commission 2012). Surface waters of the Gwydir Catchment drain the catchment to the west (Kelly & Carr 2010).

The Gwydir and Condamine catchments are similar in geological complexity. In broad terms, a mosaic of various geological formations (sandstone, siltstone, and mudstone) underlie extensive areas of geologically younger alluvial sediments in both catchments (Kelly & Carr 2010; Queensland Water Commission 2012). Alluvial aquifers comprised of fine to coarse-grained sands and gravels, interbedded with silt and clay, are commonly associated with drainage lines in both catchments. Information regarding the physical dimensions of these aquifers is limited; however, the Condamine River Alluvium, which is considered to be one of the more significant aquifers in southern Queensland, may be up to 130 metres deep (and 20 km wide) in the central Condamine region; whereas tributary aquifers linking to the main channel are relatively shallow in comparison (Queensland Water Commission 2012). Within the Gwydir Catchment, a relatively shallow alluvial aquifer between 10 to 30 metres in depth was accessed for this project: the Narrabri Formation (Barrett 2009).

Hydraulic conductivity ranges between 3 to 30 meters per day across aquifers of the Condamine Catchment (Dafny & Silburn 2014). A comparable range of hydraulic conductivity values is estimated for the Narrabri Formation, and other aquifers of the Gwydir Catchment (CSIRO 2007). The Condamine and Gwydir River floodplains are extensively developed for agricultural production, and in recent decades, groundwater extraction for crop-irrigation has caused significant water-table decline in sections of both catchments (Kelly & Carr 2010; Queensland Water Commission 2012).

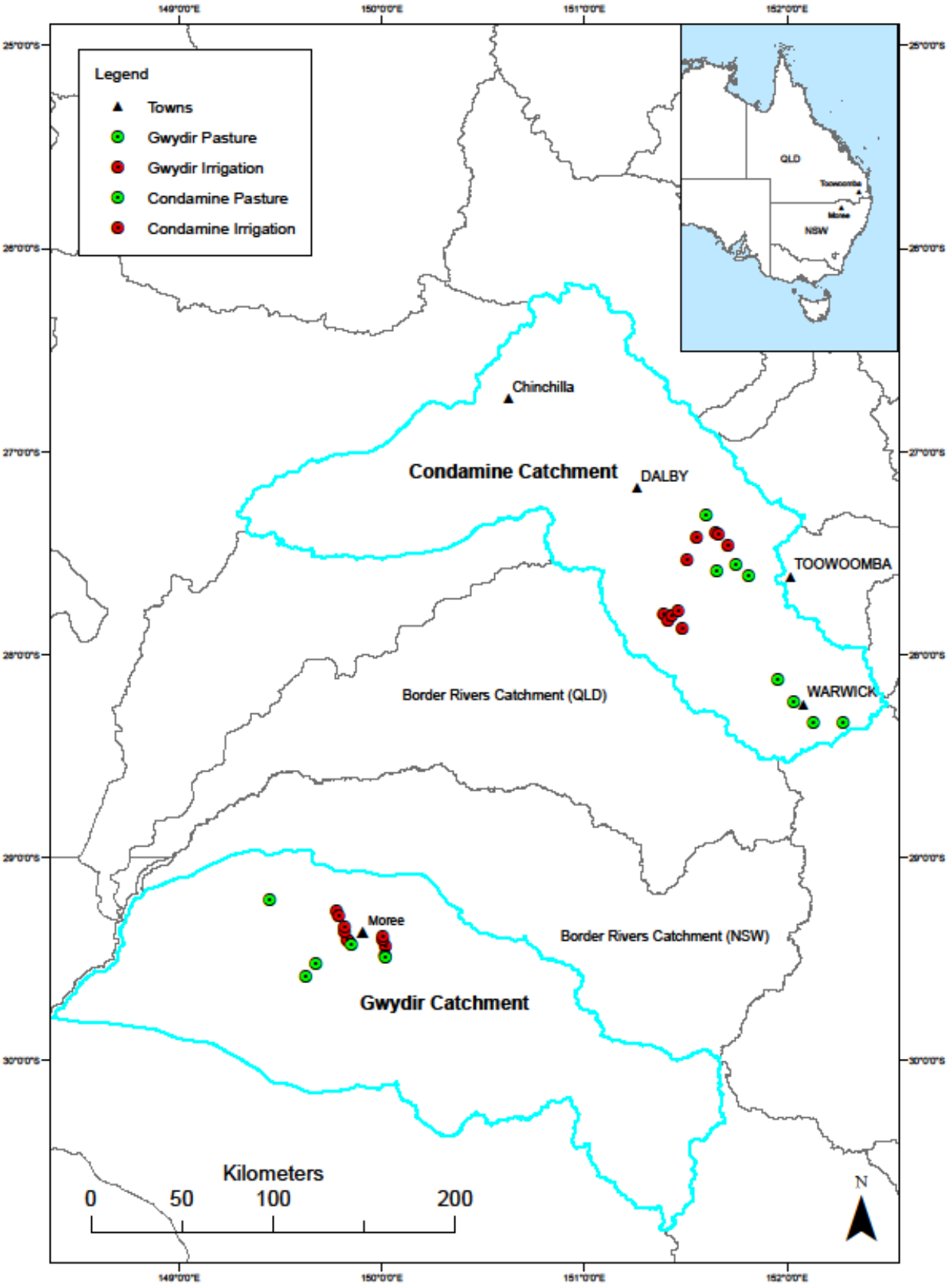

Figure 2.1 Map of the project catchments (highlighted in turquoise) and sampling sites: green markers indicate pasture sites, and red markers indicate irrigated sites

2.2 Sampling design and rationale

In order to test whether biological communities in groundwater differ under irrigated agriculture in comparison to pasture, it was important to ensure that the landscape at each sampling location conformed solely to either land-use category. Due primarily to a combination of drought conditions and legislation limiting groundwater allocations for farming, not all areas developed for irrigation in the project catchments are irrigated in any one year (Hulugalle, Weaver & Finlay 2010). Consequently, irrigated sampling sites were selected on the basis that current irrigation activity: (i.e., drip or furrow irrigation, in addition to the presence of canals, and water storage reservoirs), was the only visible land-use for several kilometres. Conversely, pasture sites were selected on the basis that there was no visible evidence of irrigation, or indeed any crop production for a distance of several kilometres.

As a consequence of historical land clearance in the Condamine and Gwydir catchments, pasture sites selected for this project could not strictly be described as undisturbed; rather, pasture sites are defined here as either unimproved grazing lands, or areas of relatively natural vegetation. Nine irrigated and eight pasture sites were selected in the Condamine Catchment, and nine irrigated and five pasture sites were selected in the Gwydir Catchment. Within the project areas, access to groundwater is dependent upon the spatial distribution of groundwater bores to sample from. The uneven sampling design here reflects the difficulty in locating areas in which groundwater bores were present, which fulfilled the land-use criteria outlined above.

The fieldwork component of this project involved collecting samples for microbial, stygofaunal, and water quality analysis from state owned groundwater monitoring bores. GPS locations of groundwater bores in the Condamine Catchment and related metadata were accessed through a web-based application that operates within the Google Earth framework: ‘The Coal Seam Gas Globe’ (Department of Natural Resources and Mines 2015). Bore metadata of interest to the sampling design of this project included recent water-level data, and bore construction records which detail the geological profile and aquifer system in-which bores are located. To maintain uniformity amongst samples, and since stygofauna are most likely to occur in porous media (Hancock et al. 2005), only bores located in alluvial aquifers were selected (as opposed to less permeable aquifer substrates: e.g., sandstone).

In addition, bore construction records detail the start and end depth of a slotted section of bore which is open to the aquifer and allows groundwater to enter. To minimise the likelihood that groundwater samples for microbial analysis were compromised with species strongly affiliated with terrestrial soils, only bores in-which the slotted section began at least five metres below the terrestrial surface were selected. Groundwater bores sampled in the Gwydir Catchment were part of an established ecological assessment program (Korbel et al. 2013a). Each bore conformed to criteria outlined above; i.e., all were located in alluvial groundwater, with a slotted section that began at least five metres below the terrestrial surface. Field sampling was conducted from February 16 to 23, 2015, which corresponded to the irrigated crop growing season (prior to harvest March - April). Rainfall in the project areas was well below seasonal averages over the weeks prior to sampling (BOM 2015); see Table A1: Appendix A.

2.3 Data collection

Water quality and stygofauna

Prior to sample collection, groundwater bores were purged with 150 L of water (2 to 3 bore volumes) using an inertia pump (Waterra, Ontario, Canada), in order to ensure that water chemistry values and all biota sampled were representative of the aquifer environment, and not the bore interior (Sundaram et al. 2009). After purging, a groundwater sample was collected for chemical analysis (major ions, nutrients and dissolved organic carbon (DOC)), by pumping directly into a sterile container, which was immediately frozen and stored for processing at the NSW Office of Water Environment Laboratory, Arncliffe, NSW, Australia. Water quality parameters: Electrical Conductivity (EC), pH, temperature (°C) and dissolved oxygen (DO), were recorded *in situ* from groundwater pumped into a 12.5 L container, using a hand-held water quality probe (TPS, Springwood, Queensland, Australia). Care was taken to ensure water exited the pump hose below the water level of the container so that aeration was minimised. Stygofauna were collected by filtering groundwater (from several plastic containers that were filled during pumping), through a 63 µm mesh sieve. Sediments and any stygofauna present in the sieve were transferred to labelled sample jars containing 100% ethanol for preservation purposes.

Microbial communities

Groundwater samples for microbial community genetic analyses were collected by pumping directly into a sterile 2 L container. The container was filled to the brim to minimise aeration of the sample and stored on ice in the field. Microbial cells were filtered from groundwater samples within the same working day at field accommodation. For each sample, aliquots of groundwater were aseptically vacuum filtered through two replicate sterile 0.45-µm mixed cellulose ester membranes (Pall Corporation, Port Washington, NV, USA). Samples were re-homogenised by inversion of the container before each filtration, and filtered for ~25 minutes. The volume of filtered groundwater ranged between 200 - 500 mL per replicate, dependent upon the sediment content of the sample, and consequently how quickly filter material became blocked with the accumulation of particles. Filters were transferred into labelled sterile containers and stored at -40°C for DNA extraction.

2.4 Laboratory processing

Taxonomic classification of stygofauna

Invertebrates were located in samples under a Motic light dissecting microscope (60x magnification) and re-stored in labelled sample jars prior to identification. Organisms were grouped on the basis of broad morphological differences and examined to determine if the characteristics of each group were stygofaunal. Individuals were classified as stygofauna according to criteria outlined in the introduction: (e.g., sightless, pigmentless, exhibiting numerous extra sensory setae). Although difficult to examine since their body parts are contained in calcite valves, Ostracoda were classified as stygofauna on the basis that this group are frequently found in groundwater ecosystems in Australia (Karanovic 2005; Halse et al. 2014; Reeves et al. 2007). Springtails (Collembola) occurred in several samples; however, these animals are ubiquitous in both terrestrial and aquatic environments (Gooderham & Tsyrlin 2002), and thus were not considered stygofauna. Oligochaeta and Acarina (mites) occur in terrestrial and subterranean aquatic ecosystems (Gooderham & Tsyrlin 2002), but were classified as stygofauna here on the basis of morphological criteria, and because they are a common component of stygofauna assemblages in Australia (Halse et al. 2014; Hancock & Boulton 2008). Invertebrate larvae or instars with obvious surface-water affinities occasionally occurred in samples, and these were identified with taxonomic keys and

removed from the dataset (Gooderham & Tsyrlin 2002). Most stygofaunal groups could reliably be identified to order or family taxonomic level using the following keys: Syncarida (Serov 2002), Amphipoda (Bradbury & Williams 1999), Copepoda & Ostracoda (Williams 2001). Due to taxonomic uncertainty, Oligochaeta and Acarina could not be identified to lower levels than Class and Subclass respectively.

2.5 DNA methods

Microbial community DNA extraction

Under sterile conditions sediments were recovered from each of two filters representing each bore sample and transferred into a pre-weighed 2 mL centrifuge tube. For a small number of filters on which relatively little sediment was present, a proportion of saturated filter material was sliced into thin strips and added to a centrifuge tube. Genomic DNA was then isolated from approximately 0.5 grams of starting material per sample using PowerSoil kits (MoBio Laboratories, Carlsbad, CA), with the following modifications to the manufacturer's protocol: during the cell lysis step, PowerBead tubes were secured vertically in a tube agitator (as opposed to MoBio vortex), agitated at speed 4 for 45 seconds, and subsequently centrifuged for 2 minutes. After addition of solution C2 and C3 steps, samples were incubated for 30 minutes and subsequently centrifuged for 2 minutes. The ethanol rinse step was repeated twice, and on each occasion samples were centrifuged for 3 minutes after C5 flow-through had been discarded. The elution step was split into two parts: firstly, a 50 µL aliquot of solution C6 was added to the centre of a spin-filter membrane and left for 1 minute at room temperature prior to centrifugation; this process was subsequently repeated with 25 µL of solution C6. Finally, eluted DNA samples were labelled and stored at -40°C prior to DNA amplification.

Microbial community DNA amplification

PCR reactions were performed in 96-well microtitre plates using an Eppendorf Mastercycler-pro PCR System. Reagent proportions in 50 µL optimised reactions were as follows: 20 µL of Milli-Q nuclease free water, 1 µL of each Primer (10 µM), 1 µL of DMSO, 2 µL of DNA template, and 25 µL of PCR Master Mix with High-Fidelity buffer (Thermo Scientific): 1.5 mM $MgCl_2$, 1 U of *Taq* DNA Polymerase, and 200 µM of each deoxynucleotide (dNTPs) in final reaction concentration.

PCR cycling conditions for bacterial / archaeal 16S rDNA consisted of 3 minutes initial denaturation at 98°C, followed by 35 cycles of denaturation at 98°C for 15 s, annealing at 50°C for 15 s, extension at 72°C for 90 s, and a final extension step at 72°C for 3 minutes (Caporaso et al. 2012). Fragments of bacterial and archaeal 16S rDNA, were amplified successfully using the primers given in Table 2.1.

Table 2.1 Primer pair and genetic marker used in this study

Gene	Primers	Sequence 5' → 3'	Reference
16S rDNA	515F	GTGCCAGCMGCCGCGGTAA	(Caporaso et al. 2012)
	806R	GGACTACHVGGGTWTCTAAT	

The resulting amplicons were analysed using a MCE-202 MultiNA Microchip Electrophoresis System (Shimadzu 2015): MultiNA DNA-1000 Reagent Kit (100 – 1000 nucleotide base pairs); note: separation buffer and marker solution quantities were determined automatically by the MultiNA instrument. SYBR® Gold Nucleic Acid Gel Stain was used for fluorescent detection of nucleic acids (working stock ratio of 99 µL TE buffer: 1 µL SYBR Gold). Amplicon fragment lengths were determined by comparison to 6 µL of Promega Phi x 174 (Hae III) DNA marker.

PCR products were purified (i.e., unincorporated dNTPs, primers, salts and contaminants removed) using an AMPure PCR purification kit (Agencourt Bioscience Corporation, Beverly, MA, USA), and Qiagen Kit elution buffer (Qiagen, Valencia, CA, USA). Following elution, DNA concentration per sample was quantified using a NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Scientific 2015). Sequencing reactions were performed by the Ramaciotti Centre for Genomics (University of New South Wales, Sydney) using Illumina® sequencing technology.

2.6 Water quality data analysis

Multivariate dataset preparation

Groundwater quality data were visually inspected to identify and remove spurious (uncharacteristically high or low) measurements prior to analysis. A seemingly high value of EC (20,100 µS cm^{-1}) was identified but not removed since it fell within the range of previously recorded values: (225 – 32,790 µS cm^{-1}) for Condamine Catchment

groundwater (Dafny & Silburn 2014). A large DOC value (29 mg/L) was removed since it was nine times greater than any other corresponding measurement.

Draftsman plot analysis was used in Primer 6.0 (Plymouth Marine Laboratory, UK) to determine correlations between variables, and those variables with correlations greater than 0.90 were excluded from further analysis (Clarke & Gorley 2006). Nitrate (NO_3^-) and Total N (nitrogen) were strongly correlated (r = 0.90); and of these nitrate was chosen further analysis, since nitrate contamination of groundwater is strongly linked to irrigated agriculture in eastern Australian catchments (Sundaram & Coram 2009). The final multivariate dataset comprised of the following variables: EC (electrical conductivity), pH, temperature (°C), DO (dissolved oxygen), Ca^{++} (calcium ion), NO_3^-, Total P (phosphorous), and HCO_3^- (bicarbonate). Dissolved organic carbon (DOC) was not included because as ~ 80% of values were reported as either '1 mg/L' or '<1 mg/L' (as opposed to decimal numbers), data values did not segregate sufficiently in the draftsman plot. Total P, Ca^{++}, EC, and NO_3^- data values were log (x+1) transformed to reduce skew, and subsequently all variables were normalised and converted to a data-matrix using a Euclidean distance measure (Clarke & Warwick 2001).

Multivariate exploratory data analysis

Statistical differences in groundwater quality profiles between catchments, and land-use within catchments (irrigated land vs. pasture) were tested by a two-factor permutational multivariate analysis of variance (PERMANOVA, Anderson 2001). A principal components analysis (PCA) ordination was used to visualise the distribution of samples in relation to change along water quality gradients.

Univariate exploratory data analysis

T-tests were performed in Microsoft Excel to compare EC (salinity), NO_3^- and Total P concentrations in groundwater under irrigated land and pasture, to assess whether nutrient and salinity levels were elevated under irrigated farmland, as is commonly reported in regard to other intensively farmed catchments in Eastern Australia (Sundaram & Coram 2009). Prior to computation, all data were tested for homogeneity of variance using Bartlett's test in *R* version 3.2.1 (R Development Core Team 2013), and log transformed if necessary to meet this assumption.

2.7 Stygofauna data analysis

Stygofaunal abundance data were square-root transformed to increase the importance of low abundance species, and converted to a data matrix using the Bray-Curtis similarity index in Primer 6.0 (Plymouth Marine Laboratory, UK). Since there were many empty (zero abundance) cells, a dummy variable of '1' was added to the dataset to correct the behaviour of the Bray-Curtis index (Clarke & Warwick 2001). A one-way PERMANOVA was used to test for differences in stygofauna community structure between land-use categories (treatments as above). T-tests were performed in Microsoft Excel to test for within catchment differences in mean stygofauna abundance between 'irrigated agriculture' and 'pasture' site groupings.

Relationships between stygofauna community structure and variation in water chemistry parameters were analysed using distance-based linear models (DistLM, Legendre & Anderson 1999). An initial analysis was performed using forward selection of all water quality variables with the goodness-of-fit examined using Akaike's information criterion (Bellchambers et al. 2011). The most parsimonious model was chosen, and the analysis then re-run using only those variables selected. A distance-based redundancy analysis (dbRDA) ordination was produced to visualise the influence of predictor variables on stygofauna assemblage composition.

2.8 Microbial community data analysis

2.8.1 16S rDNA sequence data analysis

Background and summary data

Raw 16S rDNA sequence reads trimmed to 250 base-pairs in length were assigned taxonomic identities using the Ribosomal Database Project (RDP) classifier (http://www.arb-silva.de/), and clustered into Operational Taxonomic Units (OTUs) under a 97% similarity threshold. This analysis returned 21,530 OTUs in a tabulated matrix inclusive of taxonomic and abundance data. Considered separately, the Gwydir Catchment dataset contained 12,173 OTUs, and the Condamine Catchment dataset contained 18,856 OTUs. Seventy eight percent (9502) of Gwydir OTUs were also present in the larger Condamine dataset. Hierarchical taxonomic information relating to OTUs was highly fragmented. For example, there were many OTUs unclassified at phylum level, whilst many others were ranked at all taxonomic levels through to genus.

16S rDNA dataset preparation

Visual inspection of the OTU table revealed that there were a large number of OTUs with relatively low sequence abundance totals, and many zero abundance cells. To reduce the influence of rare and inconsistent OTUs on statistical analysis, those with a total abundance of less than 200 sequences across all samples were removed from the dataset (Nielsen et al. 2014). OTUs unclassified at phylum level were also removed because there is rarely any ecological data available for novel microorganisms. These filtering steps produced a working dataset of 1108 OTUs representing both catchments.

Total sequence counts amongst samples ranged from 806 to 145,640. The median was ~40,000, and 72% of samples contained over 30,000 reads. Four samples were removed from the dataset, because at 806, 883, 2192, and 2892 sequence reads respectively, it is likely that these low counts reflect poor efficiency in the DNA extraction, PCR, or sequencing process, as opposed to true biological variation: *indicted by the red circle* (Figure. 2.2). Rarefaction curves were generated in *R* version 3.2.1 (R Development Core Team 2013) to visualise sampling efficiency for each sample. The predicted species richness of one sample was clearly much lower in compassion to the others: *indicted by the blue arrow* (Figure. 2.2), and this sample was also removed from the dataset because its inclusion may have compromised downstream statistical tests. The sample dataset was then rarefied to 9914 sequences

(the lowest retained sample total), by resampling without replacement using the 'rarefaction' function in the vegan package (Oksanen et al. 2007). The final Condamine Catchment dataset consisted of 16 samples representing 1073 OTUs, and the Gwydir Catchment dataset 11 samples representing 986 OTUs. Eighty six percent of the initial 1108 OTUs occurred in at least one sample of both datasets.

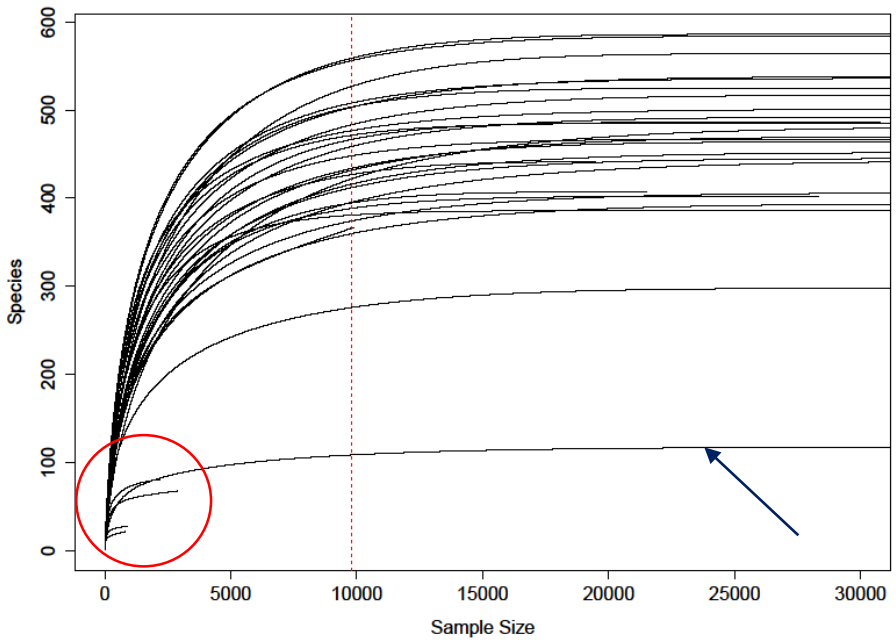

Figure 2.2 Rarefaction curves of individual samples. The red circle and blue arrow highlight five relatively low diversity samples which were removed from the dataset (see text above figure). Sample data rarefied to 9914 sequences indicated by the red vertical line on the plot.

Prior to further analysis, all OTU data were converted to presence or absence, because binary 16S rDNA amplicon data has been shown to perform more reliably than relative amplicon abundance (Logares et al. 2014a). Following conversion to presence or absence, a sample resemblance matrix was generated using the Jaccard coefficient in Primer 6.0 (Plymouth Marine Laboratory, UK). The Jaccard coefficient was chosen as a distance measure because it does not count co-absence as similarity (Greenacre & Primicerio 2014), and is thus an appropriate distance measure for binary datasets.

2.8.2 Taxonomic diversity and land-use

To determine whether the taxonomic composition of microbial communities differed significantly between irrigated and pasture site groupings, a one-way PERMANOVA (Anderson 2001) was used based on 999 permutations of the data.

2.8.3 Microbial biogeography

Permutation-based Mantel tests were conducted using the 'ade4' package (Dray & Dufour 2007) in *R* version 3.2.1, to determine whether the taxonomic composition of microbial community samples differed significantly over geographic distance. Community similarity matrices were generated in Primer 6 using the Jaccard coefficient as outlined above, and then converted to dissimilarity matrices (1 minus the similarity) in Microsoft Excel. The geodesic distance in kilometres between each sampling location was calculated in ArcGIS, and used to construct geographic distance matrices. Mantel tests based on 999 permutations of the data were performed on within-catchment datasets, and a pooled dataset inclusive of all samples from both catchments. A scatterplot incorporating a fitted linear model of the data was produced to visualise the relationship between geographic distance and sample taxonomic composition, using the 'lattice' graphics package (Sarkar 2008).

A one-way PERMANOVA was used to test for a statistical difference in community composition between catchments, and the result visualised using non-metric multidimensional scaling (nMDS) ordination.

2.8.3.1 Assessment of microbial distributions

Microbial distributions in each catchment were characterised on the basis of taxonomic groups which were most representative of the sample data overall. A dataset was constructed which comprised solely of OTUs which were present in 70% or more of samples from either catchment. The corresponding OTUs were grouped according to phylum, and stacked bar-charts were generated from the data to illustrate the proportion of OTUs per phylum and catchment, and provide an indication of which phyla the most common and widespread OTUs belonged to. (Note: the dataset constructed for this exercise was not used in any other analysis).

2.8.4 Patterns of diversity and environmental gradients

Relationships between microbial community composition and variation in water chemistry parameters were analysed using DistLM (Legendre & Anderson 1999) using the same set of variables as above (section 2.6). An initial analysis was performed using forward selection of all variables with the goodness-of-fit examined using Akaike's information criterion (Bellchambers et al. 2011). The most parsimonious model was chosen, and the analysis then re-run using only those variables selected. A distance-

based redundancy analysis (dbRDA) ordination was produced to visualise the influence of predictor variables on microbial assemblage composition.

On the basis of the outcome of DistLM analyses, a Mantel test based on 999 permutations of the data was used to test for a significant correlation between assemblage composition and EC of groundwater in the Gwydir Catchment.

2.8.5 Microbial activity and land-use

Microbial activity in groundwater at each site was assessed *in-situ* using the 'Cotton Strip Assay' (CSA). The CSA uses loss in the tensile strength of cotton strips as a surrogate measure of relative cellulolytic microbial activity between samples (Lategan, Korbel & Hose 2010). Cotton strips (4 cm x 10 cm), were submerged in each bore one metre below the water-level, and subsequently recovered after six weeks. Once retrieved, the cotton strips were divided into three sections, and loss in the tensile strength of the strips was tested for each sample in triplicate using a pneumatic tensiometer Universal Testing Machine (UTM Instron 6022 10- kN load frame; Instron Corporation, Norwood, MA, USA). T-tests with unequal variance were used to test for differences in mean loss in tensile strength of the strips between 'irrigated' and 'pasture' site groupings.

3 Results

3.1 Water quality data analysis

Multivariate exploratory data analysis

Groundwater quality profiles differed between catchments (PERMANOVA F = 3.62, p = 0.002), but not between irrigated and pasture land-uses within either catchment (PERMANOVA F = 0.63, p = 0.717). The PCA (Figure 3.1) reflects the above results showing that there was no clear separation of sites by land-use within catchments, and a degree of separation between catchments, which appears to be influenced by differences in water temperature and a combination of other variables. Since water quality profiles differed significantly between catchments, further land-use related analysis of biological data were conducted on each catchment separately.

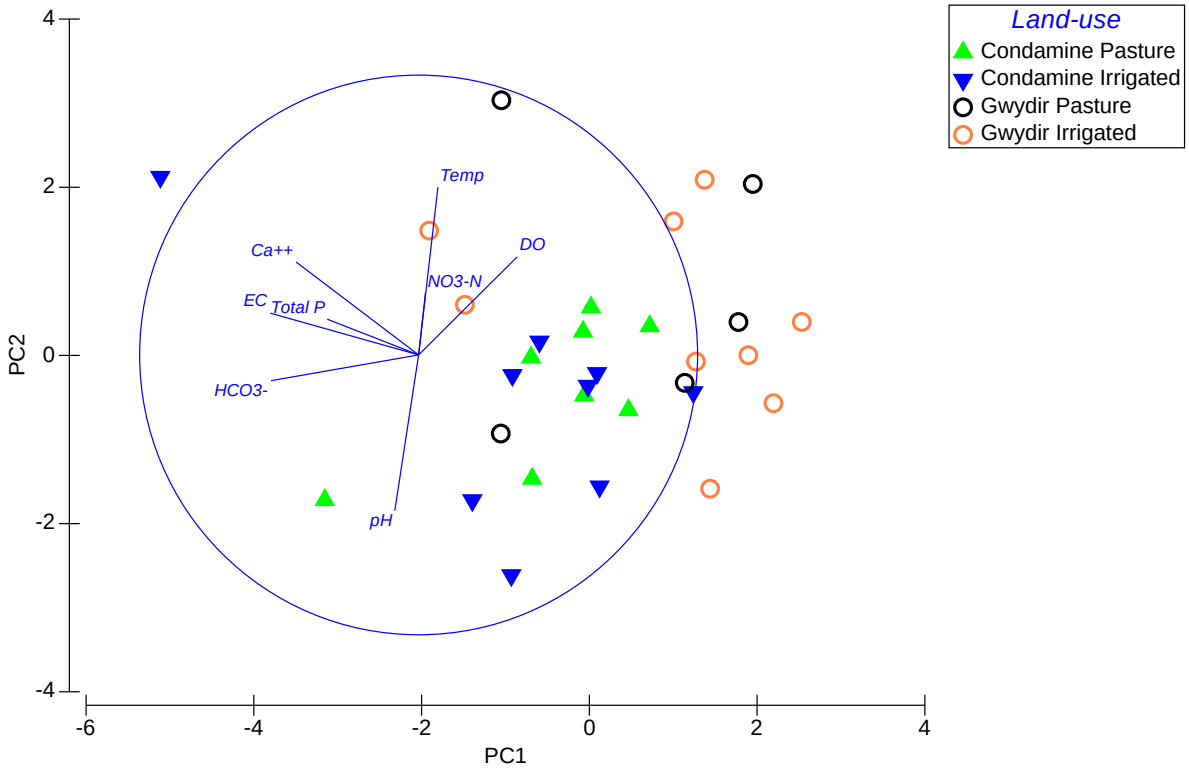

Figure 3.1 Principal components analysis plot of water quality profiles in relation to land-use. Filled triangles represent Condamine Catchment sites. Hollow circles represent Gwydir Catchment sites. Vectors indicate the direction variables are increasing.

Univariate exploratory data analysis

There was no significant difference in mean EC, nitrate, or total phosphorous concentrations between irrigated and pasture site groupings of either catchment (Table 3.1). The mean and range of all measured water quality parameters are listed in Table B1: Appendix B.

Table 3.1 T.Test results of nutrient and EC comparisons between land-use categories n = number of samples, SE = standard error, DF = degrees of freedom. NO_3^- (nitrate), P (total Phosphorous) and EC (Electrical Conductivity).

Parameter	Condamine pasture sites			Condamine irrigated sites			T Test result		
	n	Mean	SE	n	Mean	SE	p (T)	*p*	DF
NO_3^- (mg/L)		3.7	2.20		0.63	0.24	0.19	(p>0.05)	
TP (mg/L)	8	0.49	0.12	9	0.74	0.33	0.50	(p>0.05)	15
EC (µS cm^{-1})		1110	154		3472	2091	0.29	(p>0.05)	

Parameter	Gwydir pasture sites			Gwydir irrigated sites			T Test result		
	n	Mean	SE	n	Mean	SE	p (T)	*p*	DF
NO_3^- (mg/L)		2.8	2.07		1.73	0.49	0.63	(p>0.05)	
TP (mg/L)	5	0.54	0.24	9	0.4	0.06	0.62	(p>0.05)	13
EC (µS cm^{-1})		645	104		1261	555	0.31	(p>0.05)	

3.2 Stygofauna data analysis

Stygofauna collection overview

A total of 753 individuals representing 11 higher taxonomic level (family or above) groups were recovered from samples. Syncarida (Bathynellidae and Parabathynellidae), Amphipoda (Paramelitidae) and Copepoda (Cyclopoida) were the most common crustacean groups present, whilst oligochaete worms and mites (Acarina) were also relatively common. Proportionally, stygofauna occurred in more Gwydir Catchment samples (92 %) than Condamine Catchment samples (70 %), and Gwydir samples were more species-rich overall despite lower sample size. Taxon richness was greater in irrigated sites than pasture sites in both catchments (Table 3.2).

Table 3.2 Stygofauna collection summary data

land-use category	n samples	number of samples in which taxa were present	number of taxa per sample	Mean ± SE
Gwydir pasture	5	5	2 to 7	5 ± 0.81
Gwydir irrigated	9	8	0 to 7	3 ± 0.74
Condamine pasture	8	5	0 to 5	1 ± 0.59
Condamine irrigated	9	7	0 to 5	2 ± 0.57

Acarina were the most widely distributed fauna in groundwaters of the Gwydir Catchment, occurring at 66 % and 80 % of irrigated and pasture sites respectively. In terms of differences in species abundance between land-use categories: oligochaetes, cyclopoid copepods, and paramelitid amphipods were in greater abundance at irrigated compared to pasture sites. Oligochaetes were the most widely distributed fauna in groundwaters of the Condamine Catchment, occurring at 55 % and 12 % of irrigated and pasture sites respectively. In terms of differences in species abundance between land use-use categories: oligochaetes and parabathynellids were marginally more abundant at irrigated compared to pasture sites, albeit the abundance of no one species exceeded 23 individuals in samples of either land-use type. Stygofauna diversity statistics per catchment and land-use are detailed in Table C1: Appendix C.

3.2.1 Stygofauna community structure and land-use

There was no significant difference in stygofauna community structure between irrigated and pasture sites within the Condamine Catchment (PERMANOVA t = 1.1, p = 0.31), nor within the Gwydir Catchment (PERMANOVA t = 1.0, p = 0.309). Stygofauna abundance totals were greater at irrigated than at pasture sites in both catchments (342 to 290 in the Gwydir, 81 to 40 in the Condamine); however, there was no significant difference in mean stygofauna abundance between irrigated and pasture sites within the Condamine Catchment (t = 0.6, df = 15, p>0.05), nor within the Gwydir Catchment (t = 0.7, df = 13, p>0.05) (Figure 3.2).

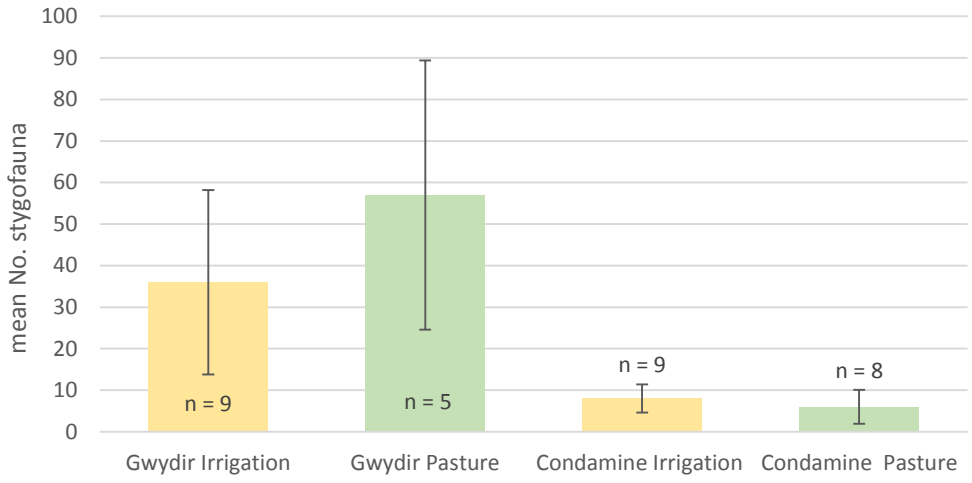

Figure 3.2 Mean stygofauna abundance in relation to land-use
n = number of sites, error bars = standard error

3.2.2 Stygofauna community structure and groundwater chemistry

DistLM analysis revealed poor correspondence between patterns of stygofauna community structure and aspects of groundwater chemistry in the Gwydir Catchment. There was no significant correlation between any of the eight water quality parameters included and stygofauna community structure (p>0.05). Under forward selection criteria, temperature was the only variable included in the most parsimonious model, explaining 10.9 % of the variation in stygofauna community structure. The resultant dbRDA ordination plot was not informative and therefore is not included here.

In the Condamine Catchment, the most parsimonious DistLM model which used EC, Total P, NO_3^-, DO and Temperature, explanied 43.9% of the total variation in stygofauna community structure (Figure 3.3).

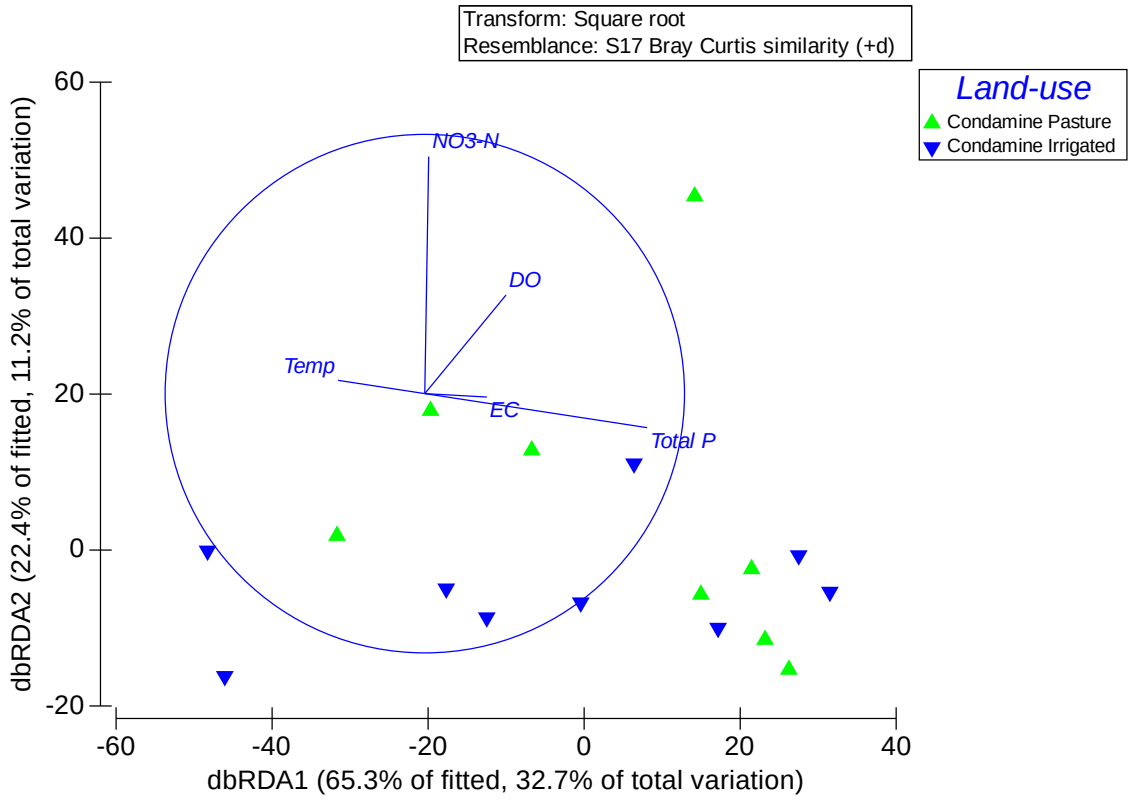

Figure 3.3 dbRDA ordination illustrating the relationship between groundwater chemistry and stygofauna community structure in the Condamine Catchment. Triangle symbols represents the community at each site; vectors indicate the direction variables are increasing.

The first dbRDA coordinate axis explained 32.7 % of the total variation in stygofauna community structure, with EC and Total Phosphorous concentration separating samples. The second dbRDA coordinate axis explained 11.2 % of the total variation, with NO_3^-, increasing temperature, and DO separating samples. NO_3^- was the only statistically significant variable included the model ($p = 0.047$, $p<0.05$).

3.3 Microbial community data analysis

3.3.1 Microbial community structure and land-use

There was no significant difference in the taxonomic composition of microbial communities in groundwater under irrigated agricultural landscapes versus pasture within the Condamine Catchment (PERMANOVA t = 1.09, p = 0.144), nor within the Gwydir Catchment (PERMANOVA t = 1.11, p = 0.108).

3.3.2 Microbial biogeography

The taxonomic composition of microbial community samples pooled from both catchments differed significantly over geographic distance (Mantel p = 0.011; r = 0.102). The Mantel correlation r = 0.102, and the slope derived from a fitted linear model of the data in Figure 3.4 below, suggest that the matrix entries are positively associated; that is, greater differences in the taxonomic composition of samples are generally seen amongst pairs of sites which are further away from each other geographically.

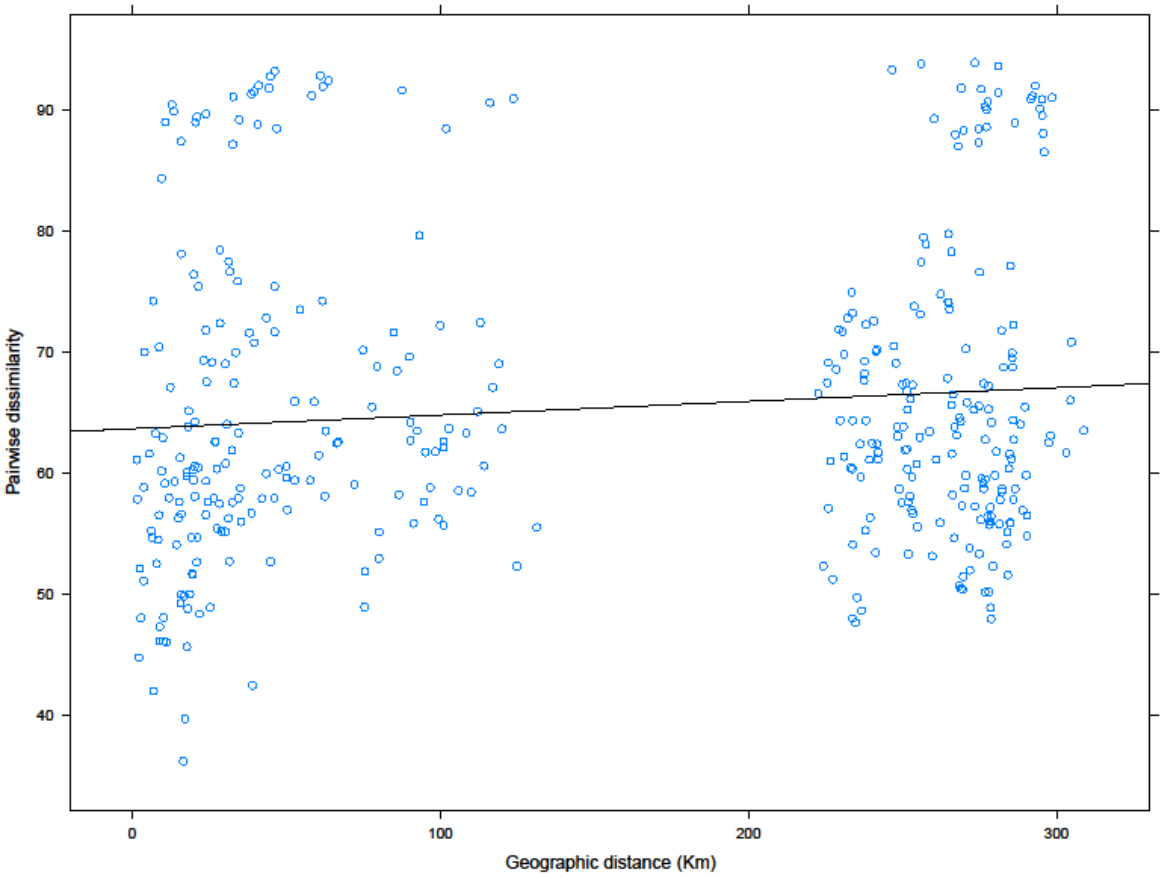

Figure 3.4 The decay of similarity over geographical distance in microbial communities of the Condamine and Gwydir Catchments (i.e., pooled data). X axis = Geographic distance in kilometres, Y axis = Jaccard dissimilarity calculated from presence / absence OTU data

Analysed separately, the Condamine Catchment geographic and community distance matrices were not significantly correlated (Mantel p = 0.458, r = -0.019); however, the Gwydir Catchment geographic and community distance matrices were significantly correlated (Mantel p = 0.01, r = 0.365): see figures 3.5 and 3.6 below.

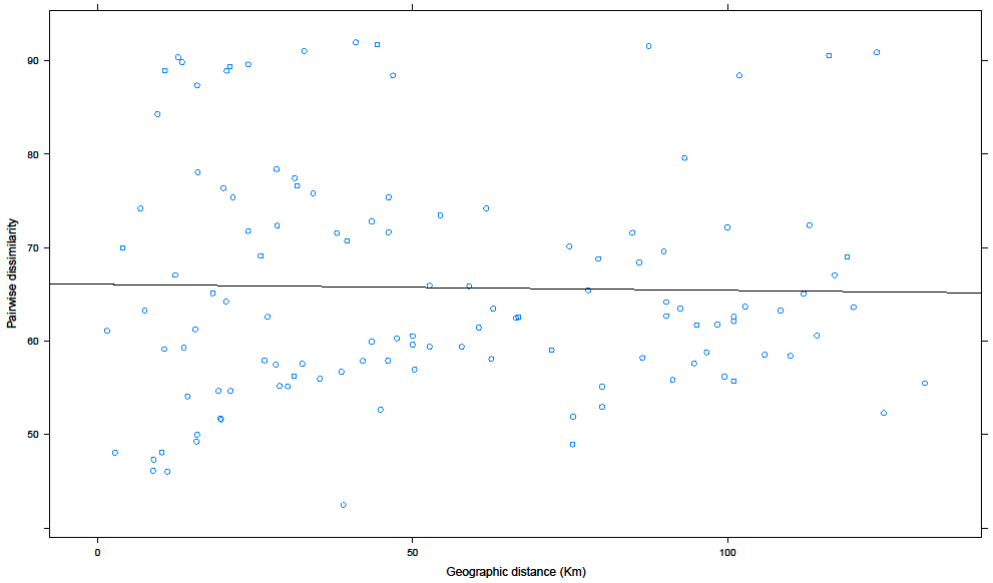

Figure 3.5 The decay of similarity over geographical distance in microbial communities of the Condamine Catchment. X axis = Geographic distance in kilometres, Y axis = Jaccard dissimilarity calculated from presence / absence OTU data. n = 16 samples

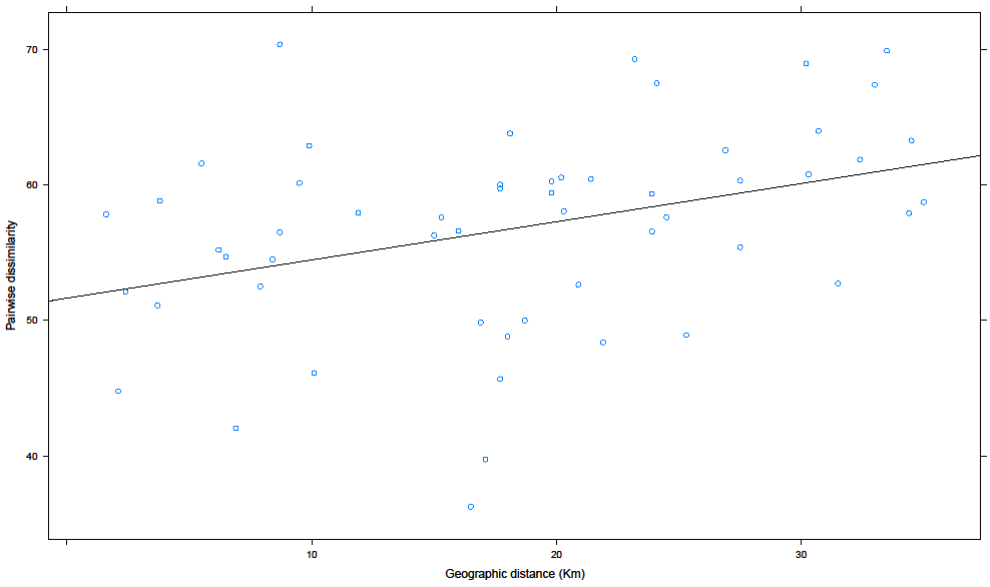

Figure 3.6 The decay of similarity over geographical distance in microbial communities of the Gwydir Catchment. X axis = Geographic distance in kilometres, Y axis = Jaccard dissimilarity calculated from presence / absence OTU data. n = 11 samples

There was a significant difference in microbial assemblages between catchments (PERMANOVA t=1.34, p = 0.001). The nMDS ordination revealed that the majority of samples from both catchments clustered relatively close together, suggesting that these samples were somewhat similar in taxonomic composition (Figure 3.5). A subset of Condamine Catchment samples are dispersed from the core cluster, suggesting that these are distinctly different in composition to the majority of samples, and each other.

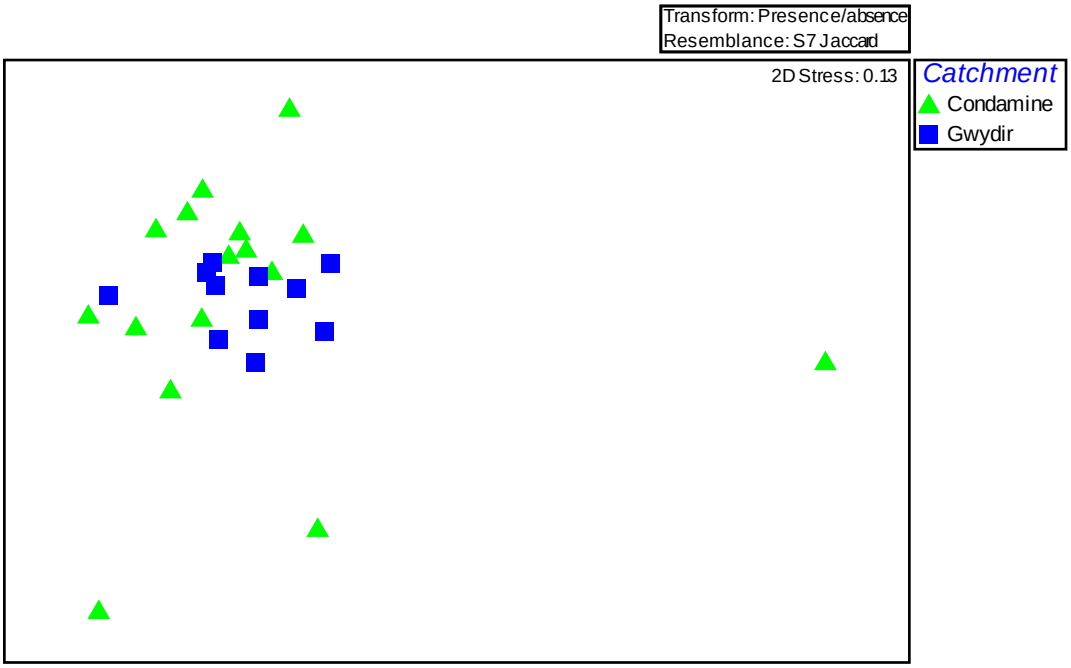

Figure 3.7 Non-metric ordination of microbial community similarity in groundwater of the Condamine and Gwydir Catchments. Condamine samples are represented by green triangle and Gwydir samples by blue square markers in the plot.

The proportion of variation (PERMANOVA average dissimilarity) partitioned between the Condamine and Gwydir catchment microbial assemblages was 70%. Considered separately, the proportion of variation amongst Condamine assemblages was 73%, and amongst Gwydir assemblages the variation was 63%.

3.3.2.1 Assessment of microbial distributions

One hundred and eighty six OTUs were present in 70% or more of Condamine Catchment assemblages, and 248 OTUs were present in 70 % or more of Gwydir Catchment assemblages. At the level of phylum, there were strong similarities in the identity and proportions of these OTUs between catchments (Figure 3.6). The Dominant phyla included Proteobacteria, Actinobacteria, Acidobacteria, Firmicutes, and Thaumarchaeota, which collectively accounted for 78 - 85 % of all OTUs detected in 70 % or more of assemblages (Figure 3.6). Eleven percent of OTUs (128 out of 1108) were present in 70% or more of assemblages in both catchments.

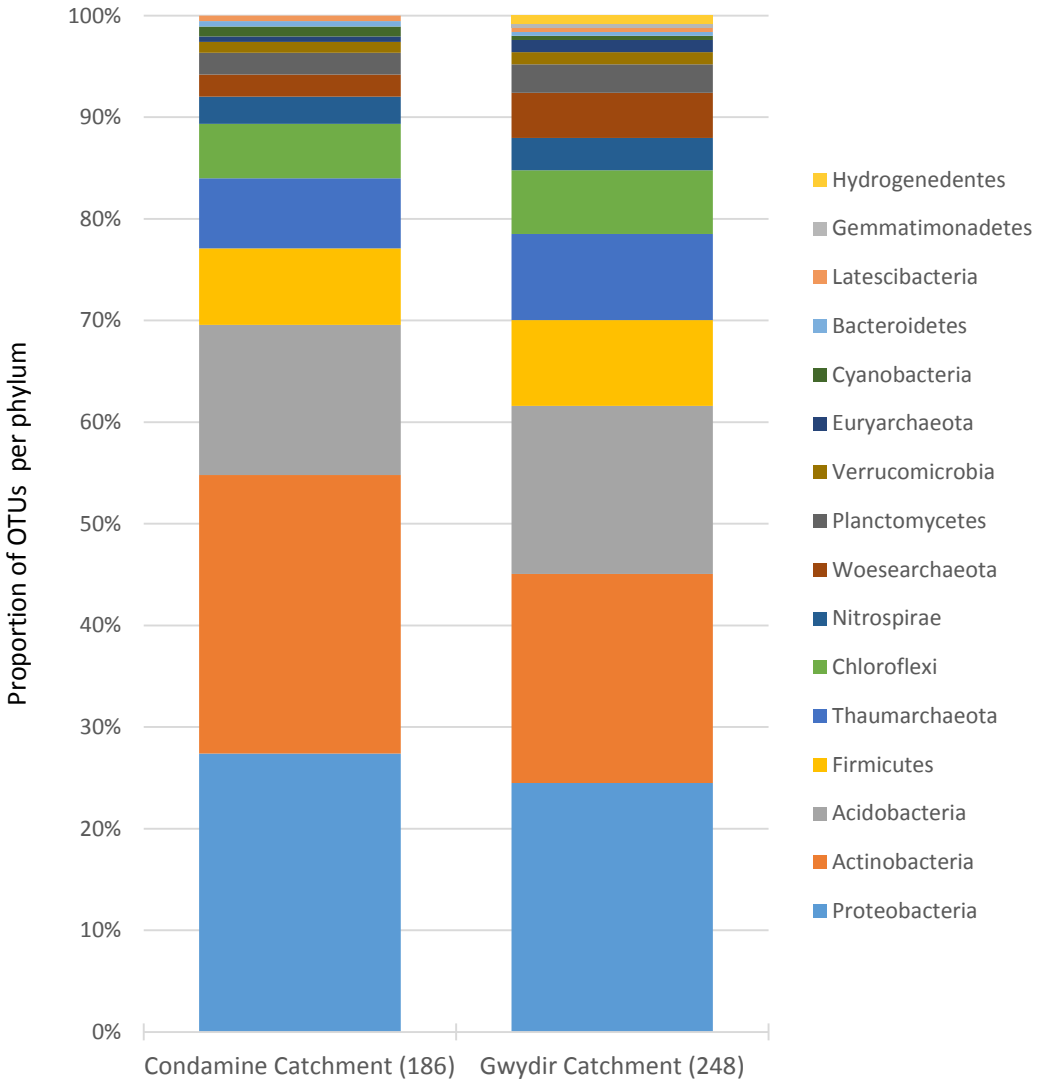

Figure 3.8 Taxonomic distribution of dominant OTUs per phylum and catchment. Bracketed values after catchment names represent the number of OTUs which occurred in 70% or more of samples. Condamine Catchment n = 16 samples, Gwydir Catchment n = 11 samples.

3.3.3 Patterns of diversity and environmental gradients

The results of DistLM analysis revealed poor correspondence between patterns of microbial community composition and variation in groundwater chemistry in the Condamine Catchment. None of the eight water quality variables included in the analysis were significant in the DistLM model ($p>0.05$). The resultant dbRDA ordination was not meaningful and therefore is not included here.

Conductivity and dissolved oxygen were the only two significant variables resulting from DistLM analysis of the Gwydir Catchment data: $p = 0.004$ and $p = 0.009$ respectively ($p<0.05$). The model explained 32.5 % of the total variation (Figure 3.7). The pattern in the resultant dbRDA ordination suggests a negative relationship between the majority of biological samples and increasing conductivity and dissolved oxygen concentration (Figure 3.7). The first dbRDA coordinate axis explained 18.2 %, and the second axis 14.3 % of the total variation in community composition in relation to water chemistry.

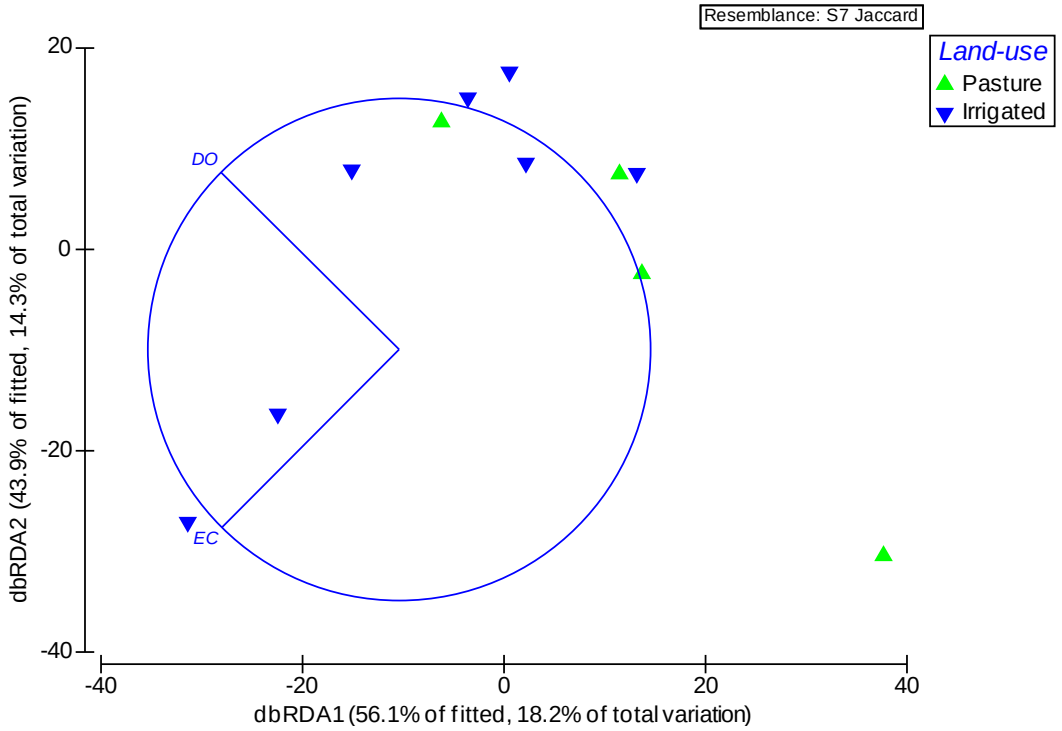

Figure 3.9 dbRDA ordination illustrating the relationship between groundwater chemistry and microbial community composition in the Gwydir Catchment. Triangle symbols represents the community at each site; vectors indicate the direction variables are increasing

In the Gwydir Catchment, microbial distributions and EC were not significantly correlated (Mantel $p = 0.07$; $r = 0.319$).

3.4 Microbial activity and land-use

There was no significant difference in mean loss in tensile strength of cotton strips between irrigated and pasture sites within the Condamine Catchment ($t = 0.09$, $df = 15$, $p>0.05$), nor within the Gwydir Catchment ($t = 0.9$, $df = 13$, $p>0.05$) (Figure 3.4). Mean loss in tensile strength was greatest overall at Condamine irrigated sites, and there is relatively little variation around the mean (Figure 3.10), suggesting marginally greater and consistent microbial cellulolytic activity relative to other groups of sites.

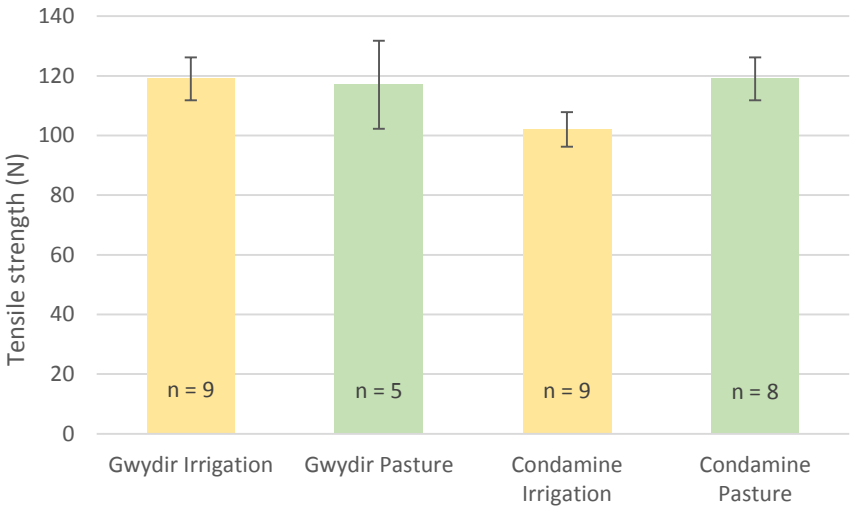

Figure 3.10 Mean loss in tensile strength of cotton strips
N = units of force, n = number of sites, error bars = standard error

4 Discussion

4.1 Water quality data analysis

Groundwater quality profiles were significantly different between catchments. This finding may reflect regional differences in geology, and marginally higher water temperatures in the Gwydir Catchment at the time of sampling. The majority of groundwater quality values were consistent with those previously recorded in the Condamine Catchment (Dafny & Silburn 2014). Within the Gwydir Catchment, water quality values fell within the range of those previously recorded with the exception of mean dissolved organic carbon concentration: (1 mg/L), which was below summer averages for both irrigated and pasture sites: ≥ 6.94 mg/L (Korbel 2012). The greatest nitrate and salinity levels were recorded in the Condamine Catchment: 18.00 mg NO_3^- /L and 20100 µS cm^{-1} (EC) respectively, and although above average, these values fall under recorded maximum values of 220 mg NO_3^- /L, and 32000 µS cm^{-1} (Dafny & Silburn 2014).

Intensive agricultural practices involving irrigation are implicated in increasing salinity and high nitrate concentrations in groundwater of several irrigated catchments in Eastern Australia (Sundaram & Coram 2009). In this study however, and contrary to expectations based on previous surveys (Korbel et al. 2013a; Sundaram & Coram 2009), relatively high nitrate and salinity (EC) values were not strongly associated with groundwater under irrigated landscapes, and mean nitrate concentration was not significantly greater under irrigated agriculture in comparison to pasture.

Following periods of intense summer rainfall, elevated nitrate concentrations have been recorded in groundwater of irrigated catchments in north-eastern Queensland (Rasiah, Armour & Cogle 2005), and elevated dissolved organic carbon levels in groundwater of irrigated catchments in north-west New South Wales (Korbel et al. 2013a). In this study, sampling coincided with the end of the summer season and crop-irrigation activity; however, rainfall in the project areas was less than a tenth that of the seasonal average over the weeks prior to sampling (Table A1: Appendix A), which may partly explain the absence of elevated chemical signals corresponding to land-use, since significant rainfall and irrigation may sometimes be necessary to transport nutrients through the soil in sufficient quantities to be detectable in groundwater.

4.2 Stygofauna data analysis

4.2.1 Stygofauna community structure and land-use

Contrary to the findings of comparable studies there was no significant difference in stygofauna assemblages, or mean faunal abundance between irrigated and pasture sites in either of the project catchments; however, total faunal abundance was greater in groundwater under irrigated landscapes in both catchments, which corresponds with results elsewhere (Korbel et al. 2013a; Korbel, Lim & Hose 2013b).

It is perhaps unsurprising that stygofauna assemblages were not strongly associated with either land-use type, since there is some uncertainty regarding the degree to which land-cover variables, agricultural or otherwise, influence the distributions of stygobitic species (Di Lorenzo & Galassi 2013; Dole-Olivier et al. 2009). Ecological conditions in the subsurface which might be altered by agricultural land-use, such as changes in groundwater chemistry, or lowering of the water-table following groundwater extraction for irrigation purposes, did not appear to influence stygobitic or stygophilic species distributions in an alluvial aquifer in Europe, although faunal abundances decreased in response to groundwater drawdown, presumably through the loss of saturated habitat (Di Lorenzo & Galassi 2013).

The distributions of stygobitic species in the Australian landscape are widely assumed to be underpinned by historical biogeographic events, and dispersal constraints: i.e., the timing and location at which ancestral organisms colonised groundwater via surface waters, and subsequent isolation by vicariance in fragmented subterranean habitats (Abrams et al. 2013; Finston et al. 2007; Humphreys 2008). Alluvial groundwater systems under Eastern Australian river basins are anticipated to be hydrologically fragmented, or discontinuous environments to some extent (Hancock & Boulton 2008). Strong population level genetic structure over relatively short distances indicated that the dispersal of stygobitic fauna was greatly inhibited by subterranean barriers to flow (such as clay bands or solid rock strata), in alluvial aquifers of New South Wales and Queensland respectively (Asmyhr et al. 2014; Cook et al. 2012). Similarly therefore, the distributions of stygobitic species in groundwater of the project catchments may be constrained by historical and physical factors, which have a greater bearing on contemporary species' distributions, than agricultural land-use related effects.

The greater abundance of stygofauna under irrigation observed in this and similar studies (Korbel et al. 2013a; Korbel, Lim & Hose 2013b), hints at a positive association between invertebrate abundance and ecological conditions in groundwater under irrigated landscapes. However, given that stygofauna species' abundances are notoriously variable over space and time (Hancock & Boulton 2009), the observed relationship may be a coincidence of geographic sampling design and faunal distributions, especially in this short study period in which sampling occurred only once.

Opportunities to collect obligate stygofauna are usually limited to the spatial distribution of groundwater bores to sample from. Since bores are often thinly distributed across a landscape, samples are a snapshot of the potential diversity present, and the inherent variability in stygofauna sampling is difficult to account for. Intensive sampling would seem an obvious approach to mitigate this variability (Hancock & Boulton 2009), although sampling effort is of course subject to time and financial resources.

Korbel et al. (2013a) found greater dissolved organic carbon concentration in groundwater under irrigated agriculture in comparison to pasture, and identified positive relationships between organically enriched groundwater, microbial activity, and stygofaunal abundance (Korbel & Hose 2015; Korbel, Lim & Hose 2013b). An increase in organic matter availability in groundwater generally leads to an increase in stygophilic species (Malard et al. 1994). Interestingly, the most abundant groups at irrigated sites in this and other studies were fauna of Oligochaeta and Copepoda (Korbel et al. 2013a), both of which comprise many stygophilic species (Dumnicka 2014; Galassi 2001). Most oligochaetes and several copepod species preferentially inhabit fine grain sediments rich in organic matter (Galassi 2001; Gooderham & Tsyrlin 2002). In terms of stygobitic species, syncarid fauna of Parabathynellidae, and amphipod fauna of Paramelitidae, were also relatively more abundant at irrigated sites. Generally, parabathynellids and paramelitids are assumed to be detritivores, feeding on bacteria and plant matter (Abrams 2012; Boulton et al. 2008). It is difficult however to associate any of the above groups with a specific habitat type, since these fauna are ubiquitous in ecologically diverse groundwater systems worldwide.

Irrespective of uncertainty regarding the nature of the relationship between intensive agriculture and associated groundwater ecosystems (Korbel et al. 2013a), it is conceivable that irrigation may increase the transport of organic carbon into groundwater under irrigated farmland, thus favouring biological communities. In

addition to the influx of plant materials as a direct food source for invertebrates, it follows that microbial populations which form the base of aquifer food-webs, should proliferate in organically enriched groundwater, forming biofilms which are a primary source of energy for several groups of stygofauna (Di Lorenzo & Galassi 2013). Furthermore, in irrigated catchments in which water for irrigation is drawn from rivers (as opposed to aquifers), groundwater levels may rise (Silburn et al. 2004); thus, stygofaunal populations may benefit from water levels kept artificially high.

4.2.2 Stygofauna community structure and groundwater chemistry

Patterns of correspondence between stygofauna community structure and variation in water quality variables were weak overall, which is consistent with the findings of several landscape and catchment scale studies (Dole-Olivier et al. 2009; Halse et al. 2014; Korbel & Hose 2015). Nitrate concentration was the only significant variable in the DistLM analysis, and accordingly, the resultant dbRDA plot indicates a negative relationship between stygofaunal assemblages and increasing nitrate concentration in the Condamine Catchment (Figure 3.3).

Interestingly, stygofauna species richness and abundance decreased with increasing nitrate concentration (range: ~ 1 to 6 mg/L) in the Gwydir River alluvial aquifer (Menció, Korbel & Hose 2014). Considerably higher nitrate concentrations however (≥ 50 mg/L), and a large degree of between site variation, appeared to have no discernible effect on stygofauna distributions in a comparable study (Di Lorenzo & Galassi 2013). It is characteristic of the relationship between stygofauna distributions and aspects of groundwater chemistry, that the findings of one study appear to contradict those of another (Di Lorenzo & Galassi 2013).

In general, long term exposure to nitrate concentrations greater than 10 mg/L is thought to adversely affect the health of epigean freshwater invertebrates (Camargo, Alonso & Salamanca 2005); thus, it seems unlikely that stygofauna are unaffected physiologically by high nitrate concentrations. Nitrate toxicity may decrease with increasing body size (Camargo, Alonso & Salamanca 2005), which suggests some stygofauna might be particularly sensitive, given that the majority (i.e., the interstitial micro-crustacean fauna) are generally smaller bodied animals in comparison to their surface water relatives (Hancock, Boulton & Humphreys 2005).

Many stygobitic species are highly specialised animals with long evolutionary histories in the Australian subsurface (Hancock, Boulton & Humphreys 2005). The high level of

physiological adaptation and narrow range paradigm which applies to many stygofauna (Humphreys 2008), may partly explain the absence of definitive relationships between their distributions in the landscape and water quality gradients; since geographically isolated populations may be adapted specifically to local physicochemical conditions, such as marine level salinities for example (Humphreys 2006; Schulz, Steward & Prior 2013), which adjacent populations may find unfavourable or intolerable.

Adaptation is rarely mentioned in studies which attempt to link patterns of stygofauna distributions to environmental gradients. The physico-chemical preferences and tolerance levels of Australian stygofauna probably vary amongst taxa as well as geographically. Thus, empirical studies to test the responses of different stygofauna to varying conditions may be necessary to develop regional, or aquifer specific guidelines.

4.3 Microbial community data analysis

4.3.1 Microbial community structure and land-use

Microbial assemblages did not differ significantly between irrigated and pasture sites within either catchment. Irrespective of this result however, it is anticipated that the effects of agricultural land-use on groundwater quality, will have some quantifiable bearing on microbial community structure which was not detected here. Changes in groundwater salinity and nutrient levels are firmly linked to irrigated agriculture (Korbel et al. 2013a, Sundaram & Coram 2009). Korbel et al (2013a) found differences in the organic carbon and nutrient status of groundwater under irrigation compared to pasture in a similar study. Given then, that microbial distributions in freshwater sediments are sensitive to nutrient and salinity gradients, and organic content (Gibbons et al. 2014; Hug et al. 2015; Sirisena et al. 2013; Smith et al. 2012), suggests that relationships between microbial diversity in groundwater and agricultural land-use are likely.

Within a catchment, groundwater generally flows from areas of higher to lower elevation, in much the same way as surface-water flows, albeit much slower (Queensland Water Commission 2012). Given that groundwater within the project areas is flowing at a rate of up to thirty meters a day (see section 2.1), it is possible that the passive dispersal of microbial cells between irrigated and pasture sites may have confounded land-use affiliated taxonomic signals. Within the Condamine Catchment for example, although some irrigated and pasture sites were separated by more than 100 kilometres, others were separated by less than 15 kilometres, and the distinction was

less in the Gwydir Catchment (Figure 2.1); which as outlined in the introduction, was a consequence of difficulties in locating groundwater bores in appropriate landscapes.

Differences between benthic microeukaryote assemblages have been identified through the analysis of presence or absence amplicon data (Chariton et al. 2015). However, given that in nutrient poor conditions, such as occur in groundwater, up to 40% of bacteria may exist in reversible state of low metabolic activity or dormancy (Jones & Lennon 2010), it may be difficult to identify meaningful differences amongst prokaryote assemblages, without an estimate of the relative abundance of the most numerically dominant, and therefore ecologically relevant taxa. If dormant diversity were a confounding factor, then a PCR free metagenomic sequencing approach might reveal patterns matching microbial abundance to ecological conditions under farmland (Smith et al. 2012), that did not emerge from the analysis of presence or absence data here.

Microbial cellulose-degrading activity did not differ significantly in groundwater under irrigated agriculture in comparison to pasture. The Cotton Strip Assay, which was employed here to assess relative cellulolytic activity, has yielded mixed results in similar studies (Korbel et al. 2013a; Korbel & Hose 2011); therefore, the absence of a clear relationship between activity and land-use is difficult to interpret in this instance.

4.3.2 Microbial biogeography

A few OTUs appeared to be widespread within each catchment whilst the majority were comparatively rare, which reflects the seemingly universal pattern observed in microbial biogeography (Nemergut et al. 2011). Unexpectedly, eighty six percent of OTUs occurred in at least one assemblage of both catchments, which implies that although most OTUs may be relatively rare on the sampling scales here, they are widely dispersed at the regional scale. Similar to the findings of comparable studies in which a core group of organisms persist in freshwater sediments on scales of tens of meters, to over a hundred kilometres (Gibbons et al. 2014; Hug et al. 2015), a small fraction of OTUs were present at the nearly all sites in both catchments. That this group co-occur at sites as far apart as 325 kilometres, seemingly irrespective of environmental gradients, suggests that ecological conditions are sufficiently similar in alluvial groundwater systems across the region, to suit the physiology of these organisms.

The finding that a large proportion of OTUs occur in at least one assemblage of both catchments might be considered unusual, since overlap in the composition of

assemblages sampled from the same type of habitat is usually low at fine-level taxonomic resolution; that is, at 97 % sequence identity in 16S rDNA, the majority of OTUs are not expected to occur in more than one assemblage (Nemergut et al. 2011). Interestingly, variation of 63 % amongst microbial assemblages within the Gwydir Catchment, and 73 % within the Condamine Catchment, is lower than the variation of 83 - 95 % found within several comparable datasets including freshwater sediment and terrestrial soil communities (Nemergut et al. 2011), which suggests there may be marginally less community heterogeneity in alluvial groundwater in comparison to other environments, perhaps because stable ecological conditions in aquifers (Gibert et al. 1994), engender relatively stable communities.

Consistent with the distance decay of community similarity paradigm which applies in general patterns of macro-organism biogeography (Nekola & White 1999), microbial assemblages became more dissimilar with increasing geographic distance. The change in community structure over distance appears to be gradual however (Figure 3.4), because as outlined above, considerable diversity reoccurs across space.

Microbial assemblages were significantly different between catchments; however, the distinction between samples appears to be minimal, and the pivotal difference might be attributed to a small number of Condamine Catchment samples which segregate from the majority in the nMDS ordination (Figure 3.5). The ordination suggests that these few samples are distinctly different in taxonomic composition to the majority, and each other, and yet they were collected at sites which were not distant from areas where most other samples were collected, which hints at unsampled diversity.

4.3.2.1 Assessment of microbial distributions

Microbial distributions of each catchment were similar in identity and proportion of the most dominant phyla; for example, Acidobacteria at 15 and 16.5 %, and Firmicutes at 7.5 and 8.5 %, respectively. At the level of phylum, the only notable difference between this and a comparable molecular analysis of soil communities on agricultural land in New South Wales (Nielsen et al. 2014) is the inclusion of archaeal Thaumarchaeota amongst the dominant phyla here. Several of the more consistent taxa at ordinal level, including Actinomycetales and Rubrobacterales within the Actinobacteria, and Rhizobiales and Burkholderiales within the Alpha and Beta-Proteobacteria respectively, play major roles in organic carbon cycling in soils (Fierer, Bradford & Jackson 2007; Geddes & Oresnik 2014; Nielsen et al. 2014).

The most widespread genera were diverse, and few were consistently represented except for Nitrospira, Nitrosopumilus and Nitrososphaera, and to a lesser extent several within the Acidobacteria (with acknowledgment that generic level taxonomy is absent for some OTUs). Members of the genus Nitrospira are nitrifying bacteria, whilst both Nitrosopumilus and Nitrososphaera are ammonia-oxidising archaea, and common to oligotrophic freshwater systems and terrestrial soils respectively (Bollmann, Bullerjahn & McKay 2014). The prevalence of the aforementioned genera indicates that nitrogen cycling is a major process performed amongst the microbial assemblages sampled in this study. Furthermore, Nitrososphaera are strongly indicative of soils modified with agricultural fertilizer (Zhalnina et al. 2013), which is interesting given that aquifers underlying intensively farmed landscapes were targeted here.

The comparisons above are drawn almost exclusively from literature focused on microbial processes in terrestrial soils; however, this reflects the limited amount of comparable research in groundwater to date (Sirisena et al. 2013), and need not necessarily imply the homogeneity of groundwater and soil communities. Given that in comparison to soils, groundwater is limited in the availability of labile organic material and nutrients, microbial communities might be expected to differ (Griebler & Lueders 2009). A compositional distinction between soil and groundwater microbes is difficult to decipher from the data here, and the degree to which populations within these two habitat types may differ, might best be investigated using a phylogenetic approach.

4.3.3 Patterns of diversity and environmental gradients

The relative effects of environmental factors which are likely to contribute to spatial variation in microbial diversity are notoriously difficult to disentangle (Gibbons et al. 2014; Martiny et al. 2006). However, the correlation of environmental and microbial community variation is observed across space; for example, salinity was identified as the major driver of community composition in a comparative analysis of several aquatic and terrestrial habitat types (Lozupone & Knight 2007). In terrestrial soils specifically, variation in community structure and pH correlate at the site and on continental scales (Lauber et al. 2009; Nielsen et al. 2014).

Gibbons et al. (2014) found a strong correlation between microbial distributions and salinity gradients, in addition to geographic distance along benthic river sediments. Similarly, distance and compositional change were positively correlated here, although EC (salinity) and assemblage composition were not, despite that EC was identified as a

significant variable in the Gwydir DistLM analysis. In their study, Gibbons et al. (2014) measured salinity from the surface waters of a continuous river channel, and found a response in microbes within a relatively narrow range of EC (414 - 1013 µS cm^{-1}). Here, the range in salinity was much greater in comparison. Salinity ranged between EC 267 - 5245 µS cm^{-1} in Gwydir Catchment groundwaters, and between EC 479 - 20100 µS cm^{-1} in Condamine Catchment groundwaters. Interestingly, the greatest variation in salinity occurred amongst Condamine samples (Appendix B), and unlike the Gwydir results, EC was not a significant variable in the Condamine DistLM analysis.

In the complex physical environment below ground, where transmissivity is relatively low, salts presumably accumulate in pockets of static or slow-moving groundwater, which may elevate salinity in certain areas and increase the degree to which salinity varies between sampling sites. The broad range of salinity values here, coupled with a relatively large degree of between site variation, may explain why a strong correlation between salinity gradients and microbial distributions failed to materialise.

Strong patterns of correspondence between microbial and environmental heterogeneity have emerged on scales of tens, to hundreds of metres in terrestrial and groundwater sediments (Horner-Devine et al. 2004; Hug et al. 2015). Given the considerable diversity and phenotypic (metabolic) versatility observed in microbial populations (Comte & Del Giorgio 2011), sampling over a smaller area at sites with a high density bore network, may recover relationships which were obscured in this relatively broad spatial scale study.

5 Conclusion

This study is one of the first in Australia to characterise microbial diversity in alluvial aquifers using high-throughput 16S rDNA sequence data. In addition to the Bacteria, Archaea were diverse and amongst the most dominant taxa, which was unexpected, since Archaea are generally less well represented in terrestrial samples. Diverse stygofauna communities were recovered from Gwydir Catchment groundwaters, and also at locations within the Condamine Catchment from which stygofauna were previously unrecorded.

The findings of this study did not support the hypothesis that stygofauna distributions vary with agricultural land-use. Stygofauna community structure did not differ between irrigated and pasture land-uses within either catchment. Molecular evidence within the

recent literature, supports the opinion that the ability of stygofauna to disperse in alluvial aquifers is largely inhibited by geological constraints. Therefore, the influence of agriculture on stygofauna distributions is likely to be limited.

Stygofauna assemblage composition and groundwater quality gradients were poorly correlated. The high level of physiological adaptation and narrow range paradigm which applies to many stygofauna, may partly explain the absence of definitive relationships between their distributions and water quality gradients; since geographically isolated populations may be adapted specifically to local physicochemical conditions, which adjacent populations may find unfavourable or intolerable.

Molecular data not support a relationship between microbial distributions in groundwater and agricultural land-use, or water chemistry gradients; however, the findings of comparable studies suggest that such relationships are likely. Microbial assemblages did not differ between irrigated and pasture land-uses in either catchment. This finding might be attributed to the passive dispersal of microbial cells in groundwater flows between sampling locations, or limitations of this analysis based on the presence or absence of OTU data. To assess the hypothesis that microbial communities differ with agricultural land-use, further studies should use a metagenomic sequencing approach to estimate the relative abundance of microbes between sites.

Microbial distributions and groundwater quality gradients were poorly correlated. Given the considerable diversity and phenotypic versatility present in microbial populations, sampling over a smaller area at sites with a high density bore network, may elucidate relationships which were obscured in this relatively broad spatial scale study.

Consistent with the distance decay of community similarity paradigm which applies in general patterns of macro-organism biogeography, microbial assemblages became more dissimilar with increasing geographic distance. The change in composition over distance is gradual however, because considerable diversity reoccurs across space.

In summary, the influence of agricultural land-use on stygofauna community structure is likely to be limited, since their distributions within the landscape are largely defined by physical limitations of the alluvial aquifer environment. In contrast, the influence of agricultural land-use on groundwater quality is likely to have a quantifiable bearing on microbial distributions, although evidence to support this hypothesis was not forthcoming from the environmental and molecular analysis here.

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Appendix A

Table A1 BOM rainfall data for the project areas over the period prior to and including sampling: Condamine Catchment: Feb 16 - 20, and Gwydir Catchment: Feb 20 - 23

Day of Month	Town : Toowoomba (Condamine Catchment) Daily Total (mm)	Town : Moree (Gwydir Catchment) Daily Total (mm)
January 29	0.2	0
30	0	0
31	0	0
February 1	0	0
2	0	0
3	0	3.2
4	0.6	0
5	0.2	0
6	0.2	0
7	0	0
8	0	0
9	0	0
10	0	0
11	1.2	0
12	0	0
13	0	0
14	0.2	0
15	0	0
16	0	0.2
17	0	0
18	0	0
19	2.2	0
20	23.8	0
21	8.6	0
22	0.8	1.0
23	1.0	0
Historical versus 2015 mean rainfall for February in the project areas		
Toowoomba	Historical Mean	107.4 mm
	2015 Mean	39 mm
Moree	Historical Mean	73.6 mm
	2015 Mean	4.4 mm

Appendix B

Table B1 Mean and range of water quality parameters for the Condamine and Gwydir Catchments. Analyte abbreviations are explained in Methods: section 2.6; n = number of samples per catchment, SE = standard error

	Condamine Catchment			Gwydir Catchment		
Parameter	n	Mean + SE	range	n	Mean + SE	range
NO_3^- (mg/L)	17	2.11 ± 1.08	0.01 – 18.00	14	2.11 ± 0.8	0.21 - 11.00
Total P (mg/L)		0.62 ± 0.18	0.01 – 3.30		0.45 ± 0	0.20 - 1.5
EC (µS cm^{-1})		2360 ± 1118	479 - 20100		1041 ± 78	267 - 5245
HCO3- (mg/L)		468 ± 47	220 - 1050		330 ± 51	150 - 725
pH		7.4 ± 0.06	6.8 - 8.1		7.2 ± 0	6.8 - 8
DO (mg/L)		4.77 ± 0.48	0.21 - 9.44		5.83 ± 0.53	0.62 - 7.89
temperature (°C),		21.48 ± 0.17	20.44 – 22.69		22.76 ± 0.26	21.12 – 24.20
Ca^{++} (mg/L)		99 ± 29	35 - 555		125 ± 78	13 - 1130
DOC (mg/L)		1.17 ± 0.09	1 - 2	13	1 ± 0.27	1 - 3

Appendix C

Table C1 Stygofauna taxonomic identity and abundance per catchment and land-use
n = number of samples

Phylum or Class	Order	Family	**Gwydir Irrigated site abundance**	Proportion of samples taxa were present	**Gwydir pasture site abundance**	Proportion of samples taxa were present
				n = 9		n = 5
Nematoda			34	44 %	10	80 %
	Syncarida	Parabathynellidae	33	44 %	63	80 %
	Syncarida	Bathynellidae	-	-	6	20%
Copepoda	Cyclopoida		150	22 %	21	60 %
Copepoda	Harpacticoida		11	22 %	25	80 %
Oligochaeta			37	44 %	3	60 %
Ostracoda		Candonidae	5	11%	-	**-**
	Amphipoda	Paramelitidae	44	22 %	-	**-**
	Amphipoda	Melitidae	1	11 %	-	**-**
Acarina sp.1			27	66%	162	80 %
			Total: 342		Total: 290	
Phylum or Class	Order	Family	**Condamine Irrigated site abundance**	Proportion of samples taxa were present	**Condamine Pasture site abundance**	Proportion of samples taxa were present
				n = 9		n = 8
Nematoda			9	33 %	-	-
	Syncarida	Parabathynellidae	19	22 %	2	12 %
	Syncarida	Bathynellidae	11	33 %	21	12 %
	Syncarida	Anaspidacea	2	22 %	1	12 %
Copepoda	Cyclopoida		7	33 %	-	-
Copepoda	Harpacticoida		6	22 %	3	25 %
Oligochaeta			23	55 %	10	12 %
Ostracoda		Candonidae	2	11 %	1	12 %
Acarina sp.2			2	11 %	2	12 %
			Total: 81		Total: 40	