METAGENOMIC INVESTIGATIONS ON THE EFFECTS OF URANIUM AND COPPER ON SEDIMENT MICROBIAL COMMUNITIES

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This thesis is presented as a partial fulfilment to the requirements for a Doctorate of Philosophy

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STATEMENT OF ORIGINALITY

This work has not previously been submitted for a degree or diploma in any university. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made in the thesis itself.

Date: 20/11/2017

Candidate's name

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PUBLICATIONS

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- **Sutcliffe, B.**, Chariton, A.A., Harford, A.J., Hose, G.C., Stephenson, S., Greenfield, P., Midgley, D.J. and Paulsen, I.T., 2018. Insights into the genomes of microbes thriving in uranium-enriched sediments. *Microbial Ecology*. 75(4): 970-984
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CONFERENCE PROCEEDINGS

Oral Presentations

- Presentation to the Tiedje Lab at Michigan State University 2016
- Presentation at the Joint Academic Microbiology Seminars in 2014
- Presentation at the Centre for Biodiversity Analysis conference in 2014

Posters

- Poster presentation at International Society for Microbial Ecology (ISME) conference in 2016
- Poster presentation at the Joint Academic Microbiology Seminar 2015

ACHIEVEMENTS

- Awarded the ISME Student Poster prize at International Society for Microbial Ecology (ISME) conference in 2016

- Awarded the Postgraduate Research Fund for conference travel from Macquarie University, Australia
- Awarded the CSIRO Oceans and Atmosphere Office of the Chief Executive (OCE) top-up scholarship for my postgraduate studies
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CONTRIBUTIONS

CHAPTER 2: EFFECTS OF URANIUM CONCENTRATION ON MICROBIAL COMMUNITY STRUCTURE AND FUNCTIONAL POTENTIAL.

Work was conceptualised by Chariton, Harford, Hose, Midgley, Paulsen and Sutcliffe. Mesocosms setup and sampling was carried out by Harford, with the help of colleagues Claire Costello, Kim Cheng and Chris Humphrey. Stephenson performed nucleic acid extractions, Sutcliffe performed nucleic acid clean-ups and prepared amplicon libraries. All sequencing was done by the Ramaciotti Sequencing Centre. All data analysis was performed by Sutcliffe, except that Greenfield ran the USEARCH pipeline and Elbourne ran the Diamond pipeline. Oytam and Hose provided guidance and assistance to Sutcliffe in performing statistical analyses. The manuscript was written by Sutcliffe with contributions from all other authors.

CHAPTER 3: INSIGHTS FROM THE GENOMES OF MICROBES THRIVING IN URANIUM-ENRICHED SEDIMENTS

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CHAPTER 4: DIVERSE FUNGAL LINEAGES IN SUBTROPICAL PONDS ARE ALTERED BY SEDIMENT-BOUND COPPER

Work was conceptualised by Chariton, Harford, Hose, Midgley, Paulsen and Sutcliffe. Pond sampling was performed by Sutcliffe and Midgely with the help of colleagues Nai Tran-Dinh and Matthew Serrett. Chemical analyses was performed by John Gouzos from the CSIRO Environmental Services branch. Sutcliffe performed all laboratory work, except that amplicon sequencing was performed at the Ramaciotti Centre. Manuscript was prepared by Sutcliffe and Midgely, with contributions by Harford and Hose.

CHAPTER 5: MICROBIAL COMMUNITIES ARE SENSITIVE INDICATORS FOR FRESHWATER SEDIMENT COPPER CONTAMINATION

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ABSTRACT

Freshwater systems are a reservoir of biodiversity and a hot spot for the biogeochemical processes underpinning global nutrient cycles. Microbial communities are a key component of these systems, forming the base of food webs and providing ecological services on which all higher trophic levels depend. Freshwater sediment microbial communities are particularly vulnerable to exposure to anthropogenic pollutants. This is due, in part, to the binding of pollutants to sediment particulates, and the intimate associations microorganisms form with sediments. To date relatively few studies have investigated the effect of metal contamination on freshwater sediment microbial communities and their function.

This work explores the effect of uranium (U) and copper (Cu) on freshwater sediment microbial communities. Concentration gradients of each metal were created by spiking the sediments in the laboratory before deploying them *in situ*. This novel approach allowed sediments with known metal concentration gradients to be exposed to natural environmental fluctuations and biotic colonisation. Metagenomic sequencing techniques were then employed to investigate compositional shifts in microbial communities along these gradients.

This work represents the first metagenomic data of sediment microbial communities along U and Cu concentration gradients in natural settings. The study demonstrates that these communities are responsive to elevated concentrations of U and Cu, and that taxonomic shifts cause functional changes. The study identifies taxa which are sensitive to elevated metal concentrations, as well as those which appear to be specialists in these environments. The ecophysiological profiles of six novel genomes, obtained from the metagenome of sediments spiked with 4 g kg^{-1} of uranium, were used to explore the life-strategies of taxa thriving under these challenging conditions. Additionally, the profiling of sediment fungal communities represents the first metagenomic survey of these taxa in freshwater sediments, and demonstrated considerable taxonomic novelty.

The findings of this study provide insights into sediment microbial communities, their complexity and the structure-function relationships underpinning this complexity. Along with their contribution to our understanding of microbial ecology, findings from this study have broad ecotoxicological implications for the monitoring of contaminated freshwater environments, and are of relevance to ecotoxicologists, regulatory bodies and policy makers.

ABBREVIATIONS

°C	degrees celcius
µg	micrograms
µm	micrometer
16Sr RNA	16S ribosomal RNA gene
ABC	ATP-binding cassestte
AEM	acid extractable metal
ANOVA	analysis of variance
BIOM	biological observation matrix
CAZY	carbohydrate-active enzymes
CDF	cation diffusion facilitators
CLLP	community-level physiological profiles
cm	centimeters
CSIRO	Commonwealth Scientific and Industrial Research Organisation
Cu	Copper
dbCAN	dataBase for automated carbohydrate-active enzyme annotation
dbRDA	distance based redundancy analysis
DDBJ/ENA	DNA data bank of Japan/ European nucleotide archive
DISTLM	distance based linear analysis
DNA	deoxyribonucleic acid
DOC	Dissolved organic carbon
EBI	European Bioinformatics Institute
EC_{50}	half maximal effective concentration
FDR	False discovery rate
g	grams
GH	glycoside hydrolases
h	hours
HME	heavy metal efflux
HUMAnN	The HMP unified metabolic analysis network
ICP-AES	inductively coupled plasma atomic emission spectroscopy
ITS1	internatal transcribed spacer 1
kg	kilograms
km	kilometers
KNP	Kakadu national park
L	litres
m	meters
MDS	multidimensional scaling
MFP	membrane fusion protein family
MFS	major facilitator subfamily
mg	milligrams
mm	millimeter
NCBI	National Center for Biotechnology Information
ng	nanograms
NPOC	Non-purgable organic carbon
NPON	Non-purgable organic nitrogen
NR	non-redundant

OMF	outer membrane factors
OTU	operational taxonomic unit
PCA	principle components analysis
PCO	principle co-ordinates analysis
PCR	polymerase chain reaction
PERMANOVA	permutational analysis of variance
ppm	parts per million
PPQ	pyrroloquinoline quinone
PTS	phosphotransferase system
QC	quality control
QIIME	Quantitative Insights Into Microbial Ecology
RDP	Ribosomal Database Project
RNA	ribonucleic acid
RND	resistance-nodulation-cell division
SEM	scanning electron microscopy
SSS	sodium-solute symporter
TOC	total organic carbon
TOC	total organic carbon
TON	total organic nitrogen
TRM	total recoverable metal
U	uranium
UNESCO	United Nations Educational, Scientific and Cultural Organization

1 INTRODUCTION

1.1 FRESHWATER ENVIRONMENTS

Freshwater environments account for just 0.8% of the Earth's surface but support a disproportionate 6% of the world's biodiversity (Dudgeon et al., 2006; Strayer and Dudgeon, 2010). In addition to this rivers, lakes and streams support surrounding biomes, supplying a source of freshwater and food for many non-aquatic animal species. Key exchanges between freshwater environments, and their surrounding terrestrial environments, include adult aquatic insects, which emerge from freshwater systems to enter terrestrial food webs. In one study, this invertebrate export was found to equate to 22.4 g m^{-2} of insect biomass annually (Ward, 1989). Flood regimes, and the resulting deposition of waterborne nutrients onto floodplains, also create bioactivity 'hotspots', which contribute to global biogeochemical processes (McClain et al., 2003) and shape plant communities in the surrounding area (Ward, 1989).

As a biome in and of itself, conservative estimates indicate that some 40% of fish species and almost 33% of invertebrates are supported by freshwater aquatic environments for at least part of their life cycle (Dudgeon et al., 2006; Strayer and Dudgeon, 2010). When also including freshwater mammals, reptiles and amphibians, the total number of non-microbial freshwater species is approximately 100,000 (Strayer and Dudgeon, 2010). It is noteworthy, however, that freshwater systems are less studied than their terrestrial counterparts (Strayer and Dudgeon, 2010), and as such, these figures are likely to be underestimates.

Several factors are expected to contribute to the high biodiversity of freshwater ecosystems. One factor is dispersal limitation. In discrete, isolated freshwater systems, certain biota are unable to colonise other environments, resulting in independent speciation. This is evidenced by, for example, the high rates of endemism in freshwater fish (Fryer, 1977; Meyer, 1993; Tedesco et al., 2012). For

other biota, for example; numerous macroinvertebrates, zooplankton and microorganisms, metacommunity studies demonstrate that species sorting (i.e. environmental control) is more influential than dispersal limitations (Heino et al., 2015). Thus, high biodiversity rates are, in part, a result of the high heterogeneity of freshwater environments (Sigee, 2005).

1.1.1 Lentic and lotic environments

Freshwater ecosystems vary in their presence or absence of water movement, with this single feature driving numerous physicochemical features within the system (Marsh and Fairbridge, 1999; Sigee, 2005). Broadly, these systems are classified as lentic, for still water bodies without water movement, and lotic, for those with water movement and turbulence of some kind. Examples of lentic systems include lakes, swamps and marshes, while examples of lotic systems are rivers, canals and streams.

A general feature of lentic systems is seasonal stratification of the water column. Without water movement, the heat transferred to the surface of lentic systems via sunlight is not readily transferred vertically. In warmer seasons, this results in two distinct strata with markedly different temperatures (Marsh and Fairbridge, 1999; Sigee, 2005). These layers are called the epilimnion (upper layer) and hypolimnion (lower layer) and are separated from each other by a third layer called the thermocline, which is characterised by a sharp temperature decline (Marsh and Fairbridge, 1999; Sigee, 2005). This temperature shift prevents the mixing of the two flanking layers by creating distinctly different density differentials for the warmer water above and cooler water below (Marsh and Fairbridge, 1999).

In receiving the greatest amount of light, the epilimnion supports a highly productive photosynthetic microbial community, which contributes oxygen to the layer while removing nutrients (Shade et al., 2008; Sigee, 2005). In lentic systems, primary producers (photosynthetic organisms) typically exhibit seasonal blooms in the epilimnion (Sigee, 2005). These microorganisms form the base of food webs,

with herbivores feeding on their biomass while detritus feeders consume the senescent organisms (Marsh and Fairbridge, 1999; Sigee, 2005). These blooming events drive physicochemical changes in the layers below, for example, the bloom biomass further obscures light from the hypolimnion, limiting the amount of photosynthesis which can be carried out in this layer. Additionally, the increased respiration in the upper layers, by biota feeding on the bloom biomass, along with a loss in oxygen generation through photosynthesis, results in depleted oxygen concentrations in the hypolimnion. In some instances, this can result in anoxia (Finlay, 1985; Shade et al., 2008). In contrast, nutrients such as nitrogen and phosphorus are more abundant in the hypolimnion, due to organic matter deposition from the overlying waters (Shade et al., 2008) and under anoxia, anaerobic metabolism results in the enrichment of reduced chemical species, for example, nitrite and sulphides.

In the presence of water movement, lotic systems have negligible stratification, with heat energy and oxygen disseminated throughout the water column. Water turbulence results in increased sediment suspension and a process called sediment scour which causes the abrasion of biota with sediment particles (Francoeur and Biggs, 2006; Marsh and Fairbridge, 1999). Sediment scour, along with the displacement of microorganisms in the water column downstream, selects against photosynthetic planktonic communities and instead favours photosynthetic organisms which attach to surfaces, forming mats or biofilms on the sediment aggregates or rock surfaces (Francoeur and Biggs, 2006; Marsh and Fairbridge, 1999; Sigee, 2005). Generally, these photosynthetic communities are less productive than those in lentic systems and thus represent a far smaller proportion of the organic matter inputs to lotic systems. Instead, organic matter in lotic systems largely originates from external sources such as leaf fall from trees growing on the banks of the water body (Marsh and Fairbridge, 1999; Sigee, 2005). Benthic heterotrophic microbes, which are able to transform these inputs into microbial biomass, therefore represent the base of food webs in lotic systems (Marsh and Fairbridge, 1999; Sigee, 2005).

An important caveat in describing the general physicochemical and ecological features of these two freshwater types, is that many systems fall somewhere in between these two categories. For example, the river continuum describes the transition of large rivers from classical lotic systems to lentic-like systems, whereby water movement slows and the river broadens as it matures (Marsh and Fairbridge, 1999; Vannote et al., 1980). This results in less turbulence, and more sun-light reaching the mature river, with a subsequent increase in photosynthetic organisms and a food-web based on this internally derived, living biomass production. This exception includes water bodies such as mature floodplains, or billabongs, where semi-stagnant pools and swamps can occur within a wider flooded context (Figure 1.1; Marsh & Fairbridge 1999).

Figure 1.1. A billabong, formed during the wet season, in the Alligator Rivers region of the Northern Territory of Australia. This system results from flooding in the region and demonstrates both lentic and lotic environmental characteristics. Image taken by the Supervising Scientist Branch (2011).

1.1.2 Freshwater habitat heterogeneity

A single freshwater system contains multiple habitats. These habitats are defined by variations in environmental features across the system, and while the environmental features may depend on

whether the system is lotic or lentic (see section 1.1.1), these variations result in niche habitats and heterogeneous biotic distributions for both types of freshwater systems. For example, the metabolic oxygen demands of different decapods determines the distribution limitations of these species (Dalosto and Santos, 2011), while distances between a given habitat and a systems' shore and/ or vegetative cover can shape fish distributions (Greenberg et al., 1996). Other environmental variables include water depth, current velocities and sediment substrates (Yozzo & Smith 1995; George & Hadley 1979; Werner et al. 1977).

As with macroorganisms, microorganisms are also heterogeneously distributed within a single freshwater system (Figure 1.2) (Beier et al., 2008; Buesing et al., 2009). Three distinct biomes are known to harbour distinct microbial communities; the water column (pelagic), sediment (benthic) and surfaces (for example, plant or rock surfaces). These biomes have strong selective pressures, with microbial communities from the same biome, but from different streams, more similar than those from different biomes but the same stream (Beier et al., 2008; Besemer et al., 2012).

1.2 MICROBIAL COMMUNITIES IN FRESHWATER ENVIRONMENTS

Microorganisms include a diverse range of taxa, spanning all three domains of life: Eubacteria, Archaea and Eukaryotes. Described as the 'unseen majority' these organisms are incredibly abundant, with studies reporting 10^8 - 10^{10} cells L^{-1} in lentic and lotic environments (Hobbie et al., 1977; Meyer et al., 1987; Sakamoto et al., 2005). Additionally, microorganisms are able to colonise challenging environments which are otherwise unconducive to life. For example, diverse microbial populations have been observed in hot spring sediments at 93°C (Hugenholtz et al., 1998) and in acidic, metal contaminated sediments with a pH of ~2 (Aguilera et al., 2006). Thus, microbial communities are abundant and ubiquitous components of all freshwater ecosystems.

The life-strategies employed by microorganisms are diverse and include primary producers, predators, saprobes, parasites and symbionts (Guerrero et al., 1986). In addition to these life-

strategies, which are largely analogues to those observed in macroorganisms, prokaryotic microorganisms possess a much greater breadth of metabolic diversity (Nealson, 1997). For example, while all macroorganisms use oxygen as an electron acceptor in respiration, numerous prokaryotes are able to respire anaerobically, employing a range of elements and compounds as electron donors (for example, metal ions, inorganic nitrogen and carbon molecules) (Nealson 1997). These reactions, along with other microbially-mediated metabolic reactions, drive chemical transformations that underpin global biogeochemical cycles (Falkowski et al., 2008). While microbial prokaryotes play critical roles in biogeochemical cycling, microbial eukaryotes undertake a host of other important ecosystem services. For example, fungi, play a major role in lignin degradation, form mycorrhizal associations with plants, and translocate nutrients over larger spatial scales than those possible by bacteria (Cooke and Rayner, 1984).

1.2.1 Spatial heterogeneity of microbes in freshwater biomes

1.2.1.1 Pelagic environment

As described in section 1.1.1, stratification in the water column of lentic freshwater systems during warmer months creates distinctly different environmental conditions within the pelagic environment. Namely, light, heat, nutrients, oxygen and reduced chemicals are distributed unevenly within the column, forming distinct layers (Marsh and Fairbridge, 1999; Shade et al., 2008). Numerous studies comparing prokaryotic communities from the epilimnion and hypolimnion layers of lakes have demonstrated these communities are distinctly different (Linz et al., 2017; Morrison et al., 2017; Okazaki and Nakano, 2016; Shade et al., 2008). Generally, putative photosynthetic taxa are highly abundant in the epilimnion, while Chloroflexi are abundant in the hypolimnion (Linz et al., 2017; Morrison et al., 2017; Okazaki and Nakano, 2016).

In addition to this, heterogeneity exists within strata, for example, light gradients occur vertically within the epilimnion, thereby structuring photosynthetic microorganisms by their light optima. This

stratification allows multiple primary producers to co-exist in separate spatial niches, defined by light exposure (Sigee, 2005; Xiao et al., 2017). An example of this can be seen in the co-existence of the buoyant high-light cyanobacteria, *Microcystis aeruginosa,* and the neutrally buoyant, low-light *Cylindrospermopsis raciborskii* (Xiao et al., 2017). The specialisation of these two species allows *M. aeruginosa* to form surface "scum" while *C. raciborskii* forms subsurface blooms.

More broadly, a plethora of heterogeneity exists at the microscale within all pelagic environments, across all seasons, and in both lentic and lotic systems. Specifically, a number of microscale events happen in rapid succession within the environment (Azam and Malfatti, 2007; Stocker, 2015). For example, zooplankton densities in freshwater systems range from 100 - 22,400 individuals L^{-1} (Pace, 1986; Reckendorfer et al., 1999), and the release of waste by these individuals results in a sudden enrichment of nutrients and organic matter of an area in the order of 100 μm^{-3} around these individuals (Figure 1.2) (Stocker 2015). These materials then rapidly disseminate into the surrounding environment. Ultimately, this process represents a substantial nutrient input for microorganisms, creating a patchwork of nutrient gradients. In order for an organism to exploit this sudden increase in heterogeneous resource availability, it must respond rapidly to such events. This has been observed in freshwater environments where the release of biotic excretions has resulted in a 5.9-fold increase in cell abundance, and the enrichment of substrate-specific microbial taxa (Dann et al., 2016).

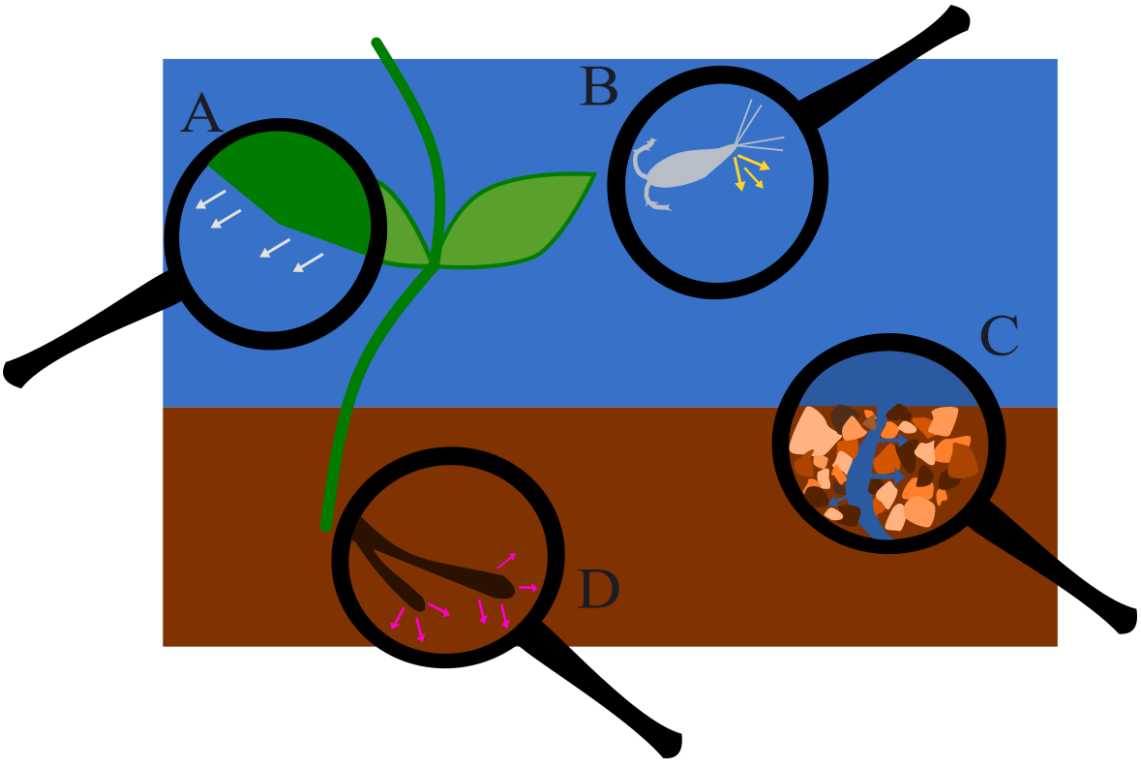

Figure 1.2. Representation of microgradients and nutrient hotspots in freshwater environments. Coloured arrows indicate gradients from the source into the surrounding environment. A) represents a leaf surface, from which molecules such as oxygen evolve and can dissolve outward into surrounding water. B) represents the excretion of organic material, such as urea, from multicellular eukaryotes. In C) a vertical pore within the sediment matrix is represented, from this, horizontal gradients of water, and molecules dissolved within the water, disseminate into the sediment matrix. D) represents plant roots, from which organic molecules are excreted and deposited into the surrounding sediment.

In addition to biotic excretions, numerous other microscale events may lead to heterogeneous nutrient releases. Such events may include lysis events and leaky feeding by biota, both of which have been observed to induce distinct microbial community clusters in marine environments (Azam and Malfatti, 2007; Seymour et al., 2017; Stocker, 2015). These studies ultimately demonstrate that the pelagic environment is heterogeneous, not only in its nutrient distributions, but also microbial abundance and taxonomic distributions. This is analogous to the distributions of macroorganisms (see section 1.1.2), but at a finer scale few have investigated for freshwater systems. The global biogeochemical relevance of these processes in marine systems (Seymour et al., 2017), however, suggests that these are ecologically relevant distinctions.

1.2.1.2 Benthic environment

As with the pelagic environment, both macro- and microscale heterogeneity exists within the benthic environment. A well-defined macroscale gradient within sediments is the vertical redox profile (Figure 1.3) (Nealson, 1997). This gradient is mediated by chemical diffusion and microbial activity, and is dictated by a principle which asserts that the most energetically favourable electron acceptor is normally used in preference to less energetically favourable acceptors (Capone and Kiene, 1988; Nealson, 1997). Oxygen has the greatest energetic yield of all electron acceptors, and thus, when oxygen is available, aerobic respiration is carried out. This respiration ultimately leads to the depletion of oxygen, and thus microorganisms inhabiting the sediments below, utilise, and then deplete, the next most energetically favourable electron acceptor (nitrate). This process results in a fairly consistent cascade of electron acceptor utilization with sediment depth, i.e. oxygen, nitrate, sulfate, metal ions and finally, organic and inorganic carbon compounds in methanogenesis (Capone and Kiene, 1988; Nealson, 1997). Anaerobic respiration typically dominates sediment depth profiles, with oxygen generally being depleted in the top <5 mm of sediment (Kappler et al., 2004; Sweerts et al., 1991).

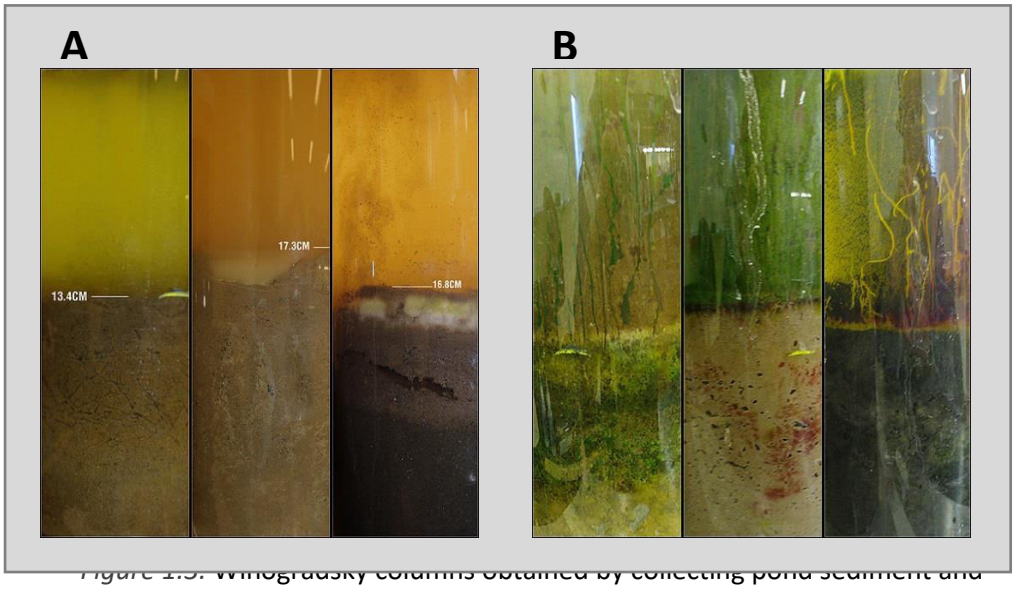

Figure 1.5. Winogradsky columns obtained by collecting pond sediment and allowing it to settle for A) one week and B) seven weeks. The clear vessels allow different redox strata to be observed across the depth profile. Adapted from figures created by Tjmhay (2006).

In addition to electron acceptor availability, microorganisms occupying specific redox zones are characterised by the respiration products they evolve (Capone and Kiene, 1988; Nealson, 1997). These products also diffuse into upper sediment layers, shaping microbial communities and processes in the upper strata. For example, the production of methane in deeper sediment layers, provides a carbon source for aerobic microorganisms in upper sediment layers (He et al., 2012; Sweerts et al., 1991). As such, redox zones are dynamically influenced by other zones within the profile, and are characterised by the microbial activities of multiple communities along this profile.

Redox gradients are also observed at the micro-scale. Sediments have a range of pore sizes between different particle aggregates, and within single aggregates. These differences in size result in different nutrient and electron acceptor diffusion rates between larger pores (between aggregate pores, >6 µm) and the smaller, within aggregate pores (<6 µm; Ranjard & Richaume 2001). Thus, micro-scale redox gradients occur horizontally within a vertical, macroscale, redox zone, i.e. within an oxic layer, anoxic micro-niches can exist. Indeed, sulfate-reducing 'hotspots' have been observed

in oxic sediments in freshwater and marine environments (Fukui and Takii, 1990; Jørgensen, 1977; Widerlund and Davison, 2007).

Another, broad, macroscale gradient in sediments is the biochemical forms of detritus available with depth. Organic matter decomposition and dissolution starts in the water column and continues as material in the water column descends to the sediment surface, and becomes buried within the sediment due to overlying deposition of other materials. During decomposition, microorganisms utilise different substrates within organic matter at different rates (Coleman et al., 1983; Cummins and Klug, 1979). Simple molecules which require little to no modification in order to be transported across a cell membrane, for example, sugars and amino acids, are quickly absorbed by microbial communities or lost to the water column through solubilisation. Once these substances are depleted, microorganisms require more specialised enzymatic machinery in order to first fragment complex molecules into simpler substrates, before they can be absorb by the microbe (Arnosti, 2011; Flint et al., 2008). This process is slower due, in part, to the fact that fewer members of a microbial community are able to produce these metabolically costly enzymes (Arnosti, 2011). Without the introduction of new detritus in lower layers, these layers are left with organic matter, rich in complex biomolecular structures that could not be readily utilised in the upper layers.

An important consideration for the two macro-gradients described here, is that they are subject to disturbance by abiotic and biotic events. Examples of biotic events include the activity of benthic fauna, for example, bioturbation and biodeposition activities (Figure 1.2). Bioturbation is achieved through burrowing, the construction of tubes, and the irrigation of these burrows and tubes (Mermillod-Blondin, 2011). Biodeposition occurs when invertebrates deposit waste, either on the surface of the sediment, or below the surface during burrowing activities (Kuzyakov and Blagodatskaya, 2015; Mermillod-Blondin, 2011). These biotic disturbances result in a plethora of physiochemical changes across the sediment depth profile, including increased oxygen penetration, nutrient exchanges with the water column, movement of surface organic matter to greater sediment

depths, changes in pore sizes and the prevention of sediment compaction (Kuzyakov and Blagodatskaya, 2015; Mermillod-Blondin, 2011; Nogaro et al., 2006). In addition to these fauna-mediated processes, biopores through sediment strata are also produced by plants via root growth (Figure 1.2) (Kuzyakov and Blagodatskaya, 2015). These roots are conduits of oxygen, allowing for the oxygenation of anoxic zones within the sediment (Armstrong, 1964). Additionally, roots release nutrients, mucilage, exudates and border cells into the surrounding sediment, transferring labile material to these lower depths (Philippot et al., 2013).

Each of these biotic disturbances to sediment stratification stimulates microbial activity, creating "hotspots" on, and within, the sediment matrices (Kuzyakov and Blagodatskaya, 2015; Mermillod-Blondin, 2011; Philippot et al., 2013). This increased microbial activity additionally effects redox gradients by increasing electron acceptor demands at sites increased organic matter inputs (Mermillod-Blondin, 2011). Thus, macro-scale redox and organic molecule gradients are complex and will inevitably fluctuate over temporal and spatial scales.

In addition to disturbances in sediment macrogradients, plants represent a specialised microhabitat for microorganisms (Figure 1.2). For benthic communities, this specifically pertains to the area extending <3 mm from root surfaces, called the rhizosphere, which is characterised by distinctly disparate microbial assemblages and processes when compared with bulk sediments (Philippot et al., 2013). For example, the rhizosphere is a key zone for coupled nitrification and denitrification rates in sediments, and are associated with differences in the relative abundances of ammonia-oxidising bacteria and archaea (Herrmann et al., 2009). In wetland systems, metal speciation and mineralogy within plant rhizospheres is associated with specialised microbial biofilms which appear to mediate metal complexation (Hansel et al., 2001). Furthermore, fungal plant symbionts form vesicular arbuscular (VA) mycorrhizae structures with plant roots, resulting in a concentration of Ascomycota and Glomeromycota taxa around these roots (Sondergaard & Laegaard 1977; Khan & Belik 1995; Philippot et al. 2013). While soil rhizospheres are more extensively studied than those in

aquatic environments, it is likely that a similar enrichment of the prokaryotic phyla occurs in aquatic rhizospheres, an analogue to that of terrestrial rhizospheres (Philippot et al., 2013).

1.2.1.3 Surfaces

Diverse and dynamic microbial communities are known to form on fixed surfaces within freshwater ecosystems. These surfaces include rocks, sediment particles, leaves and detritus. Numerous and taxonomically diverse microorganisms are incorporated into these communities (Sigee, 2005), and in one study, cell densities of 2×10^{10} cm^{-2} and 5×10^{5} cm^{-2} for bacterial and algal cells, respectively, were achieved in just 60 days on substrates introduced to a freshwater stream (Romani and Sabater, 1999).

Initially, surfaces are colonised by microorganisms with adhesive characteristics. These may be structural, for example, specialised appendages like adhesive pads, stalks or tubes; or biochemical, with the production of sticky extracellular polymeric substances (EPS), or the combined implementation of both (Roemer et al., 1984). These early colonisers have rapid growth rates, horizontally colonizing surfaces at exceptional rates (Bott and Brock, 1970; Lawrence and Caldwell, 1987). In addition to this, adhesive structures facilitate a vertical dimension to the colony, trapping successions of microorganisms and particulate and organic material and creating 'upper' and 'under' storeys within the biofilm (Roemer et al., 1984).

Biofilms are broadly classed based on the surface they are attached to, with those attached to living plants called epiphyton, and those attached to sediments or rocks called epipelon (Roemer et al., 1984). Further, when a biofilm, either epiphyton or epipelon, has an abundance of photosynthetic microorganisms within their community, the biofilm is called periphyton. Periphyton are known to be complex ecosystems with a high amount of biodiversity and numerous functional guilds (Uz et al., 2007). Among these, photosynthetic bacteria appear to play a crucial role in structuring the community and mediating the activity of non-photosynthetic members. For example, they produce

oxygen, provide fixed carbon to heterotrophic bacterial and fungal members (Kuehn et al., 2014) and stimulate bacterial growth through cellular excretions (Murray et al., 1986). In a broader ecological context, these photosynthetic microorganisms fix ≈50% of the carbon which is eventually assimilated into fish, either through fish directly grazing upon periphyton communities, or via their consumption of zoobenthos (Vadeboncoeur et al., 2002). Periphyton carbon fixation also influences global biogeochemical cycles. For example, in a 2003 study of lake annual production, investigators found that epipelon periphyton were responsible for ~77% of the carbon fixation in clear lakes, with a total yield of ~100 g C m^{-2} yr^{-1} (Liboriussen and Jeppesen, 2003). Thus, periphyton communities are an ecologically relevant component of freshwater food webs.

Micro-scale redox gradients, similar to those described for sediments in section 1.2.1.2, exist within biofilm communities. While these communities may be growing on surfaces in oxic layers, microbes on the upper story of biofilms exhaust available oxygen rapidly. This has even been observed in periphyton communities in which community members are actively producing oxygen (Lyautey et al., 2005; Teissier et al., 2002). Thus, both aerobic and anaerobic microorganisms and processes exist within these communities, stratified by redox conditions and resource availability (Uz et al., 2007).

Biofilm communities growing on macrophyte surfaces, epiphyton, exhibit complex, bimodal redox and substrate gradients due to the activity of the macrophyte (Figure 1.2). While the diffusion of electron acceptors and nutrients from the uppermost biofilm layers inwards is impeded, the macrophytes themselves excrete nutrients at leaf surfaces in an analogue of rhizosphere excretions (section 1.2.1.2), as well as oxygen during photosynthesis (Goulder and Baker, 1991). This contributes to distinctly different microenvironments on these surfaces compared with inert surfaces (e.g. detritus and sediment particulates). In one study, oxygen concentrations at the leaf surface fluctuated from 33% of the concentration in surrounding bulk water at night, to 241% during the day when photosynthesis was active (Sand-Jensen et al., 1985). The majority of oxygen depletion at night occurred within the 300 µm biofilm covering the leaf surface, and was similarly

apparent in reverse during the day, in which only 61% of the oxygen generated at the leaf surface was measured at the surface layer of the biofilm (Sand-Jensen et al., 1985). Thus, it is unsurprising that the microbial communities occupying epiphytic microhabitats are distinctly different to those inhabiting detritus and sediment surfaces (Buesing et al., 2009).

The distribution of bacterial communities on plant surfaces varies greatly. For example, bacterial densities are greater on stems compared to leaves (Rimes and Goulder, 1986). Additionally, leaf surfaces have heterogenous topography, with bacterial communities varying along these surfaces (Andrews, 1992; Goulder and Baker, 1991). The microenvironments, and thus the microbial communities on leaves may also differ among plant taxa. For example, some aquatic plants have hydrophobic surfaces, which maintain a gaseous layer around the leaf through convective flow from parts of the plant which emerge from the water (Goulder and Baker, 1991). Whilst others have leaves in which the surface is wholly hydrated (Goulder and Baker, 1991). While this field is less studied than its terrestrial counterparts (Goulder and Baker, 1991), it is clear that plant associated biofilm communities are dynamic, heterogeneously distributed within freshwater environments and play important ecological roles within these systems.

1.2.2 Microbial diversity in freshwater sediments

Diversity is a complex and nuanced concept in ecology (Kenchington and Kenchington, 2013; Mace et al., 2012). In this section, diversity refers to taxonomic diversity, namely the range of microbial taxonomic groups detected in surveys of freshwater sediments. Diversity metrics from such surveys distil this information into a quantifiable index, and offer a means of comparing different communities. A number of these metrics have been developed and applied over the last century, however, the most common capture one of three features: total species richness, the evenness of the community, and a combination of these two in the form of a biodiversity indices (reviewed in Kenchington and Kenchington, 2013). These metrics are routinely calculated for macroorganisms

such as invertebrates (Gallardo et al., 2011) and fish (Sarkar et al., 2012), and allow communities at different trophic levels, sites and temporal scales to be compared.

Total species richness is the number of species detected in a given sampling effort (Gotelli and Colwell, 2001; Kenchington and Kenchington, 2013). Community evenness refers to the abundance of individuals within a community, with low values indicative of a large disparity in abundances among community members, while higher values indicate similar abundances across the community (Kenchington and Kenchington, 2013). Finally biodiversity indices such as Shannon's diversity index are calculated from species richness and evenness, and offers a single metric which summarises multiple community features (Kenchington and Kenchington, 2013).

Broadly, freshwater benthic microbial communities are substantially less studied than pelagic or marine communities (Zeglin, 2015; Zinger et al., 2012). Among those studies investigating these communities, lentic habitats have been interrogated more often than lotic, with the focus more often on bacterial communities rather than Archaea or Fungi (Zeglin, 2015; Zinger et al., 2012).

Investigations using early molecular surveying techniques described the most dominant microbial taxa in freshwater sediments (Auguet et al., 2010; Briée et al., 2007). These surveys, however, demonstrated steep sampling curves which did not reach a taxonomic plateau for prokaryotes, indicating that a number of less abundant taxa could not be detected by these techniques (Auguet et al., 2010; Briée et al., 2007). With the advent of next-generation, high-throughput sequencing, rare taxa could be detected and their subsequent application to freshwater sediment communities showed a long tail of rare prokaryotic taxa existed within these communities, and that the communities were extremely diverse (Wang et al., 2012; Zeglin, 2015; Zhang et al., 2015). The application of these techniques in freshwater fungal surveys is far less prevalent (Zeglin, 2015), with a number of aquatic fungi appearing to be highly novel and poorly understood (Bärlocher and Boddy, 2016; Grossart et al., 2016).

1.2.2.1 Prokaryotic diversity

In an early survey of global prokaryotic diversity patterns, DNA reassociation was used to estimate that pristine marine sediments had >10,000 unique genomes per gram of sediment (Torsvik et al., 2002). This phenomenally high amount of diversity exceeded that seen in soils from a range of habitats (Torsvik et al., 2002). Until recently, reports on freshwater sediment microbial diversity were based on under-sampled communities, which suggested freshwater sediments were also highly diverse but lacking the sampling power to quantify this (Auguet et al., 2010; Briée et al., 2007; Tamaki et al., 2005). Recent high-throughput next-generation molecular surveys report Shannon's biodiversity indices of >7 for freshwater sediments. These values are substantially greater than those reported for invertebrates and fish in similar habitats. For example, Shannon's biodiversity indices of 0.06 - 1.93 were reported for macroinvertebrates in 9 sites along the Ebro river floodplain in Spain (Gallardo et al., 2011), while this indices ranged from 1.44 - 3.59 for fish along the river Ganga in India (Sarkar et al., 2012). In studies which compare prokaryotic communities from marine and lake sediments, those from freshwater sediments appear to contain greater microbial diversity than marine sediments (Wang et al., 2012; Zhang et al., 2015), making prokaryotic freshwater communities the most diverse communities in the world.

The most commonly detected and abundant prokaryotic taxa observed in lentic and lotic environments include members from the phyla Acidobacteria, Bacteroidetes, Chloroflexi, Nitrospira, Proteobacteria and Verrucomicrobia (Spring et al., 2000; Wang et al., 2012; Zeglin, 2015; Zhang et al., 2015). Substantially fewer surveys report archaeal taxonomic profiles for freshwater environments (Zeglin 2015), however, a recent survey reported the detection of 982 - 1,793 archaeal OTUs from 13 Chinese lakes (Zhang et al., 2015). Of these, numerous taxa were broadly distributed among the Euryarchaeota and Crenarchaeota phyla, however a substantial portion of the community (20-80%) could not be assigned to a phylum, indicating that a high degree of novelty exists within the community (Zhang et al., 2015).

1.2.2.2 Fungal diversity in freshwater sediments

Estimates of total aquatic fungal diversity range from 300 to 4,145 species (Bärlocher and Boddy, 2016). These are substantially smaller estimates than those reported for terrestrial systems, and likely reflect the disparity between fungal surveys in aquatic and terrestrial environments. For example, in the first ten volumes of Fungal Ecology, only 8% of papers concerned aquatic fungi (Bärlocher and Boddy, 2016). Additionally, a number of these aquatic fungal surveys are concerned with submerged plant material, for example wood (Hyde and Goh, 1998), leaf litter (Duarte et al., 2014) and pollen (Wurzbacher et al., 2014) and thus, do not explore resident fungal communities within bulk sediments (Bärlocher and Boddy, 2016).

Of those aquatic fungi identified, the majority of isolates belong to the phylum Ascomycota (Shearer et al., 2007). Examples of apparently ubiquitous aquatic fungi include *Massarina ingoldiana, Aniptodera chesapeakensis, Aniptodera lignatilis, Annulatascus velatisporus, Halosarpheia retorquens* and *Nais inornata* (Shearer et al., 2007). Molecular surveys, however, suggest that Chytridiomycota may also account for a substantial proportion of the fungal communities, with a recent meta-analysis of these data suggesting that chytrids are as abundant as ascomycetes in sediments (Panzer et al., 2015). In one high-throughput molecular survey of lake sediments specifically, Chytridiomycota from the three orders Chytridiales, Rhizophydiales and Rhizophlyctidales were detected and vastly outnumbered ascomycetes (Monchy et al. 2011). There are also few aquatic representatives of the Basidiomycota phylum, of which terrestrial isolates are numerous (Shearer et al., 2007).

1.3 METHODS FOR INTERROGATING MICROBIAL COMMUNITIES

Over the past 50 years, methods involved in the exploration of microbial ecology have transitioned through several stages as technologies have advanced and scientific trends have emerged and disappeared. Early microbial ecological studies were characterised by the visualisation of

microorganisms under the microscope (Bostrm et al., 1989; Hobbie et al., 1977; Johnson et al., 1989; Meyer et al., 1987), and the isolation and characterisation of cultivable bacteria (Baldi et al., 1989; Harrison et al., 1972; Jones et al., 1984). Such techniques demonstrated that a plethora of microorganisms inhabit freshwater environments, and the estimates of cell densities and biomass determined through microscopy are still relevant today. In particular, photosynthetic microorganisms can be distinguished based on their autofluorescence, and thus, a combination of auto- and epifluorescence microscopy has been used to elegantly demonstrate temporal and vertical variations in photosynthetic and non-photosynthetic microbes in lakes (Bostrm et al., 1989) and interactions between photosynthetic organisms and benthic invertebrates (Johnson et al., 1989).

The cultivation of particular taxa from freshwater environments has also contributed to our understanding of microbial ecology and metabolic potential. For example, the isolation and characterisation of a *Vibrio* sp. from lake sediments demonstrated its ability to alter fermentation products through the reduction of ferric ions, and was the first demonstration of cross-feeding between microbes engaged in these two distinctly different anaerobic metabolic pathways (Jones et al., 1984). Additionally, bacterial isolates in another study where shown to release organic acids in the presence of algal biomass, liberating phosphate from a range of metal complexes and demonstrating a strategy for inorganic nutrient uptake in microorganisms (Harrison et al., 1972). In ecotoxicological studies, the quantity of cultivable microorganisms has also been used to distinguish between perturbed and unperturbed environments (Bååth, 1989). A commonly used technique for this was the measurement of colony forming units (CFUs) (Bååth 1989). Unfortunately, as with other environments, few of the microorganisms viewed under the microscope were cultivable (Torsvik et al., 2002) and as prokaryotic organisms show modest morphological differences compared with eukaryotes, taxonomic resolution based on microscopy and colony formation was limited (Bååth, 1989).

Community-level functional assays have also been used to profile microbial communities from a range of environments (Bååth, 1989; Winding et al., 2005). These functional assays predominately report the evolution of carbon dioxide in the presence of carbon source amendments (Bååth, 1989; Sinsabaugh, 1994; Winding et al., 2005) or, the evolution of other enzyme products indicative of microbial metabolism, for example nitrite production as a measure of nitrate reductase (Jones, 1979; Sinsabaugh, 1994). Ecologically, these assays have contributed to our understanding of metabolic stratification in sediments, for example, the activity of nitrate reductase at depths of 10 - 15 mm and within sediment particles (Jones, 1979). Additionally, in ecotoxicological studies soil respiration rates have been used to distinguish metal contaminated soils from controls (Bååth, 1989; Winding et al., 2005). These assays have the benefit of broadly discerning the functional capability of whole communities, even uncultivable members, however, there are limitations. For example, measurable differences in carbon dioxide evolution typically take place under carbon amendments which greatly exceed those which occur naturally (Winding et al., 2005), calling into question the ecological relevance of these methods. Also, enzymes are not necessarily coupled with living microbial activity, with soil-bound enzymes shown to remain active (Burns, 1982).

More recent advances in molecular techniques have assisted in the taxonomical profiling of environmental communities. One such method is the profiling of lipid biomarkers (Torsvik and Øvreås, 2002; Winding et al., 2005; Zelles, 1999). The benefit of these biomarkers is that they are able to provide both quantitative and qualitative information, linking biomass measurements with taxonomic profiles (Winding et al., 2005; Zelles, 1999). In addition to this, where functional characteristics are closely associated with taxonomically distinct clades, distinguishable through known fatty acid biomarkers, structure-function relationships could be established. An elegant example of this is the quantification of methane-oxidising bacteria at different depths within the water column, and sediment, of a lake (Mancuso et al., 1990). Additionally, this method could be used in ecotoxicology surveys to distinguish between different microbial community compositions

(Winding et al., 2005). Despite this, the taxonomic resolution of fatty acid biomarkers is poor, and genus level differentiations are rare (Zelles, 1999).

In addition to fatty acid biomarker profiles, taxonomic profiling through nucleic acid marker genes was also gaining traction in the 1990's. This was largely driven by the broad adoption of the polymerase chain reaction (PCR) method (Saiki et al., 1988), in which a targeted region of a genome (i.e. a marker gene) could be amplified exponentially. Importantly, this method could be applied to DNA extracted from environmental samples, theoretically amplifying marker genes from all genomes within the sample (Anderson and Cairney, 2004; Torsvik and Øvreås, 2002). The biological variability of these marker genes represented the genetic diversity within an environmental sample, and thus techniques were developed to qualify biodiversity (Anderson and Cairney, 2004; Torsvik and Øvreås, 2002). Initially methods for analysing this involved running amplified target regions through a gel, and visualising their migration under variable conditions (Anderson and Cairney, 2004; Torsvik and Øvreås, 2002). This technique generally separated marker genes based on size and GC content. Alternatively, sequence variation could be observed through the digestion of marker genes with restriction enzymes, before running the samples on a gel (Anderson and Cairney, 2004; Torsvik and Øvreås, 2002). The direct sequencing of amplified DNA was also possible, initially with the cloning of genes in order to isolate each gene individually and later (Anderson and Cairney, 2004; Torsvik and Øvreås, 2002), high-throughput sequencing of multiple amplicons, termed "metagenomics" (Gilbert et al., 2010; Hudson, 2008).

Microbial ecology is now firmly in the era of metagenomic sequencing (Caporaso et al., 2012; Fierer et al., 2012; Gilbert and Dupont, 2011; Hudson, 2008; Lewin et al., 2013; Temperton and Giovannoni, 2012). This work has revolutionised our understanding of the taxonomic diversity of prokaryotes, demonstrating that previous measurements had grossly underestimated the biodiversity of these organisms and that a large portion of the prokaryotic phylogenetic tree had been missing (DeLong, 2009; Hug et al., 2016; Temperton and Giovannoni, 2012). High-throughput sequencing analysis was

also extended to include untargeted whole sequencing of environmental DNA, allowing researchers to explore the functional potential of a microbial community, in addition to taxonomy. The identification of novel proteins through this approach, has provided insights into the ecology and physiology of microorganisms, as well as global biogeochemical processes (Gilbert and Dupont, 2011; Temperton and Giovannoni, 2012). Further some of these proteins have potential industrial applications and substantial economic value (Berini et al., 2017; Gilbert and Dupont, 2011; Temperton and Giovannoni, 2012).

With sufficient sequencing coverage, genomes can be constructed from metagenomic sequencing (Baker et al., 2015; Hess et al., 2011; Hua et al., 2015; Tyson et al., 2004; Vavourakis et al., 2016). This technique allows researchers to explore structure-function relationships within microbial communities, identifying who, is doing what. This has proven to be of significant ecological relevance, with genome reconstructions demonstrating essential metabolic pathways are partitioned among community members, with rare taxa undertaking critical community processes (Garcia et al., 2015; Hua et al., 2015; Pernthaler et al., 2008). These findings provide a potential exploration for why axenic cultures are so difficult to achieve, and emphasises the importance of understanding microorganisms at the community level.

1.4 STRESSORS ON MICROBIAL COMMUNITIES

The CSR ecological theory describes three ecological strategies for species propagation and survival: competitors (C), stress-tolerant (S) and ruderal (R) (Grime, 1977). This theory succeeded the r-K continuum theory in plant ecology (MacArthur and Wilson, 1967; Pianka, 1972), in which r-strategists are ruderals and K-strategists are a less nuanced combination of stress-tolerant and competitor strategy types (Grime, 1977). Ruderal organisms are described as fast-growing, short-lived organisms which produce abundant offspring. These organisms are described as pioneer taxa and often dominate community assemblages after a disturbance, taking advantage of resource rich

environments which lack competition. Over time, these ruderals are replaced by competitors which are slower growing, longer-lived and invest more heavily in fewer offspring. Competitors invest into efficient resource utilization as well as competitive strategies, i.e. structural, biochemical or behavioural features which serve to provide a competitive advantage to the organism. Finally, stress-tolerant organisms are adapted to persist under a particular environmental stress. They are typically slower growing than ruderals, however, unlike competitors they do not invest into competitive strategies and may not be efficient in their use of resources. Instead, this ecological strategy capitalises on lowered competition under a given environmental stress.

Along with plants, evidence for each of these ecological strategies can be observed in animals and fungi (Cooke and Rayner, 1984; Grime, 1977). Specific fungal examples include ruderals belonging to the Mucorales, which have fast spore germination and mycelial growth rates (Cooke and Rayner, 1984). In contrast with this, Gigasporaceae are arbuscular mycorrhiza which show a strong competitive advantage as a carbon sink for their plant symbionts in comparison with other arbuscular mycorrhiza (Chagnon et al., 2013). In comparison, arbuscular mycorrhiza belonging to the Acaulosporaceae family become more abundant in acid soils, demonstrating a stress-tolerance life strategy (Chagnon et al., 2013). Additionally, Neocallimastix fungi capable of inhabiting invertebrate digestive tracts, and subsequently exposed to digestive acids, also exemplify a stress-tolerance life strategy (Cooke and Rayner, 1984; Gordon and Phillips, 1998).

A number of prokaryotic taxa also conform to ruderal, stress-tolerant or competitive descriptions. For example, some Betaproteobacteria and Bacteriodetes are classed as ruderals, based on their early colonisation of nutrient-rich materials in ecological settings and fast growth rates using simple sugars (Fierer et al., 2007). Some slow-growing Actinobacteria, capable of producing extracellular enzymes for polymer catabolism, as well as antibiotics in order to lyse other bacterial cells, may also be classed as competitors (Goodfellow and Williams, 1983; McCarthy and Williams, 1992). Finally,

Ho et al., (2013) described type II methane oxidising bacteria as possessing stress-tolerant strategies, showing increases in abundance after heat stress.

The CSR model provides a framework for understanding broad community responses to naturally occurring environmental fluctuations. For example, when a new and nutrient-rich substrate becomes available, i.e. in the form of a senescent leaf making contact with a sediment surface, it will first be colonised by ruderals. These organisms will first rapidly colonise the material, but quickly be replaced by efficient resource using competitors (Grime, 1977). These successional changes are well documented for microbial communities colonising and decomposing leaf litter in terrestrial and freshwater systems (Gessner et al., 1993; Torres et al., 2005; Voříšková and Baldrian, 2013). Additionally, the CSR theory predicts that when an environmental stress is applied to a system, stress-tolerant taxa will broadly outcompete competitors and ruderals.

1.5 METALS

Metals are a naturally occurring component of the lithosphere. In the vast majority of environments, however, these occur at fairly low concentrations (Figure 1.4). For example, trace metals such as copper, zinc, nickel and chromium each account for less than 0.1 mg kg^{-1} of the Earth's crust. All organisms require some metals for normal metabolic functioning (Bruins et al., 2000; Lemire et al., 2013), with these elements referred to as 'essential metals'. This dependency on metals relates to their use as protein cofactors, with almost 50% of enzymes relying on metals as either protein stabilisers or reactive centres (Figure 1.5) (Andreini et al., 2008). A microorganisms metal requirements depend on the organism's metabolic processes and the metal in question (Figure 1.5). While iron, zinc, calcium and copper are essential for all living things, many metals and metalloids, for example, silver, mercury and uranium are non-essential (Lemire et al., 2013). There are some metal-reducing microorganisms, however, which are capable of using uranium as terminal electron

acceptors (Cologgi et al., 2011). Hence, metals are ubiquitous in nature and many are essential or metabolically relevant for life.

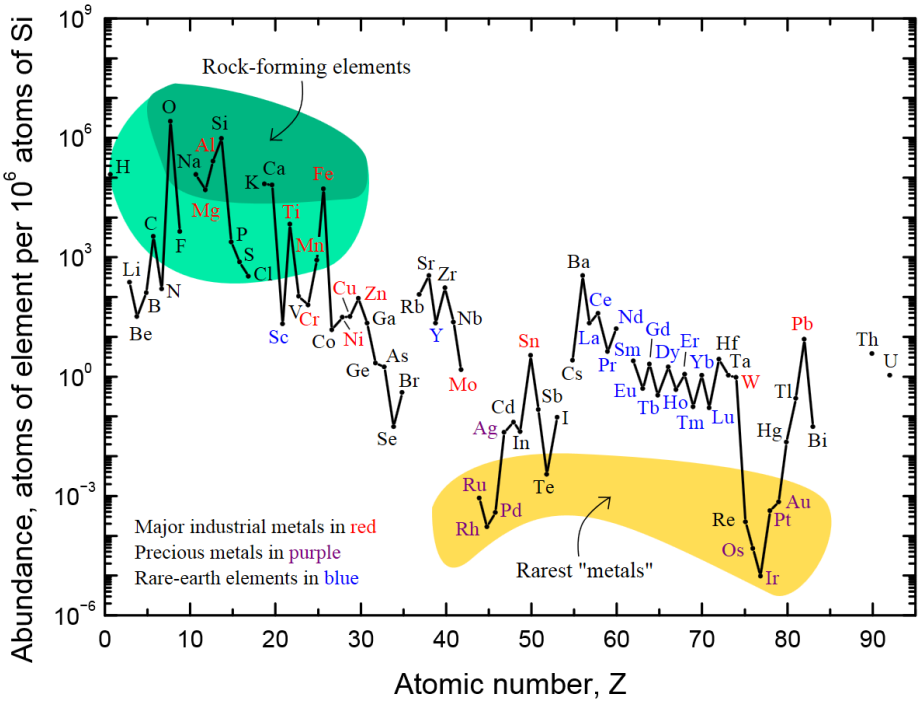

Figure 1.4. Relative abundance of elements in the Earth's upper crust. Not shown: Noble Gases, Tc(43), Pm(61), and all elements after Bi(83), except for Th(90) & U(92). Figure created by Gordon B. Haxel, Sara Boore and Susan Mayfield from United States Geological Survey (2003).

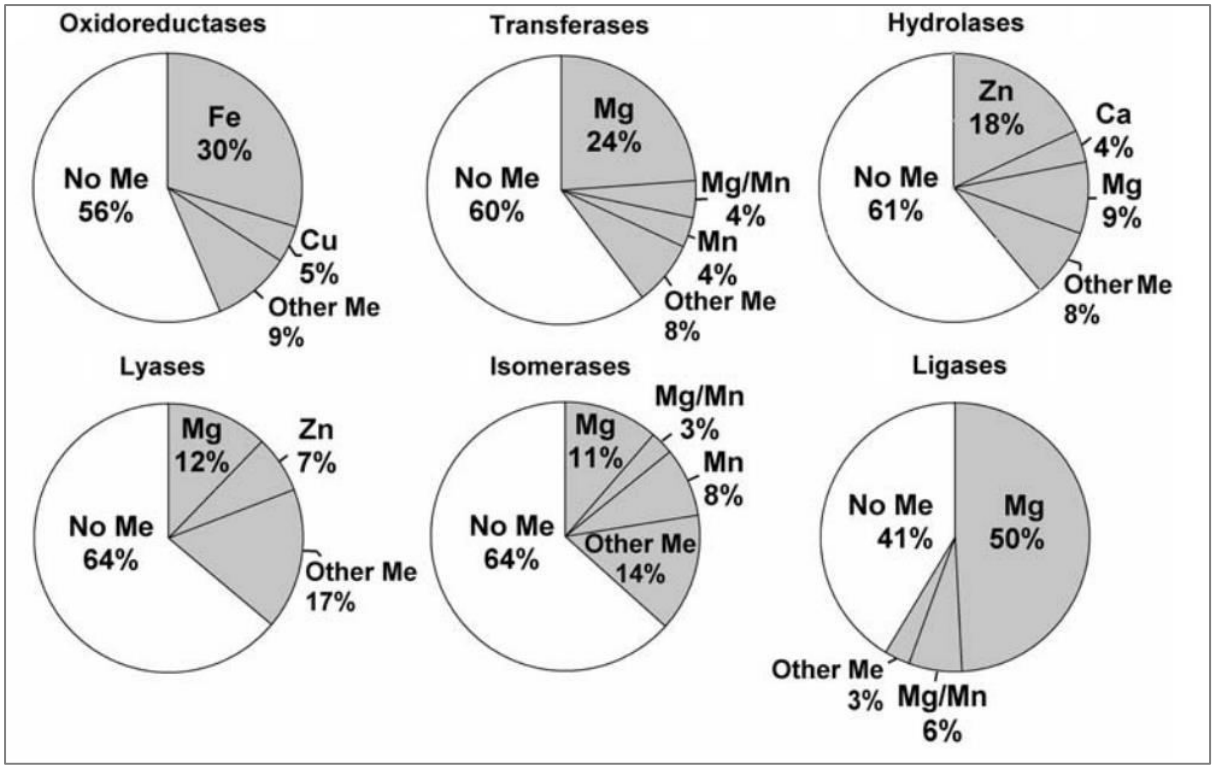

Figure 1.5. Proportion of enzymes from six enzyme families with catalytic metal ions. Figure sourced from Andreini et al. (2008).

Elevated metal concentrations, those concentrations greater than the averages outlined in Figure 1.4, can result from natural events, such as volcanoes, or through anthropogenic activities, for example, pesticide use and the deposition of mining waste (Newman and Jagoe, 1996). These elevated concentrations can be regarded as an environmental stressor, inducing toxicity for many organisms and enriching for organisms which are more tolerant (stress-tolerators). As observed in plants, however, stress tolerators are not necessarily as productive as ruderal or competitor taxa (Grime, 1977) and losses in productivity and biodiversity are well documented for environments with relatively high metal concentrations (for example, Galbraith et al., 1995; Spurgeon and Hopkin, 1996; Tarras-Wahlberg et al., 2001).

1.5.1 Metal toxicity in microorganisms

The mechanisms behind metal toxicity in microorganisms broadly involve oxidative stress, enzyme inhibition, cell membrane disruption and starvation (reviewed in Lemire et al. 2013). Oxidative stress

involves the production of free radicals, which are toxic to cells and rapidly exhaust cell antioxidants (Lemire et al., 2013). Metal-induced enzyme inhibition results from the binding of metal ions to an enzyme, or enzyme cofactor. For a number of metals (e.g. copper, silver, nickel and zinc), one enzymatic target is the exposed Fe-S bonds of certain enzymes, for example fumarase A, isopropylmalate isomerase and 6-phosphogluconate dehydrogenase (Lemire et al., 2013; Macomber and Imlay, 2009). In other instances, ion mimicry occurs, a process which involves the displacement of a metal ion cofactor by another non-specific metal ion which is unable to support enzymatic function. An example of this is the displacement of calcium ions by the uranium ion, uranyl, in the pyrroloquinoline quinone (PQQ) cofactor (Vanengelen et al., 2011). This cofactor is required by a number of dehydrogenases and, in *Pseudomonas aeruginosa*, the inhibition of PQQ ultimately results in cell death (Vanengelen et al., 2011).

Metal toxicity via cell membrane disruption is poorly understood, and is likely to be more relevant for eukaryotes than prokaryotes (Lemire et al., 2013). This process involves the binding of metal ions to electronegative groups on membrane polymers, resulting in lipid peroxidation of membrane polyunsaturated fatty acids (Lemire et al., 2013). This process destabilises the cell membrane and causes cell death, for example, in fungi exposed to high concentrations of copper (Baldrian, 2003). Finally, cell starvation results when transporters of essential organic and inorganic substrates are overwhelmed with the non-specific uptake of metals. For example, Cr(VI) inhibits the uptake of sulfate by sulphate transporters Sul1 and Sul2 in yeast (Pereira et al., 2008).

1.5.2 Metal resistance in prokaryotes and fungi

A number of broad metal tolerance strategies have been observed in prokaryotic organisms. Strategies which reduce metal bioavailability within cells include reduced uptake, complexation (also known as sequestration) and efflux (Figure 1.6) (Bruins et al., 2000; Lemire et al., 2013). Alternative strategies which counter toxic metal effects include by-passing metabolic pathways in which essential enzymes have been inhibited, and an increased investment into repair pathways to counter

metal-induced damage (Lemire et al., 2013). Depending on the metal in question, the molecular mechanisms underpinning these strategies may be well understood, for example copper-tolerance strategies (Bondarczuk and Piotrowska-Seget, 2013), or poorly understood, for example in the case of uranium (Merroun and Selenska-Pobell, 2008). Additionally, prokaryotic pathways are generally better understood than fungal (Lemire et al., 2013).

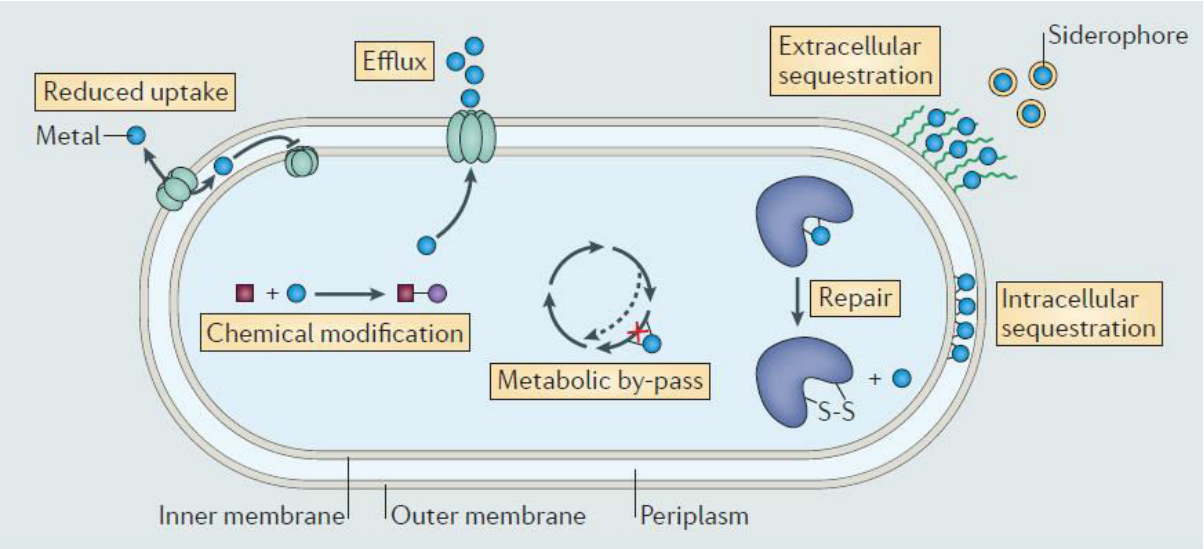

Figure 1.6. Overview of metal resistance strategies in microorganisms. Figure sourced from Lemire et al. (2013).

The reduced uptake of metals can be achieved through tight regulation, expression or activity of metal ion transporters (Cervantes and Gutierrez-Corona, 1994; Lemire et al., 2013). This process was elegantly demonstrated for zinc *in vitro*, using the *E. coli* regulatory proteins Zur and ZntR, which regulate zinc uptake and export, respectively (Outten et al., 1999; Outten and O'Halloran, 2001). When Zn(II) is in an over-abundance, it forms a complex with the Zur protein. This process increases the protein's affinity for the *znuC* promoter, causing it to bind to the region and prevent transcription of the *znuABC* operon responsible for a zinc high affinity ABC transporter (Outten and O'Halloran, 2001). Excess Zn(II) also binds with the ZntR, a protein bound to the spacer region of the *zntA* gene responsible for the ZntA zinc exporter (Outten et al., 1999). When bound to this zinc ion, ZntR changes the conformation of the DNA region making it more accessible to RNA polymerase and

thus, allowing it to be transcribed (Outten et al., 1999). This model demonstrates the intimate and tightly regulated control of metal ion uptake, however, this strategy is inadequate in the case of non-essential metals. In this instance, non-essential metals are mistakenly taken up by transporters of other elements and thus, the regulation of these transporters is related to their intended substrate's concentration, and not that of the non-essential metal ion (Lemire et al., 2013).

A number of diverse biotic processes results in the complexation of metals. These processes may involve the complexation outside the cell or within the cell. In external complexation, metal ions may be bound to cell membrane or wall structures (Beveridge et al., 1982; Cervantes and Gutierrez-Corona, 1994; Lemire et al., 2013). These include carboxyl, amide and phosphate groups in lipids and proteins of prokaryotic structures (Lemire et al., 2013), and the polysaccharides chitin and chitosan have putatively been assigned this role in fungi (Cervantes and Gutierrez-Corona, 1994). In the fungus *Trichoderma viride,* cultures exposed to elevated copper concentrations have cell walls up to five times thicker than those of controls (Cervantes and Gutierrez-Corona, 1994).

Alternative extracellular complexation mechanisms include the excretion of metal complexing agents, such as glutathione, oxalates, phosphate and siderophores into the surrounding environment (Bruins et al., 2000; Cervantes and Gutierrez-Corona, 1994; Lemire et al., 2013; Schalk et al., 2011; Silver and Phung, 1996). For example, the production of siderophores is known to increase resistance to a number of metals in strains of *Pseuodomonas aeruginosa* and *Streptomyces tendae* F4 (reviewed in Schalk et al., 2011). Some microbes also excrete enzymes which mediate the release of complexing agents into the environment, for example the excretion of phosphatases by *Citrobacter* sp., which release phosphate from organic molecules in the extracellular environment, precipitating U(VI) (Macaskie et al., 2000). Intracellular complexation via similar processes has also been reported, with the complexation of metals to polyphosphate granules and metallothioneins reported in both prokaryotes and fungi (Cervantes and Gutierrez-Corona, 1994; Lemire et al., 2013; Nies, 1999). For example, the production of metallothioneins, small thiolate-rich proteins which bind

to metals, in the cyanobacterial genus *Synechococcus* are known to confer zinc resistance in this taxa (reviewed in Silver and Phung, 1996).

The final mechanism for reducing the internal cellular bioavailability of metals, is efflux (Bruins et al., 2000; Lemire et al., 2013). A multitude of efflux systems are involved in the export of metals from prokaryotic cells. These include members of membrane transporter families: resistance-nodulation-cell division (RND), membrane fusion protein family (MFP), outer membrane factors (OMF), cation diffusion facilitators (CDF) and P-type ATPases (reviewed in Nies, 2003). RND transporters typically function in association with MFP and OMF proteins, with their genes often occurring within the same operon (Nies, 2003). For example, the *czcCBA* operon, which is found on the pMOL30 megaplasmid and mediates resistance to Co(II), Zn(II) and Cd(II), encodes the OMF CzcC, MFP CzcB and RND CzcA (Nies et al., 1989). While the deletion of the RND or MFP gene renders this system ineffective, the loss of *czcC* (OMF gene) is less significant (Nies et al., 1989), this appears consistent with other RND, MFP and OMF systems (Nies, 2003). Metal-resistance genes encoding CDF and P-type ATPase transporters are ubiquitously distributed among prokaryotes and eukaryotes, suggesting that these transporters are part of normal metal homeostasis processes. Examples include the five CDS transporters present in the yeast *Saccharomyces cerevisiae* (ZRC1, COT1, MSC2, MFT1 and MFT2), which are associated with different cellular organelles. For example, ZRC1 and COT1 are located on vacuole membranes and mediate the compartmentalisation of Zn(II) and Cd(II) in vacuoles. The MSC2 appears to mediate Zn(II) efflux from the nucleus while MFT1 & 2 are in the mitochrondrial membrane and are assumed to transport Fe(II) (Nies, 2003, 1999). The reduced distribution of heavy metal ion efflux systems from the RND family (HME-RND) among microorganisms, as well as their presence in taxa possessing exceptional metal-tolerances, suggests that these transporters confer enhanced metal resistance (Nies, 2003; von Rozycki and Nies, 2009).

1.5.3 Metal bioavailability in freshwater environments

When metals are introduced into an aquatic environment they typically adsorb to the sediment fraction (Flemming and Trevors, 1989; Newman and Jagoe, 1996). Adsorption is driven by the binding of metal cations to organic matter, humic acids and clay particles and the sedimentation of these metal-associated particles (Flemming and Trevors, 1989; Newman and Jagoe, 1996). Most historically contaminated sites have sediment metal concentrations far greater than those of the water column (Newman and Jagoe, 1996). In some instances, these sediment metal concentrations can be up to 5,000 times greater than water concentrations (Newman and Jagoe, 1996). This partitioning of metals into sediments has broad ecological significance, due to the fact that metal toxicity in macroorganisms correlates with the concentration of soluble metals species (Newman and Jagoe, 1996). Thus, the complexation and sedimentation of metals into insoluble forms reduces their bioavailability, and thus toxicity to macrobiota. This decrease in toxicity for sediment-bound metals is mirrored with the disparity in environmental trigger values for metals. For example, in Australia, a low-impact copper concentration threshold of 65 mg kg^{-1} exists for sediments, while in the water column this threshold is 1 µg L^{-1} (Simpson et al., 2013).

For microorganisms, the principles dictating metal bioavailability are less clear (Flemming and Trevors, 1989). This knowledge gap is primarily driven by a lack of research in this area, and the complexity of microbial communities. Microbial metabolic diversity is vast, with a number of enzymatic capabilities not available to macroorganisms. As such, microorganisms are able to access metals which are unavailable to macroorganisms, and thus, have a greater sensitivity to the metal. For example, while copper-bound, insoluble ligands are less toxic than free metal ions to both fish and invertebrate taxa (Newman and Jagoe, 1996), the two forms have equivalent toxic effects in many microorganisms (Fitzgerald and Faust, 1963). In fact, in some instances, these ligand complexes are more toxic, due to the microbes utilisation of the organic material as a nutrient source (Lighthart, 1980). Additionally, microorganisms produce highly effective siderophores, which

are able to sequester metals from complexes (Lemire et al., 2013), while the production of acids by certain microbial metabolic processes (e.g. fermentation) are likely to cause microscale pH gradients, which may liberate metals from particulate complexes with a binding optima of pH 7 (Flemming and Trevors, 1989). Conversely, as described in sections 1.4.2 and 1.4.3, microorganisms are also able to create metal complexes, actively altering metal speciation and reducing their exposure to soluble metal forms. Thus, it is clear that metal bioavailability to microorganisms depends on the metabolic activity of that organism, and those around them, resulting in heterogeneous microscale gradients (Flemming & Trevors 1989; Mondani et al. 2011) which are difficult to measure, but ecologically relevant to microbial communities. Future work is needed to both resolve the principles underlying microbial metal bioavailability, and measure bioavailable metal concentrations in the environment.

1.6 URANIUM AND MICROORGANISMS

Uranium (U) is a trace metal, and hence is generally of low abundance in the Earth's crust (Figure 1.4). In natural settings, U typically represents 1 ppm of the Earth's crust, with the majority in the ^{238}U isotopic form (>99%) (Gavrilescu et al., 2009). Anthropogenic activities, however, can result in substantially elevated concentrations of U, and in some instances, an increase in the isotopes ^{234}U and ^{235}U due to their enrichment in nuclear energy production (Gavrilescu et al., 2009). In some instances, substantially elevated U concentrations occur naturally, for example, in Villard, France, where concentrations of ~25g kg^{-1} have been reported (Mondani et al., 2011). While U is radioactive, its radiotoxicology is low, and it's mechanism of toxicity is typically analogous to that of other non-essential metals (Zavodska et al., 2008).

In total, there are six U redox states (Zavodska et al., 2008). Only two of these, the U(VI) and U(IV) ions, are stable enough to be of ecological relevance (Zavodska et al., 2008). U cycling involves the flux of U between U(VI) and U(IV) states, which is mediated by both abiotic and biotic factors. Due to U(IV)'s proclivity towards forming insoluble complexes, this form is of lesser ecological concern, as it

results in decreased U mobility and thus, dispersion in the environments, as well as toxicity to macroorganisms (Zavodska et al., 2008). In contrast, U(VI) is soluble (Zachara et al., 2013). As such, much of the literature pertaining to U contamination in aquatic systems addresses how U(VI) can be immobilised via its reduction to U(IV). Numerous microorganisms are known to carry out this process, including species of the *Geobacter, Desulfotomaculum* and *Shewanella* (Richter et al., 2012; Tebo and Obraztsova, 1998).

In U-contaminated underground water systems (aquifers), labile carbon sources have been provided to stimulate U-reducing taxa (Zachara et al., 2013). The increased abundance of these taxa, as a result of amendments, positively correlates with U removal from the water column, thus demonstrating the efficacy of this bioremediation strategy in controlled, and closely managed, systems (Zachara et al., 2013). It is noteworthy, however, that when carbon source amendments cease in the aquifers, U quickly transitions back to the water column (Zachara et al., 2013). In such instances, known U-reducing taxa become minor constituents of the microbial community. In natural freshwater environments, the availability of labile carbon sources in sediments is variable. As with amended aquifers, however, this availability has been shown to strongly correlate with U reduction, as well as the abundance of known U-reducing bacteria (Dang et al., 2018). Thus, labile carbon source availability appears to control, in part, microbially-mediated U reduction and thus, these reductions may be rare in natural environments with low labile carbon source availability.

Microbial surveys of U contaminated sites report high abundances of novel Acidobacteria, Gammaproteobacteria and Bacteroidetes taxa (Islam et al., 2011; Mondani et al., 2011; Rastogi et al., 2010; Suzuki et al., 2005; Zachara et al., 2013). In studies where comparisons are made with control communities, clear shifts in microbial community structure are reported (Dhal et al., 2011; Islam and Sar, 2011; Mondani et al., 2011; Rastogi et al., 2010; Suriya et al., 2016; Suzuki et al., 2005). Additionally, investigations into microbial functions have demonstrated a reduction in enzymatic activities, for example urease, dehydrogenase and cellulase, as well as declined overall

respiration and plant biomass degradation (Antunes et al., 2011; Kenarova et al., 2014; Meyer et al., 1998). Using metagenomic sequencing technologies to infer community functional potential, an increase in membrane transporters and a decrease in amino acid transporters and enzymes involved in carbohydrate catabolism have been observed for microbial communities inhabiting U enriched environments (Hemme et al., 2010; Yan et al., 2016).

In addition to the well-documented reduction of U(VI) to its tetravalent form, numerous investigations suggest that U-tolerance strategies may be diverse within these communities. For example RND and P-type ATPase transporters, along with other metal transporters appear to be enriched in U-tolerant communities (Hemme et al., 2010; Kumar et al., 2012; Liang et al., 2012; Martinez et al., 2006; Yan et al., 2016). Bacterial isolates capable of U sequestration are also observed (Kumar et al., 2012; Nedelkova et al., 2007). While there is a predilection in the literature towards bioremediation and U-reduction, these results demonstrate that other U-tolerance strategies may be ecologically relevant.

1.7 COPPER AND MICROORGANISMS

Both naturally occurring and anthropogenically derived copper (Cu) enrichments have been reported for numerous aquatic settings. For example Kendrick (1962) measured Cu concentrations of up to 68,000 mg kg^{-1} in undisturbed swamp sediments, while concentrations of 7,650 mg kg^{-1} have been reported for mine-impacted lake sediments in the United States of America, and 12,000 mg kg^{-1} for estuarine sediments in Norway (Flemming and Trevors, 1989; Kendrick, 1962). Cu has four redox states (I, II, III and IV), although only the Cu(I) and Cu(II) are of significant ecological relevance (Flemming and Trevors, 1989). The Cu(I) is more toxic to prokaryotes than the Cu(II), but is less abundant in oxic environments (Beswick et al., 1976; Macomber and Imlay, 2009). Fewer laboratories undertake anaerobic culturing, however, and as such most studies of Cu toxicity pertain to Cu(II).

1.7.1 Copper and prokaryotes

As with most toxins, the toxicity of Cu to microorganisms varies greatly among taxa. For Cu, the effective concentrations in which 50% of isolate growth is reduced (EC_{50}) range from 160 µg L^{-1} and 1,100 µg L-1 for an unidentified freshwater bacterium and *Pseudomonas putida*, respectively (Stauber and Davies, 2000). Taxa which exhibit extremely high Cu tolerances include *Cupriavidus metallidurans, Pseudomonas paucimobilis* and *Ralstonia* spp. (previously *Alcaligenes* spp.) (Dressler et al., 1991; Monchy et al., 2006; Nies, 2003). The underlying molecular mechanisms facilitating Cu tolerance in each of these taxa is relatively well understood (reviewed in Bondarczuk and Piotrowska-Seget, 2013). Four operons have been identified as conferring Cu resistance in Gram-negative bacteria. These include two chromosomal operons, *cue* and *cus*, and two plasmid-borne operons *pco* and *cop*. The proteins encoded within these operons are involved with Cu oxidation, transforming Cu(I) to the less toxic Cu(II) (i.e. CueO and PcoA), Cu chaperons which bind the ion, preventing it from interacting with other cell components (i.e. CusF, PcoC and CopC) and efflux (i.e. the CusCBA complex composed of an OMF (CusC), MFS (CusB) and HME-RND (CusA), as well as a P-type ATPase (CopA)) (Bondarczuk and Piotrowska-Seget, 2013; Nies, 2003). Their distribution among different taxa is unclear, however, Cu-enriched environments are associated with increased abundances of *cusA* and *copA* in marine sediments (Besaury et al., 2013), and intra-genera horizontal gene transfer has been documented (Stall et al., 1985).

At the community level, freshwater planktonic communities have EC_{50} values of 28 – 100 µg L^{-1} of Cu based on the incorporation of labelled amino acids (Stauber and Davies, 2000). For attached microbial communities, even lower Cu concentrations decreased the diversity of established freshwater protozoan communities on plastics (0.42 mg L^{-1}; Cairns et al. 1980) and bacterial biomass of established leaf litter communities (3 mg L^{-1}; Duarte et al. 2009). An elegant study design metering cupric sulphate into a stream system, demonstrated the long-term (2 years) effect of just 12 ng L^{-1} of Cu to the functions of attached communities (Leland and Carter, 1985). In this study, periphyton

were cultivated in periphytometers, plastic vessels which sit within the water column, allowing water flow. The rates of photosynthesis, carbon and sulfate fixation by these communities, as well as the decomposition of leaf litter within the stream, were all reduced under Cu enrichment compared with controls (Leland and Carter, 1985). Nitrogenase activity also declined, but returned to levels comparative to that of controls after nine months (Leland and Carter, 1985).

In contrast with these studies, others have found Cu concentrations have a positive effect on microbial communities, both planktonic (Havens, 1994) and periphyton (Gardham et al., 2015). These researchers have attributed their findings to a corresponding decline in invertebrates which graze on microbial communities (Gardham et al., 2015; Havens, 1994). This demonstrates the benefit of capturing a range of environmental features, and shows the limitation of studies which exclude other trophic levels. Taken together, these studies suggest that while Cu has a negative impact on planktonic and pelagic bacterial communities, these negative effects may be countered by a decline in predatory pressures. Determining the combined effect of these contrasting effects, however, is difficult to determine from short-term studies.

Relative to the body of work pertaining to elevated Cu concentrations in the water column, few studies have specifically addressed the impact of sediment Cu concentrations on microbial communities. The only study to address this effect in freshwater ecosystems was conducted by Gardham et al. (2015), who reported an increase in photosynthetic biomass for periphyton communities under sediment Cu concentrations of ≥62 mg kg^{-1}, as well as an increase in surface sediment decomposition rates at 650 mg kg^{-1} of Cu (Gardham et al., 2015). Another study, which looked at suspended, metal-rich particulates in an underground freshwater system, report a high diversity of prokaryotic taxa associated with these aggregates (Stein et al., 2002).

A handful of studies have also explored the effect of sediment Cu enrichments in marine systems. These studies found sediment microbial biodiversity remained the same at 44 mg kg^{-1} acid extractable Cu (Gillan et al., 2005) but increased at 1,150 mg kg^{-1} (Sorci et al., 1999) and declined at

1,600 mg kg^{-1} (Besaury et al., 2014) for total sediment Cu concentrations. Together, these studies suggest microbial communities in marine sediments may be more tolerant to Cu than their pelagic and biofilm forming counterparts.

1.7.2 Copper and fungi

Cu tolerant fungi have been isolated from a number of Cu enriched environments. Terrestrial isolates include entomopathogenic fungi *Isaria farinosa* and *Beauveria bassiana* from the Hypocreales, and multiple species from the *Verticillium* and *Penicillium* genera (Arnebrant et al., 1987; Stoke and Lindsay, 1979; Yamamoto et al., 1985). For freshwater river sediments, Cu-tolerant isolates belong to five genera *Alternaria, Aspergillus, Fusarium, Geotrichum, Penicillium* and *Trichoderma* (Ezzouhri et al., 2009; Iskandar et al., 2011) A number of these isolates have been grown on media containing > 1,000 mg kg^{-1} Cu (Arnebrant et al., 1987; Iskandar et al., 2011).

In contrast with these Cu-tolerant organisms, growth inhibition has been reported in media containing just 100mg kg^{-1} for basidiomycetes *Agaricus bisporus*, *Stropharia* spp. and *Mycena galericulata* as well as ascomycetes *Sordaria* sp., *Pyrenophora* sp. and *Chaetomium* sp. (Hartikainen et al., 2012). A handful of studies have also reported the sensitivity of species within the saprotrophic zygomycete genus, *Mortierella* (Arnebrant et al., 1987; Hartikainen et al., 2012), with multiple isolates demonstrating sensitivity at Cu concentrations of <60 mg L^{-1} (Arnebrant et al., 1987). Additionally, chytrid isolates *Terramyces* sp., *Rhizophlyctis rosea*, *Chytriomyces hyalinus* and a *Gaertneriomyces* species showed declines in growth and reproduction at Cu concentrations of 30 mg L^{-1} (Henderson et al., 2015).

At the community level, few fungal surveys of Cu enriched environments include comparisons to control communities, and thus it is difficult to infer the significance of their findings. Further, only a couple of these comparative studies have been conducted in freshwater systems (Duarte et al., 2009; Roussel et al., 2008). These studies found that water Cu concentrations of up to 3 mg L^{-1} did

not affect fungal biomass on leaf litter in freshwater environments, however, at 3 mg L^{-1} of Cu, community structural changes were observed (Duarte et al., 2009; Roussel et al., 2008). The fungal taxa most adversely effected by Cu, exhibiting a reduction in sporulation, and were *Anguillospora filiformis*, *Flagellospora curvula* and *Tricladium spendens* (Duarte et al., 2009). This shift in fungal structure, along with alterations to the prokaryote communities also in association with leaf litter, may have contributed to the decline in leaf litter decomposition (Duarte et al., 2009).

1.8 SCOPE OF THESIS

The current study describes the application of metagenomic sequencing techniques to sediment microbial communities along spiked, metal concentration gradients. Two gradients were explored, a U gradient of 0 – 4,000 mg kg^{-1} in tropical billabong sediments and a Cu gradient of 0 – 540 mg kg^{-1} in subtropical lentic pond sediments. The spiking of metals into sediment mesocosms in a laboratory setting allowed for control over the metal gradient, while limiting the occurrence of confounding co-contaminants. The subsequent *in situ* deployment of these mesocosms into the field, provided exposure to natural environmental fluctuations, such as biotic colonisation, temperature changes etc.

This study is the first investigation to apply 16S rRNA amplicon and shotgun metagenomic sequencing to sediment microbial communities along a spiked U concentration gradient. Chapter 2 describes the compositional changes of these communities in response to U, both at the taxonomic and functional level. In Chapter 3, the assembly and genome binning of shotgun metagenomic reads from the highest U spiked treatment (4,000 mg kg^{-1}) allowed for the ecophysiological profiling of the most abundant genomes in this sediment.

Investigations into the effects of Cu-bound sediment on microbial communities included amplicon metagenomic sequencing of both the fungal marker gene, ITS1, and the prokaryotic marker 16S rRNA. This study also deployed cellulose baits onto the sediment surfaces, in order to determine

whether sediment-bound Cu altered baited cellulolytic communities. Chapter 4 is the first metagenomic investigation into freshwater sediment fungi, and thus, this chapter explores the biodiversity of an understudied group of aquatic microbes, in addition to the effects of Cu on these microbial communities. Chapter 5 is an ecotoxicological study on the impact of sediment-bound Cu on freshwater prokaryotic communities, with an emphasis on whether current international sediment quality guideline values are sufficient to protect microbial communities.

Thus, the current study aimed to determine:

1. How sediment-bound uranium concentration impacts the genetic composition of prokaryotic communities in tropical sediments, and at what concentrations do these alterations occur.
2. What ecological strategies are employed by abundant prokaryotes inhabiting sediments with elevated uranium concentrations.
3. How sediment-bound copper concentration impacts the fungal communities from three biomes within freshwater ponds: pelagic, benthic and cellulose-associated, and at what concentrations do these alterations occur.
4. Whether copper effects the structure and function of microbial communities in freshwater ponds, as assessed using multiple lines of evidence.

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2 EFFECTS OF URANIUM ON MICROBIAL COMMUNITY STRUCTURE AND FUNCTIONAL POTENTIAL

Sutcliffe, B., Chariton, A. A., Harford, A. J., Hose, G. C., Greenfield, P., Elbourne, L. D. H., Oytam, Y., Stephenson, S., Midgley, D. J., & Paulsen, I. T. (2017). Effects of uranium concentration on microbial community structure and functional potential. *Environmental Microbiology*, 19(8), p. 3323-3341.

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3 INSIGHTS FROM THE GENOMES OF MICROBES THRIVING IN URANIUM-ENRICHED SEDIMENTS

Sutcliffe, B., Chariton, A. A., Hartford, A. J., Hose, G. C., Stephenson, S., Greenfield, P., Midgley, D. J., & Paulson, I. T. (2018). Insights from the genomes of microbes thriving in uranium-enriched sediments. *Microbal Ecology* , 75, p. 970-984.

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4 DIVERSE FUNGAL LINEAGES IN SUBTROPICAL PONDS ARE ALTERED BY SEDIMENT-BOUND COPPER

Sutcliffe, B., Chariton, A. A., Harford, A. J., Hose, G. C., Greenfield, P., Midgley, D. J., & Paulsen, I. T. (2018). Diverse fungal lineages in subtropical ponds are altered by sediment-bound copper. *Fungal Ecology*, 34, p. 28-42.

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5 MICROBIAL COMMUNITIES ARE SENSITIVE INDICATORS FOR FRESHWATER SEDIMENT COPPER CONTAMINATION

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5.1 ABSTRACT

Anthropogenic activities, such as mining and agriculture, have resulted in many freshwater systems having elevated concentrations of copper. Despite the prevalence of this contamination, and the vital ecological function of prokaryotes, just three studies have investigated prokaryote community responses to copper concentration in freshwater sediments. To address this, the current study investigated these communities in outdoor mesocosms spiked with varying copper concentrations. We profiled the prokaryotic communities at the taxonomic level, using next-generation high-throughput sequencing techniques, as well as their function, using baiting with leaf analogues, and Biolog Ecoplates for community-level physiological profiling. Sediments containing just 46 mg kg^{-1} of copper, had distinctly different microbial communities compared with controls, as determined by both DNA and RNA 16S ribosomal RNA gene (rRNA) profiling. In addition to this, sediment communities displayed a greatly reduced utilization of carbon substrates under elevated copper, while the communities recruited onto leaf analogues were also disparate from those of control ponds. Given the vital role of prokaryotes in ecosystem processes, including carbon cycling, these changes are potentially of great ecological relevance, and are seen to occur well below the 'low risk' sediment quality guideline values (SQGV) used by regulatory bodies internationally.

5.2 CAPSULE

Sediment prokaryotic microbial communities were found to alter, both structurally and functionally, with just 46 mg kg^{-1} of sediment-bound copper. Additionally, the structure of communities recruited onto cellulose baits resting on this sediment was also altered, showing a remarkable sensitivity to copper.

5.3 INTRODUCTION

Microbial communities play a fundamental role in all ecosystems. They are the foundation of all food webs and support many organisms directly as a food source and indirectly by making otherwise unavailable food sources available through their roles as autotrophs, decomposers and parasites (Graça, 2001; Norderhaug et al., 2003; Sigee, 2005; Winding et al., 2005). Prokaryotes (Eubacteria and Archaea) also perform biogeochemical transformations of nutrients such as carbon, nitrogen and phosphorus, providing important nutrients to Eukaryotic organisms (Falkowski et al., 2008). Thus, microbial communities are ecologically vital and perturbations to these communities will have consequences for ecosystem structure and functioning.

Copper is an essential trace metal (Macomber and Imlay, 2009) but can be toxic at concentrations detected in the environment - both naturally occurring and anthropogenically elevated (Flemming and Trevors, 1989; Newman and Jagoe, 1996; Papagiannis et al., 2004). While the response of a small number of aquatic macrobiota to copper contamination has been well studied, only a handful of studies have reported the response of freshwater microbial communities to copper and these have shown that planktonic and biofilm communities are sensitive to low concentrations of soluble copper ions (Duarte et al., 2009; Stauber and Davies, 2000). In laboratory studies, median effect concentrations (EC_{50} values) of 28 – 100 µg Cu L^{-1} were reported for freshwater planktonic communities (Stauber and Davies, 2000), while declines in bacterial biomass on leaf litter occurred at 3 mg L^{-1} of copper (Duarte et al., 2009). Leland and Carter (1985) reported a decline in rates of photosynthesis, carbon and sulfate fixation and leaf litter decomposition at just 12 ng Cu L^{-1} in a stream over a two year period. In contrast, Havens (1994) observed that 140 µg Cu L^{-1} of copper increased planktonic bacterial biomass, which was a secondary response to a decline in zooplankton abundance and consequent reduced predation pressure. Importantly, these studies highlight the need to also consider higher trophic levels in studies investigating microbial responses to disturbance.

Only three studies have specifically addressed the effects of sediment-bound copper on freshwater benthic microbial communities (Gardham et al., 2015; Sutcliffe et al., 2018; Yang et al., 2017). This paucity of information is significant because copper ultimately partitions into the sediments of aquatic environments and is present in the water column for a limited time (Flemming and Trevors, 1989; Newman and Jagoe, 1996). For example, in Lake Montana, the addition of copper sulfate as an algaecide over a period of 80 years resulted in sediment concentrations of 300 – 1,000 mg Cu kg^{-1}, but water column concentrations of just 3 µg Cu L^{-1} (Flemming and Trevors, 1989). Sediment partitioning decreases the bioavailability, and hence toxicity, of copper to macroorganisms (Flemming and Trevors, 1989; Newman and Jagoe, 1996) but the bioavailability of sediment-bound copper to microorganisms is poorly resolved (Flemming and Trevors, 1989).

Yang et al. (2017) identified 22 copper-sensitive sediment prokaryotic taxa, which declined in relative abundance at just 60 mg Cu kg^{-1} of copper in sediment. In a recent study investigating the effect of copper on fungal sediment communities, concentrations of just 46 mg Cu kg^{-1} resulted in the decline of sensitive fungal taxa and an overall shift in community structure (Sutcliffe et al., 2018). In contrast, Gardham et al. (2015) demonstrated that sediment-bound copper at concentrations of >200 mg Cu kg^{-1} increased both photosynthetic biomass in periphyton and decomposition rates of submerged carbon. These observations were attributed to a decline in invertebrate grazing on microbial communities (Gardham et al., 2015) and suggests that while a subset of sediment microbes are sensitive to copper, functional redundancy and ecological complexity may mitigate the ecological significance of their loss.

Microbial communities, specifically prokaryotes, are considered to have a high degree of functional redundancy (Nannipieri et al., 2003; Yin et al., 2000). For example, numerous studies have shown that while decomposition increases with increasing microbial diversity, a plateau is reached within 50 species of either bacteria or fungi (reviewed in McGuire and Treseder, 2010). In comparison,

uncontaminated sediments are estimated to contain >11,000 genomes (Torsvik and Øvreås, 2002), and thus, not all of these microorganisms are necessary to maintain normal ecological functioning.

DNA-based methods have made robust assessments of microbial assemblages in sediments possible. However, cell-free DNA can persist in soils and sediments, while much of the cellular DNA may be associated with non-active microbial spores (Anderson-Carpenter et al., 2011; Blagodatskaya and Kuzyakov, 2013; Carini et al., 2016). Thus, DNA-based molecular surveys may detect non-living and metabolically inactive taxa along with ecologically active and relevant community members. Indeed, the DNA from inactive and/or non-viable microbes may account for ~40% of the microorganisms detected in sediment and soil communities (Blagodatskaya and Kuzyakov, 2013; Carini et al., 2016). One method for addressing this is the analysis of RNA, with the added advantage that RNA surveys (using the 16S ribosomal RNA marker gene, 16S rRNA), can have greater sensitivity to anthropogenic disturbance than DNA surveys (Girvan et al., 2003; Zhang et al., 2014). Despite this, there are a number of technical difficulties with environmental RNA work, which, together with functional redundancy, have led to the limited use of microbial communities in applied ecological fields, such as ecosystem modelling and ecotoxicology (Allison and Martiny, 2008; Giller et al., 2009).

In the current study, we aimed to determine whether sediment-bound copper effects the structure and function of microbial communities in freshwater ponds. We implemented multiple techniques in order to address concerns regarding i) functional redundancy and ii) remnant cell-free DNA. We aimed to determine how much influence each of these factors had on the findings derived solely from a DNA-based survey approach. We hypothesize that sediment-bound copper effects sediment microbial communities both taxonomically and functionally.

5.1 METHOD

5.5.1 Ponds

A system of outdoor artificial pond mesocosms were established in 2010 by Gardham et al. (2014a) at Macquarie University, Sydney, Australia (Supplementary material S5.1). Briefly, four copper amended treatments were achieved by spiking replicate pond sediments with $CuSO_4.5H_2O$ to achieve nominal copper concentrations of 32, 65, 270 and 540 mg kg^{-1}. Each of these treatments had four replicates. Additionally, four unamended ponds were used as controls. The addition of $CuSO_4.5H_2O$ caused an acidification of sediments and the pH was adjusted using garden lime as described in Gardham et al. (2014a).

5.5.1 Experimental overview

Cellulose baits were deployed into the ponds and left *in situ* for 118 days. Following this, baits were collected and subjected to three lines of investigation. Firstly, from each pond, three replicate baits were subjected to microbial community DNA profiling. Another replicate baits were dried and weighed for weight loss measurements, and finally, one bait from each pond was used in scanning electron microscopy (SEM). Water samples were also collected for microbial community DNA profiling, as well as chemical analysis.

Sediment samples were subjected to chemical profiling, and microbial communities were profiled using both DNA and RNA templates. Sediments were also used to inoculate Biolog Ecoplates for substrate utilisation profiling.

5.5.1 Water and sediment sampling and chemistry

In mid-December 2015, sediment and water samples were collected as per Sutcliffe et al. (2018). Briefly, for each pond, microbial biomass was collected from 1L of water filtered onto a 0.22 µm PVDF filter. Sediment cores (ten 2 cm × 2 cm cores) were collected and homogenized, with three 1 mL sediment subsamples each combined with 1 mL of RNA Bacteria Protect solution (Qiagen) and

flash frozen. Approximately 20 g of this homogenised sediment was dried at 37°C for 48 h and sent for chemical analyses, along with 150 mL of pond water.

All chemical analyses were performed by the CSIRO Environmental Services branch, Sydney. Details of these analyses are described in Sutcliffe et al. (2018). Specific to metal concentration measurements: sediment total metals were measured as per US EPA method 3051A (1998) and water cations/ metals were measured following APHA 3120. A complete list of water and sediment chemistry measures are provided in Supplementary material S5.1.

5.5.1 Baiting for cellulolytic communities

Bait preparation and sampling was conducted as per Sutcliffe et al. (2018). Briefly, sterile ~5 × 8 cm cellulose paper (Whatman) baits were weighed and then being aseptically sealed into mesh bags (pore-size 63 µm) with sterilised weights. Seven bags were deployed into each pond in direct contact with the sediment surface.

Following a 118 day *in situ* deployment, the baits were collected. For each pond, three baits were combined with 1 mL of RNA Bacteria Protect solution (Qiagen) and snap frozen, a fourth bait was fixed in 3% glutaraldehyde in 1X phosphate buffered solution (PBS) and the remaining three baits were dried at 37°C room for 48 h before being weighed.

Fixed cellulose baits were prepared for scanning electron microscopy (SEM) following the method described by Vick et al. (2016). Two 1 cm × 1 cm squares were cut from each filter and mounted so that each side of the paper could be gold plated and imaged by SEM. Imaging was carried out at the Macquarie University Microscopy Unit.

5.5.1 Biolog community-level physiological profiling

At the time of sediment sampling, two 1 mL homogenised sediment subsamples were separately combined with 99 mL of pond water filtrate. The sediment:water mixtures were placed on a shaker at low speed for 20 minutes before being left to settle for 2 – 10 minutes. The overlying liquid was

then used to inoculate Biolog Ecoplates with 150 µL per well. Plate lids were sealed with parafilm and the plates left to incubate for a total of 7 days (168 hours) after which absorbance was measured at 595 nm using a Synergy HT plate reader (BioTek).

5.5.1 Nucleic acid extractions and reverse transcription reactions

Nucleic acid extractions are described in Sutcliffe et al. (2018). Briefly, sediment DNA and RNA were extracted using an optimized method of the PowerMax Soil DNA isolation kit (Sutcliffe et al., 2018). DNA from the water filter and cellulose bait samples were extracted using the MoBio PowerWater DNA Isolation Kit, as per the manufacturer's instructions.

For sediment samples, a subsample of the nucleic acid extract (44 µL) was subjected to a DNase digest using the MoBio DNase Max kit following the manufacturer's protocol. The resulting RNA extracts were split into two: with one used as an RNA control in subsequent PCRs and the other converted to cDNA using the High Capacity cDNA Reverse Transcription Kit following the manufacturer's protocol (Applied Biosystems).

5.5.1 PCRs and sequencing

The 16S ribosomal RNA (16S rRNA) gene was amplified from all sediment, water filter and cellulose bait DNA samples, as well as the cDNA from sediments, as per Sutcliffe et al. (2017a). Specifically, the Earth Microbiome Primers, 515F (GTGCCAGCMGCCGCGGTAA) and 806R primer (GGACTACHVGGGTWTCTAAT) with custom barcodes were used to amplify a ~300 bp fragment of the V4 region within the 16S rRNA gene (Caporaso et al., 2012). Each PCR reaction was in a final volume of 30 µL and contained: 1X MiFi High Sensitivity PCR Mix (Bioline), 200 nM of each primer and 2 µL of each sample. RNA controls were also subjected to 16S rRNA amplification as a control for the DNase treatment, and each PCR plate contained a positive (*Pseudomonas aueruginosa* DNA) and negative (nuclease-free water) control.

PCR products were quantified using the Quant-it Picogreen assay (ThermoFisher Scientific), before being pooled at equimolar amounts. This pool was then purified using the AMPure XP purification kit

(Beckman-coulter). A TruSeq DNA PCR-Free library preparation was performed before paired-end 250 bp sequencing on the MiSeq platform across two sequencing lanes (Ramaciotti Centre, Sydney).

5.5.1 Bioinformatics

The amplicon sequence data was processed as described in Sutcliffe et al. (2017a) using the GHAP pipeline (https://doi.org/10.4225/08/59f98560eba25). All OTUs without a Domain-level assignment were removed from the dataset, along with OTUs assigned to the Cyanobacteria/ Chloroplast phylum with a >80% blastn similarity to known chloroplast sequences.

A total of 50 rarefactions to 5,000 counts per sample was performed on this OTU table. The average rarefied count per OTU (n=50) for each sample was then used for a final OTU table. This process was achieved using an in-house Python script.

5.5.2 Accessioning

This Transcriptome Shotgun Assembly project has been deposited at DDBJ/ENA/GenBank under the accession KBZE00000000. The version described in this paper is the first version, KBZE01000000.

5.5.1 Statistical analyses

5.5.1.1. Water and sediment chemistry

Statistical testing was performed as per Sutcliffe et al. (2018). Briefly, an in-house Python script using the packages *Scipy* and *Statsmodels* (Jones et al., 2001; Seabold and Perktold, 2010), was used to perform Benjimani-Hochberg false-discovery rate (FDR) adjusted ANOVA *p* values for each measure. This was followed by Tukey-Kramer posthoc analysis on measures found to vary significantly (Supplementary material S5.2). An alpha value of 0.05 was used to determine statistical significance. Pearson's correlation co-efficients were also calculated for each measure against the measured sediment copper concentration (Supplementary material S5.2).

5.5.1.2. Community analyses

Univariate and multivariate community statistics were performed as per Sutcliffe et al. (2018) using PrimerE v7.0 (Clarke and Gorley, 2006). To determine whether biodiversity indices (OTU richness, Pielous' evenness and Shannon's diversity) and phyla relative abundances altered between treatments, the in-house Python script described above was used to generate FDR-adjusted ANOVA *p* values and perform Tukey-Kramer analyses. Phyla relative abundances were transformed prior to this using the arcsine square root transformation (Underwood, 1997).

Non-metric multidimensional scaling (nMDS) plots generated in PrimerE were based on Bray-Curtis similarity matrices of Hellinger transformed data (Clarke and Gorley, 2006). Differences among a) sample types and b) copper treatments within sample types, were explored.

5.5.1.3. Biolog community substrate utilization profiles

For each Biolog Ecoplate, the average absorbance from control wells was subtracted from all other well absorbance values. The resulting absorbance values were averaged for each carbon substrate replicates from each plate. Community level physiological profiles (CLPPs) were visualised using principal components analyses using PrimerE (Clarke and Gorley, 2006). A PERMANOVA was also performed after first converting the table to a Euclidean similarity matrix (Clarke and Gorley, 2006). As described above, FDR-adjusted ANOVA *p* values and Tukey-Kramer comparisons were performed for each carbon substrate.

5.5.1.4. Bait mass loss measures

Final bait masses were calculated as a percentage of the initial mass. Treatment mass loss percentages were compared using an ANOVA and Tukey-Kramer analysis in the NCSS10 software (NCSS LLC, 2015).

5.6 RESULTS

5.6.1 Physicochemistry of the ponds

Copper was below detection limits in all water samples (Table 5.1). With the exception of the ponds spiked with a nominal 32 mg Cu kg^{-1} (40%), the measured values of copper in sediment were within 10% of the nominal copper concentrations (Table 5.1). Henceforth, we will refer to copper treatments by their average measured concentration (to 0 decimal places): 46, 60, 257 and 487 mg Cu kg^{-1}. There was no significant difference in water dissolved organic carbon (DOC) and sediment total organic carbon (TOC) concentrations between control ponds and treatments. The highest copper treatment, however, had a substantially higher DOC when compared to the control (average 6.2 mg L^{-1} for the control and 11.6 mg L^{-1} for the 487 mg Cu kg^{-1} treatment). The initial ANOVA p-value suggested that there was a significant difference in this metric, but upon false-discovery rate (FDR) correction, this p value was adjusted to become in significant ($p < 0.12$). The overall mean (±SE) DOC and TOC for the ponds were 7.4% (± 0.7) and 1.1% (± 0.04), respectively.

Table 5.1. Water and sediment chemistry. Values represent the mean of four replicate measurements (one from each replicate pond) ± standard error. Asterisks indicate measurements in which the treatment is found to be significantly different to the control measurement ($p < 0.05$).

Measurements below detection limits (n.d.)

	Water chemistry averages (± standard error)				Sediment chemistry averages (± standard error)			
	pH	EC (mS/m)	Dissolved organic carbon (%)	Copper (mg L^{-1})	pH	EC (mS/m)	Total organic carbon (%)	Copper (mg kg^{-1})
Control	8.0 ± 0.2	12.5 ± 1.0	6.2 ± 1.2	0.0 ± 0.0	7.6 ± 0.2	9.1 ± 0.6	1.0 ± 0.0	0.0 ± 0.0
46 mg kg^{-1}	8.1 ± 0.2	18.1 ± 2.4	6.5 ± 0.6	0.0 ± 0.0	8.2 ± 0.0	10.6 ± 0.5	1.1 ± 0.1	45.7 ± 2.7
60 mg kg^{-1}	8.2 ± 0.2	17.4 ± 4.1	7.0 ± 1.0	0.0 ± 0.0	8.0 ± 0.1	11.1 ± 0.7	1.0 ± 0.1	59.7 ± 4.4
257 mg kg^{-1}	8.2 ± 0.3	15.1 ± 0.7	5.9 ± 0.4	0.0 ± 0.0	8.3 ± 0.0	10.6 ± 0.1	1.1 ± 0.1	257.3 ± 14.5
487 mg kg^{-1}	7.9 ± 0.2	16.2 ± 1.8	11.6 ± 2.1	0.0 ± 0.0	8.3 ± 0.0	10.3 ± 0.5	1.0 ± 0.1	486.5 ± 13.8

As described in Sutcliffe et al. (2018) sediment pH, total carbon, calcium carbonate, and calcium concentrations were found to significantly differ between copper treatments (Supplementary material S5.2). Of these, all but the sediment pH showed a strong correlation with sediment copper concentration (Pearson's $r > 0.8$, Supplementary material S5.2).

5.6.2 Microbial communities in sediment: DNA vs RNA templates

Microbial communities were profiled using both DNA and RNA templates. Community diversity metrics (OTU richness, Pielous' evenness and Shannon's diversity) were all lower in the sediment RNA-based microbial communities when compared with the DNA-based communities (Supplementary material S5.3). Despite this, there was substantial overlap between the two communities with an average of 87% of OTUs common to both communities (Supplementary material S5.4). Of the remaining OTUs, 11% were found only in the DNA-based communities and 2% were detected only in RNA-based communities (Supplementary material S5.4).

DNA-based microbial communities in sediments had a high average relative abundance of Proteobacteria (43%), Unassigned Bacteria (13%), Acidobacteria (12%), Bacteroidetes (7%) (Supplementary material S5.5). In sediment RNA-based communities, the most abundant phyla were Proteobacteria (46%), Cyanobacteria (16%), Unassigned Bacteria (10%) and Bacteroidetes (7%). The Acidobacteria and Cyanobacteria showed consistent differences in relative abundances between DNA and RNA-based communities, across all copper treatments. Namely, Acidobacteria was substantially more abundant in the sediment DNA-based communities than the RNA-based communities, while an inverse relationship was observed for the Cyanobacteria (Supplementary material S5.5).

5.6.3 Effect of copper on sediment DNA and RNA-based microbial communities

Broadly, diversity metrics for sediment DNA and RNA-based communities decreased with increasing copper concentration (Supplementary material S5.3). For sediment DNA-based communities, mean Pielous' evenness and Shannon's diversity indices were significantly lower in the all copper treatments (46, 60, 257 & 487 mg Cu kg^{-1}) compared to the control. Mean Pielous' evenness and Shannon's diversity indices for sediment RNA-based communities were not significantly ($p > 0.05$) different for controls compared with treatments (Supplementary material S5.3).

Community composition of both DNA and RNA-based communities in control sediments were significantly different to those of all copper treatments (Supplementary material S5.6). Additionally, all copper treatments were significantly different to one another ($p < 0.05$; Supplementary material S5.4). Ordination plots (Figure 5.1A-B) support these PERMANOVA findings as they show clear separation between replicate samples from all treatment groups compared with control replicates.

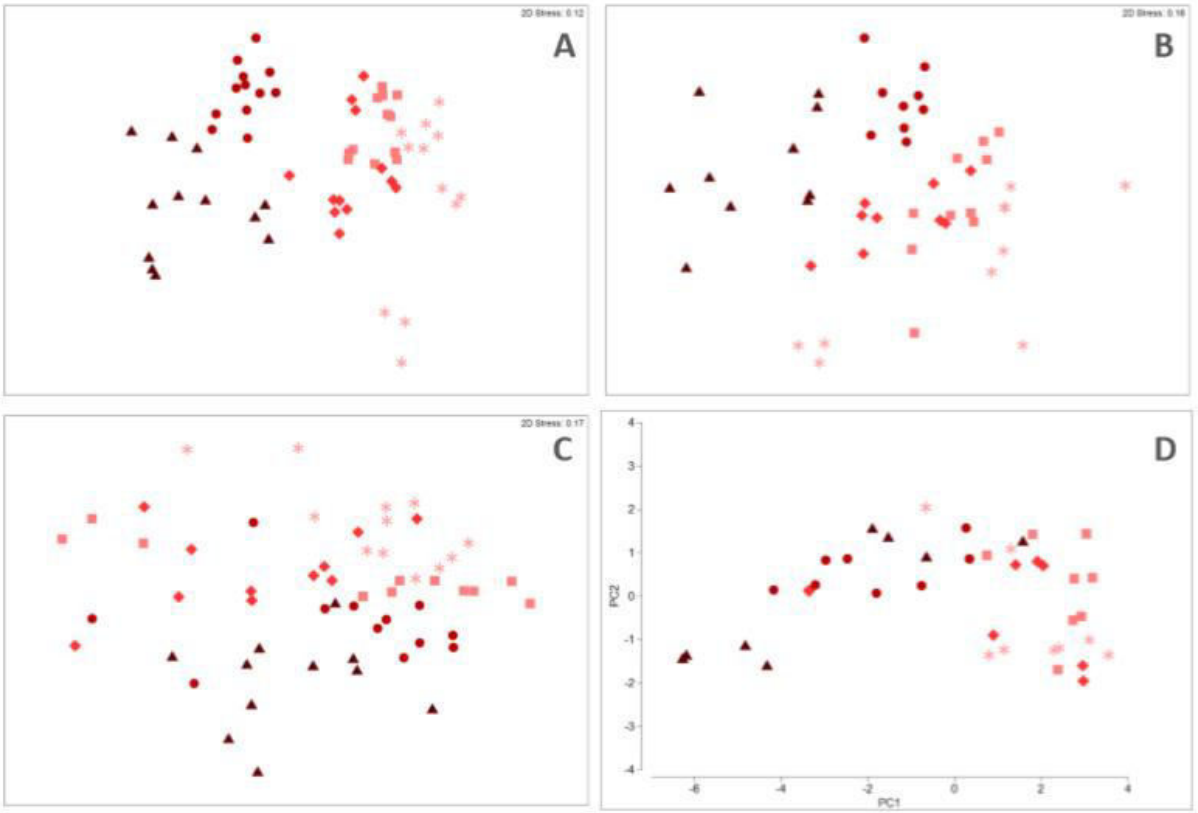

Figure 5.1. Non-metric multidimensional scaling (nMDS) plots showing the similarities of sample OTU composition in A) sediment DNA samples, B) sediment RNA samples and C) cellulose bait DNA samples. D) is a principal co-ordinate analysis plot showing the similarities of carbon utilization profiles. The colour of each point corresponds with the sediment copper treatment of the corresponding sample: * control, ■ 46 mg Cu kg^{-1}, ◆ 60 mg Cu kg^{-1}, ● 257 mg Cu kg^{-1}, and ▲ 487 mg Cu kg^{-1}.

Numerous phyla, in both the DNA and RNA sedimentary microbial communities, differed in their relative abundance in controls compared to the treatments (FDR-adjusted *p* value <0.05; Figure 5.2). The greatest number of phyla was observed in the sediment DNA-based communities, with the

relative abundance of 18 phyla altered under copper treatment, as well as unassigned Archaea and Bacteria (taxa which could not be assigned to a known phyla). Many of the most responsive phyla in the sediment DNA-based communities also showed similar trends in sediment RNA-based communities (Figure 5.2). For example, in sediment DNA and RNA-based communities, the bacterial phyla Fibrobacteres, Latescibacteria and Planctomycetes, as well as the archaeal phylum Woesearchaeota, generally decreased in relative abundance with increasing spiked copper concentration (0 > 46 > 60 > 257 > 487 mg Cu kg^{-1}; Figure 5.2). Conversely, in the same dataset, Acidobacteria, Gemmatimonadetes, Nitrospirae, SR1 and Thaumarchaeota phyla were found to have a significantly greater relative abundance in one or more of the two highest copper treatments (257 & 487 mg Cu kg^{-1}) compared with the control (Figure 5.2).

Interestingly, numerous phyla differed significantly in their relative abundance between treatments and controls in only the DNA, or RNA, sediment communities (but not both). For example, in the RNA-based communities the bacterial Hydrogenedentes and Poribacteria declined in relative abundance with increasing copper concentration, but these phyla did not differ significantly in the DNA-based communities (Figure 5.2). Conversely, in the DNA sediment communities, phyla Candidatus Saccharibacteria, Euryarchaeota, Microgenomates, Unassigned Archaea and candidate division WPS-1 had significantly lower relative abundances in two or more copper spiked treatments compared with the controls, but were only rarely detected in the RNA-based communities and thus, did not differ significantly in these communities (Figure 5.2).

	Phylum	FDR adjusted p value	Copper treatment (mg kg^{-1}) 0	46	60	257	487
Sediment DNA	Acidobacteria	1.0E-08			*	*	*
	Actinobacteria	5.5E-04				*	
	Bacteroidetes	1.6E-04					*
	Candidatus Saccharibacteria	7.7E-05		*		*	*
	Euryarchaeota	1.1E-02			*	*	*
	Fibrobacteres	1.0E-04		*	*	*	*
	Gemmatimonadetes	4.2E-09				*	*
	Latescibacteria	7.1E-19		*	*	*	*
	Microgenomates	4.1E-09		*	*	*	*
	Nitrospirae	3.7E-09				*	*
	Omnitrophica	9.1E-04			*		
	Pacearchaeota	7.8E-10				*	*
	Planctomycetes	6.7E-05				*	*
	SR1	6.7E-05					*
	Thaumarchaeota	1.8E-03				*	
	Unassigned_Archaea	1.0E-03		*	*	*	*
	Unassigned_Bacteria	2.1E-06					*
	Verrucomicrobia	2.8E-04				*	
	Woesearchaeota	1.2E-07			*	*	*
	candidate division WPS-1	1.8E-03				*	*
Sediment RNA	Acidobacteria	3.3E-06				*	*
	Actinobacteria	1.4E-02				*	
	Armatimonadetes	2.0E-02					*
	Crenarchaeota	1.3E-02					*
	Deinococcus-Thermus	7.0E-03					*
	Fibrobacteres	4.7E-04		*	*	*	*
	Gemmatimonadetes	6.7E-05					
	Hydrogenedentes	1.3E-02		*	*		*
	Ignavibacteriae	1.4E-02		*			
	Latescibacteria	2.0E-09		*	*	*	*
	Lentisphaerae	3.9E-02		*			
	Nitrospirae	8.0E-10				*	*
	Pacearchaeota	6.6E-03			*		
	Planctomycetes	1.9E-02			*		*
	Poribacteria	3.2E-02			*	*	*
	SR1	7.5E-04					*
	Thaumarchaeota	1.5E-02				*	
	Woesearchaeota	1.6E-02				*	*
Bait DNA	Acidobacteria	1.3E-03		*			*
	Cyanobacteria/Chloroplast	4.4E-02			*		
	Deinococcus-Thermus	2.8E-02					*
	Ignavibacteriae	5.3E-03			*		
	Planctomycetes	4.4E-02		*	*		
	Verrucomicrobia	5.3E-03			*		

Figure 5.2. Phyla found to significantly differ between one or more copper spikes when compared with the control. The relative average abundance of each phylum for each treatment is represented by colour. Pale yellow represents the lowest relative phyla abundance and red, the highest relative abundance. Asterisks denote significant differences between a treatment and the control.

5.6.4 Effect of copper on sediment community-level physiological profiles

Community-level physiological profiles (CLPPs), as determined using Biolog Ecoplates, were significantly different at day 7 for all copper treatments when compared to the control, except the 60 mg Cu kg^{-1} treatment (Supplementary material S5.6). The 60 mg Cu kg^{-1} treatment was significantly different to both the 257 and 487 mg Cu kg^{-1} treatments, but not the control or 46 mg Cu kg^{-1} treatment (Supplementary material S5.6). Ordination plots of these profiles on day seven separated the majority of 257 and 487 mg Cu kg^{-1} samples from controls on the first axis (Figure 5.1D). Samples from the 46 and 60 mg Cu kg^{-1} treatments were generally separated from Controls on the second axis. The 60 mg Cu kg^{-1} samples appeared highly diverse in this ordination, however, with one pond clustering with 487 mg Cu kg^{-1} samples and another three closest to controls (Figure 5.1D).

Of the 31 carbon substrates tested, 24 (77%) had significantly reduced rates of utilisation under one or more copper treatments compared to the controls (Figure 5.3; Supplementary material S5.7). Overall, the amino acids were metabolised less by sediment microbial communities of the 257 and 487 mg Cu kg^{-1} treatments, compared with those of the lower concentration treatments and controls (Figure 5.3; Supplementary material S5.7). Despite this, not all ecologically or chemically similar substrates showed similar trends (Supplementary material S5.7). For example, D-cellobiose and D-xylose – a disaccharide, and pentose monosaccharide from plant material – differed in their usage; at day seven, D-cellobiose utilization was similar between copper treatments and controls, while D-xylose utilization in 257 and 487 mg Cu kg^{-1} treatments was less than the control and all other treatments (Figure 5.3). Further, the utilization of 2-hydroxybenzoic acid was significantly reduced in all treatments relative to controls, whereas the utilisation of 4-hydroxybenzoic acid was only impaired at 487 mg Cu kg^{-1} (Figure 5.3).

Carbon substrate	FDR adjusted p value	Copper treatment (mg kg^{-1})				
		0	46	60	257	487
2-Hydroxybenzoic Acid	4.8E-04		*	*	*	*
4-Hydroxybenzoic Acid	4.8E-04					*
α-D-Lactose	4.1E-03					*
α-Ketobutyric Acid	4.6E-02					*
D-Galactonic Acid γ-Lactone	3.2E-03				*	
D-Galacturonic Acid	1.1E-04				*	*
D-Glucosaminic Acid	4.2E-04				*	*
D-Malic Acid	4.8E-04			*		*
D-Mannitol	3.7E-03					*
D-Xylose	4.1E-03				*	*
Glucose-1-Phosphate	9.3E-03					*
Glycogen	8.9E-03				*	*
Glycyl-L-Glutamic Acid	9.2E-03					*
L-Arginine	5.9E-06				*	*
L-Asparagine	3.0E-03					*
L-Phenylalanine	2.6E-02					*
L-Serine	1.1E-04				*	*
L-Threonine	7.1E-04				*	*
Phenylethylamine	4.8E-04				*	*
Putrescine	5.4E-06					*
Pyruvic Acid Methyl Ester	4.8E-04				*	*
Tween 40	9.5E-05					*
Tween 80	4.8E-04					*
i-Erythritol	4.8E-04				*	*

Figure 5.3. Biolog Ecoplate carbon substrates which had a significantly different utilisation rate at day 7 between at least one treatment and the control. Average dye development is used to represent the amount of substrate utilised by a microbial community. This figure represents the relative amount of dye development for each substrate by colour. Pale yellow represents the lowest amount of dye developed, and red, the highest amount. Asterisks denote significant differences between a treatment and the control.

5.6.5 Effect of copper on baited microbial communities

Baited microbial communities were distinctly different to both the water and sediment microbial communities (Supplementary material S5.4 & S6). A subset of microbial OTUs were enriched on these baits, many of which belonged to the proteobacterial Myxococcales order, although the specific OTUs from this Order varied between copper treatments (Supplementary material S5.5).

Copper did not have an effect on the univariate metrics for bait microbial communities ($p > 0.05$; Supplementary material S5.3). However, the composition of the communities was altered by copper, with all treatments significantly different from the control ($p < 0.05$), and all other treatments

(Supplementary material S5.4; Figure 5.1C). Six phyla differed significantly in their average relative abundance for one or more copper treatments when compared to control samples. Of these, trends along the copper gradient were inconsistent in their statistical significance. For example, the Acidobacteria phylum was more abundant in baited communities from all treatments compared with controls, however, only the 257 and 487 mg Cu kg^{-1} treatments were found to be significant (Figure 5.2).

SEM imaging of cellulose baits revealed that extensive eukaryotic and prokaryotic colonisation of the baits under all copper treatments (Figure 5.4). A variety of morphologically distinct organisms (eukaryotes) and cell types (eukaryote and prokaryote) were observed on these baits but differences in these morphotypes between copper treatments and controls were difficult to infer (Figure 5.3C-F). When comparing different treatments qualitatively, no difference could be observed. There were few instances in which the cellulose fibres of the baits could be observed, as putative biofilm-like structures had formed over their surface (Figure 5.4C-F). Where the fibres were exposed, pitting could be observed, a sign of cellulose decomposition (Figure 5.4G). Within these pits were rod shaped cells (Figure 5.4G).

5.6.6 Effect of copper on cellulose bait decomposition

Changes to bait mass over the time of deployment (118 days) were not significantly different between copper treatments and controls (ANOVA p value > 0.05; Supplementary material S5.8). Changes to mass were highly variable, with a number of baits increasing in mass (final mass >100% of initial mass), while one bait deployed into a 46 mg Cu kg^{-1} pond had a final mass of ~30% of its initial mass (Supplementary material S5.8).

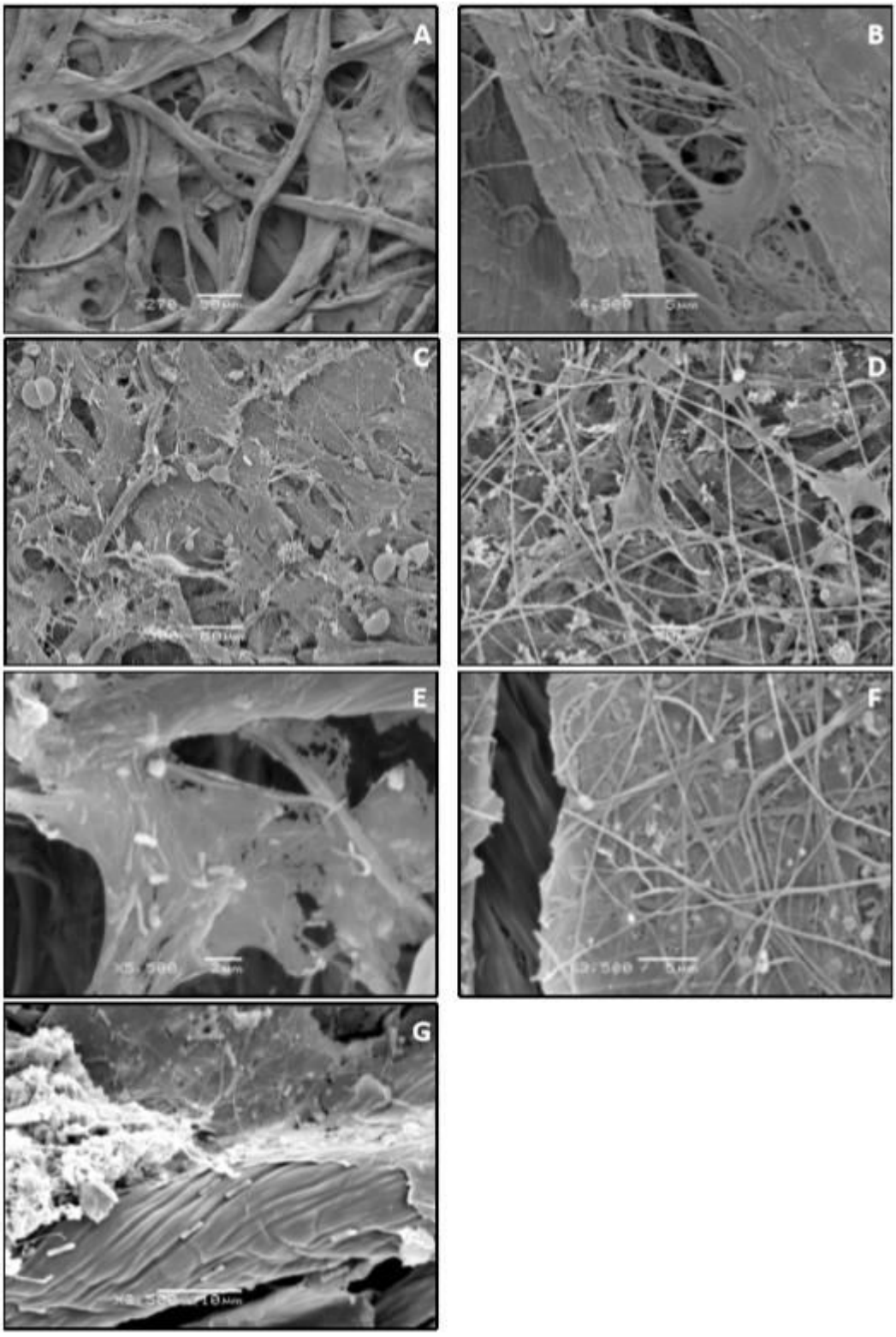

Figure 5.4. Scanning electron microscopy images of cellulose baits. A) and B) are images of blank cellulose papers at different magnifications. An overview of biofilm formation and eukaryotic colonisation of the baits can be seen in C) and D) which were deployed into control and 46 mg kg^{-1} copper treated ponds, respectively. E) and F) are images of biofilms at greater magnification, showing

an assortment of prokaryotic cell morphologies. These baits were from 46 mg kg^{-1} and 487 mg kg^{-1} copper treated ponds, respectively. G) is of a bait from a 257 mg kg^{-1} copper treatment and shows an exposed cellulose fibre with evidence of cellulolytic degradation i.e. furrows caused by microbial activity. All deployed baits were left in ponds for a total of 118 days.

5.6.7 Effect of copper on water microbial communities

The total microbial community composition of water samples were significantly different to those in sediment and bait samples (Supplementary material S5.4). These communities were also generally unaffected by copper concentration, with no phyla shown to significantly differ in composition between controls and treatments (data not shown).

5.7 DISCUSSION

Collectively, our findings clearly demonstrate that sediment microbial communities display a remarkable sensitivity to sediment-bound copper. Copper altered the composition of both DNA and RNA sediment microbial communities under all treatment concentrations, the lowest treatment containing just 46 mg Cu kg^{-1}. Additionally, the microbial communities colonising leaf analogues on sediment surfaces were also altered by all copper treatments, while the community-level physiological profiles (CLPP) were altered in most treatments (46, 257 & 487 mg Cu kg^{-1}). This suggests that at relatively low concentrations, particulate copper not only alters microbial community composition, but also their recruitment onto deposited organic material and community-level physiology.

5.7.1 Effects of copper on microbial taxa

The taxa that were sensitive to copper enrichment in both DNA and RNA-based communities were from the phyla Fibrobacteres, Latescibacteria, Planctomycetes and Woesearchaeota (Figure 5.2). Members of the Fibrobacteres and Latescribacteria were particularly sensitive, showing statistically significant decreases in relative abundance for all treatments compared to the controls. Several members of the Fibrobacteres and Latescribacteria appear to be highly specialised primary

decomposers. For example, *Fibrobacter* isolates possess specialised metabolic strategies for the catabolism of plant polymers (Ransom-Jones et al., 2012), while genomic data for the Latescibacteria phylum indicates a specialisation in algal biomass catabolism, along with plant polymer catabolism (Farag et al., 2017; Youssef et al., 2015). Thus, the decline of these taxa in response to sediment-bound copper may indicate a corresponding decline in primary decomposition.

Taxa belonging to the Planctomycetes and Woesearchaeota are reported to be involved in symbiotic or parasitic interactions (Bengtsson and Øvreås, 2010; Cai et al., 2005; Castelle et al., 2015; Jetten et al., 2003). For example, Planctomycetes are overwhelmingly associated with photosynthetic organisms as epiphytic biofilm taxa, using exudates from primary producers for energy (Bengtsson and Øvreås, 2010; Cai et al., 2005; Jetten et al., 2003). A number of Planctomycetes are also uniquely capable of anaerobic ammonium oxidation (Kuenen, 2008). Thus, the decline of these prokaryotic organisms may be indicative of a disruption to carbon and nitrogen transformations and translocation within the food web, or the loss of photosynthetic eukaryotes not captured in this study.

In contrast to the above taxa, a number of taxa increased in relative abundance under copper treated conditions, including Acidobacteria and Nitrospirae. Many of the acidiobacterial OTUs showing the greatest increases under elevated copper concentrations are affiliated with the metal-reducing Holophagaceae family, e.g. *Geothrix*-like OTUs (Supplementary material S5.5; Coates et al., 1999; Liesack et al., 1994; Sutcliffe et al., 2017b). Thus, these taxa may be directly benefitting from the presence of copper in the sediments, using the metal - or other metals displaced by the copper - to respire. In comparison, while many *Nitrospira,* from the Nitrospirae, have been detected from metal enriched environments (Ehrich et al., 1995; He et al., 2008), to our knowledge, they are not associated with metal-respiration. This suggests that *Nitrospira*-like taxa are not energetically benefitting from the presence of copper, but rather are more resilient than other taxa within the community. Importantly, members of the Holophagaceae and Nitrospirae appear to have limited

catabolic potential (Coates et al., 1999; Ehrich et al., 1995; Liesack et al., 1994; Nunes-Alves, 2016; Sutcliffe et al., 2017b), and thus, are unlikely to fulfil the role of the declining putative primary decomposers discussed above i.e. Fibrobacteres, Latescibacteria and Planctomycetes. Together, these findings demonstrate that while functional redundancy is present in a robust, healthy ecosystem, this does not ensure that all ecological niches are filled when a system is disturbed.

5.7.2 Effects of copper on cellulose bait colonisation and decomposition

The communities detected on cellulose baits after *in situ* deployment into copper treated mesocosms were compositionally different when compared with control mesocosms. Additionally, these communities differed between treatments, showing a concentration dependant response (Figures 2). The majority of taxa identified in baited communities were recruited from the sediment (Supplementary material S5.4), and thus, the altered composition of baited communities may be an indirect effect of a copper-altered sediment community. Despite this, communities inhabiting baits were expected to have less exposure to copper than communities in sediments, and thus this sensitivity is noteworthy.

Interestingly, while microbial community structure was highly responsive to copper, cellulose decomposition rates did not change between treatments. Some baits were undoubtedly degraded (Supplementary material S5.8), with clear cellulose fibre pitting observed microscopically (Figure 5.4). In addition to this, known prokaryotic cellulose-degraders were detected in bait communities (for example, the increase in members from the Bacteroidetes), along with cellulolytic fungi in Sutcliffe et al. (2018). In contrast, thick biofilms were also observed on bait surfaces, which may explain the mass gain of several baits (Figure 5.4; Supplementary material S5.8). These findings suggest that dual processes were occurring on the baits, one being cellulolytic degradation and the other being the utilisation of cellulose as a scaffold for biofilm formation.

Cyanobacteria, a group of prokaryotes which fix carbon through photosynthesis, were detected at relatively high abundance in bait communities. This finding shows that the study's baiting technique

was not specific for cellulolytic microorganisms and instead harboured complex periphyton communities. A potential explanation for this is the simplicity of the baits used. Typically, natural cellulose-containing materials will contain numerous other polymers, including DNA, amino acids and lipids, which contain carbon, but also nitrogen and phosphorus. The pure cellulose baits did not contain nitrogen or phosphorus, but a microbe cannot subsist on cellulose alone and must presumably access these nutrients from other sources. In periphyton communities, photosynthetic Cyanobacteria fix nitrogen and this is disseminated to other heterotrophic microbes within the biofilm (Sigee, 2005). Similarly, fungal members on these bait communities potentially gained nitrogen and phosphorus through predation (Sutcliffe et al., 2018). Therefore, nutritional limitations of cellulose baits may have produced a more dynamic and complex microbial community than expected. An alternative method for assessing the decomposition rates of biopolymers would be the use of naturally-occurring, more complex, plant material (for example leaf litter). This material, however, is likely to attract an array of microbial functional types, able to utilise any number of substrates found within these materials, including DNA, protein, hemicellulose, pectin etc. and thus, the interpretation of these communities would also harbour difficulties.

Importantly, periphyton biofilms are recognised as distinct biomes for microbial communities in freshwater systems. They are the foundation of food webs, contributing to the production of these environments (Buesing et al., 2009; Liboriussen and Jeppesen, 2003; Vadeboncoeur et al., 2002; van Dam et al., 2002). Thus, the copper-induced changes described here may result in significant ecological consequences, which warrant further investigation.

5.7.3 Effects of copper on sediment community-level physiological profiles

In addition to altering the taxonomic composition of microbial communities, particulate copper also altered sediment CLPPs (Figure 5.3). In general, carbon substrate utilisation declined as sediment copper concentrations increased. The greatest change in utilization was observed for 2-hydroxybenzoic acid; its utilisation being reduced in all copper spiked treatments (Figure 5.2).

Aromatic compounds such as these are found within lignin polymers - a primary constituent of plant vascular tissues and wood (Kirk and Farrell, 1987; Milstein et al., 1983), and are also common components of plant secondary metabolites (Khadem and Marles, 2010). Further to this, the utilisation of xylose was found to be lower for sediment communities in the 257 and 487 mg Cu kg^{-1} treatments (Figure 5.3). Xylose is a plant pentose sugar that is prevalent in cellulolytic material and supplied to microbes involved in syntrophic relationships with plants (Kogel-Knabner, 2002). These two trends are in accordance with the use of copper to prevent wood decay (Freeman and McIntyre, 2008), and indicate that plant biomass turn-over in these systems may be reduced, even at sediment copper concentrations of just 46 mg kg^{-1}. A reduction in the utilization of phenolic compounds such as 2-hydroxybenzoic acid has also been proposed as a broader ecotoxicological issue. Doelman et al. (1994) hypothesized that sites co-contaminated with metals and polyaromatic hydrocarbons will be more resistant to hydrocarbon bioremediation efforts.

5.7.4 Comparing microbial community profiling approaches

We hypothesized that RNA-based microbial community profiling would be more sensitive to sediment-bound copper, when compared with DNA-based microbial community profiles. This hypothesis is based on high amounts of extracellular environmental DNA (Anderson-Carpenter et al., 2011), and estimates that indicate a large portion of the microbial population are inactive (Blagodatskaya and Kuzyakov, 2013). Thus, these reservoirs of “background” DNA may obscure copper-induced changes to living biomass, as detected through DNA profiling. In contrast, RNA is actively transcribed by a cell, and thus, microbial communities detected through RNA profiling are presumably active at the time, or just prior to, sampling. This reasoning is consistent with previous studies reporting compositional shifts in RNA-based communities precede those in DNA-based communities (Girvan et al., 2003; Zhang et al., 2014). Our results, however, did not support this hypothesis. Indeed, DNA-based microbial communities were more sensitive than RNA-based communities when considering univariate biodiversity metrics (Pielous’ evenness and Shannon’s biodiversity indices), while the composition of both DNA and RNA-based communities were sensitive

to all copper treatments (Figure 5.1; Supplementary material S5.3). Interestingly, 87% of microbial taxa identified in the sediment samples occurred in both the DNA and RNA-based communities (Supplementary material S5.4). This is greater than the estimated 60% of microbial communities thought to be associated with active/ potentially active members (Blagodatskaya and Kuzyakov, 2013; Carini et al., 2016) and is suggestive of a highly diverse, metabolically active microbial community within this system.

Further to the above hypothesis, we also hypothesised that molecular surveying approaches would be more sensitive than physiological assays. The basis for this is the purported functional redundancy within these communities, whereby a copper-sensitive taxa could be replaced by another copper-resilient taxa with the same functional capabilities. As discussed above, decomposition rates could not be determined using the cellulose baiting technique employed in this study, but the CLPP approach demonstrated a similar sensitivity to the DNA- and RNA-based community profiling, i.e. most copper treatments tested showed significant differences to controls. Notably, however, the 60 mg Cu kg^{-1} treatment was not statistically different to the control using a CLPP approach (Supplementary material S5.6). Ordination plots of the CLPPs (Figure 5.1D) suggest that this treatment showed an overall difference to the control, but that three of the eight replicates did not. This variability within the 60 mg Cu kg^{-1} treatment may have been caused by technical error, or genuine biological variation, and future studies should be carried out to resolve this further. Additionally, future studies which investigate a gradient of lower concentrations, for example 0 to 70 mg Cu kg^{-1}, with smaller increments between treatments, would further clarify both structural and functional threshold concentrations for microbial community responses to copper. Broadly, however, this study does not support the conclusion that molecular profiling surveys are more sensitive than CLPP approaches and comparisons across different environments, and environmental disturbances, is needed. Additionally, lower copper concentrations should be tested to interrogate limits of detection for these particular techniques.

5.8 SIGNIFICANCE

Our results demonstrate significant compositional changes to microbial communities in sediments containing just 46 mg kg^{-1} of copper. This finding applies to both fungal (Sutcliffe et al. 2018) and prokaryotic communities (this study). Additionally, sediment CLPPs, as well as the subset of microbes recruited onto cellulose baits, differed at 46 mg kg^{-1} of copper when compared to controls. These findings demonstrate a remarkable sensitivity in sediment microbial communities to copper, with 46 mg kg^{-1} of copper well below values reported to effect eukaryotic benthic communities in these same mesocosms (Gardham et al., 2014b). This concentration is also well below the guideline sediment value of 65 mg kg^{-1} used in Australia, New Zealand and Hong Kong, and 51 mg kg^{-1} used in Norway (Bakke et al., 2010; Burton, 2002; Simpson et al., 2013).

An important consideration for this study is the correlation between sediment copper concentration and sediment total carbon, calcium carbonate and calcium concentrations (Supplementary material S5.3). This finding is consistent with the spiking method employed in this study, namely, the addition of garden lime ($CaCO_3$) to ensure that the sediment pH was not lowered with the addition of copper (Gardham et al., 2014a). Additionally, copper is known to be adsorbed to calcium carbonate (Papadopoulos and Rowell, 1989; Pickering, 1983), and thus the addition of calcium carbonate is likely to have played a role in maintaining high sediment-bound copper concentrations in the highest treatments, through immobilisation. A review of the literature suggests that the effects of calcium carbonate on microbial communities have not been investigated, and thus, we cannot confirm that the findings in the current experiment are not influenced by an effect of calcium carbonate. It is noteworthy, however, that prokaryotic communities are known to mineralise calcium carbonate in a number of natural settings (Beveridge et al., 1982; Cacchio et al., 2003; Douglas and Beveridge, 1998; Dupraz et al., 2009).

5.9 CONCLUSIONS

This is the first molecular survey to investigate the response of prokaryotic communities to sediment-bound copper exposures of >1 year. We demonstrated that both DNA and RNA-based microbial communities in sediment were significantly altered by sediment-bound copper enrichment at, and above, 46 mg kg^{-1} total extractable copper. In addition to this, sediment communities showed a significant reduction in the breadth and relative rate of substrate utilisation under these elevated copper concentrations. These findings suggest that current sediment quality guideline values used in many countries (e.g. Australia, New Zealand, Hong Kong and Norway) to protect aquatic ecosystems may not protect microbial communities and their function. Given the importance of microbial communities in maintaining key ecological and functional processes, it is critical that they are considered in ecological risk assessments and the setting of environmental quality criteria.

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6 DISCUSSION

6.1 URANIUM ALTERS THE TAXONOMIC AND FUNCTIONAL POTENTIAL OF TROPICAL SEDIMENT MICROBIAL COMMUNITIES

Due to the presence of a U mine in the Alligator Rivers region of the Northern Territory of Australia, U is a relevant ecological and ecotoxicological concern for this area. Chapter 2 aimed to determine how sediment-bound uranium concentration impacts the genetic composition of prokaryotic communities in tropical sediments, and at what concentrations these alterations occur. This represents the first study to investigate the *in situ* effects of U on indigenous sediment microbial communities in Australia, and more broadly, is the first study to apply a controlled U gradient to microbial communities in the field anywhere in the world. Such an approach allowed for the capture of complex, natural, physiochemical and biological fluctuations in the environment, while also allowing for the investigation of responses to a broad range of known U concentrations with a general omission of confounding factors. While no single study design is without caveats, these features are important in assessing the effect of U concentration microbial communities in an ecologically meaningful manner. Additionally, the controlled concentration gradient provides a baseline study for future inter-study comparisons, contributing to our understanding of environmental features shaping U toxicity in prokaryotic communities, and how the presence of co-contaminants effect this U toxicity.

Data from Chapter 2 indicated that across a U concentration gradient of 0 - 4,000 mg kg^{-1}, numerous taxonomic and functional changes occurred in the sediment prokaryotic community. Taxa belonging to the Acidobacteria Groups 1 and 3, as well as the Rhizobiales, appear to be particularly sensitive to U concentration, and may be useful indicator taxa for future ecotoxicological monitoring efforts. Functions which appeared to decline at U concentrations ≥1,500 mg kg^{-1} included aerobic respiration and the scavenging of sugar oligosaccharides from plant-derived polysaccharide breakdown. Both of

these trends may be explained by a decline in bioturbation and biodeposition by macroinvertebrates, highlighting the value of the *in situ* approach undertaken in this study, by showing the importance of capturing biological interactions and co-dependencies between different trophic levels.

Along the U gradient, a number of anaerobic taxa and functional genes also increased in relative abundance, highlighting a shift from aerobic to anaerobic metabolic processes. These included putative metal-reducing taxa and methanogenic archaea, as well as genes involved in methanogenesis. Additionally, nitrogen fixation genes which convert nitrogen gas to ammonia, increase in relative abundance under elevated U concentrations. Together, these trends suggest that U contamination has important implications for nitrogen and carbon cycling in sediments, potentially leading to a decrease in the rates of primary decomposition and an increase in ammonia and methane production. These processes, in addition to altering the biogeochemistry of the sediments, would alter the forms in which nitrogen and carbon are available to other members of the microbial community. For example, these processes would favour prokaryotic taxa which utilise ammonia and methane, further altering microbial assemblages. These alterations may affect higher trophic levels, as microbes form the base of food webs and thus, an alteration to these communities represents an alteration to the food source of many invertebrate and vertebrate biota.

Some of the putative metal-reducing taxa found to increase in relative abundance under U enrichment, for example *Geobacter, Geothrix* and *Holophaga* spp., are associated with the reduction of U(VI) to U(IV). As discussed in Chapter 1, U(IV) readily forms insoluble complexes, a characteristic which is known to decrease the bioavailability, and hence toxicity, of metals towards macrobiota. In addition to this, the complexation of U(IV) in sediments, immobilises the metal, limiting its distribution within aquatic systems. Anthropogenic stimulation of *Geobacter* and *Geothrix* taxa in U contaminated aquifers, via labile carbon amendments, results in the immobilisation of U within the system (reviewed in Zachara et al., 2013). Thus, the increased abundance of these taxa without

targeted stimulation may indicate a natural process occurs within these sediments which would limit overall toxicity and mobility of U within this billabong system.

6.2 LIFE UNDER URANIUM ENRICHMENT – INSIGHTS FROM THE GENOMES OF URANIUM TOLERANT TAXA

Chapter 3 aimed to determine what ecological strategies are employed by abundant prokaryotes inhabiting sediments with elevated uranium concentrations. The assembly and contig binning allowed multiple individual genomes to be identified from data for the highest U treatment (~4 g kg^{-1} of U). Of the genomes identified, three were assigned to the genera: *Geobacter, Geothrix* and *Dyella*, however, they appeared to be novel species and were given the identities: *Geobacter* sp. GB1*, Geothrix* sp. GB2 and *Dyella* sp. GB3, respectively. Two more assembled genomes were identified and found to belong to a novel class within the Bacteroidetes (Bacteroidetes sp. GB4) and a new order within the Anaerolineae (Anaerolineae sp. GB5). Finally, a mixed species bin was found to contain multiple genomes from members of the methanogenic archaeal genus, *Methanocella*. The depth of sequence coverage for these genomes, along with 16S amplicon data, suggest that these were the most abundant microorganisms in sediments from the 4 g kg^{-1} U treatment, and that all but Anaerolineae sp. GB5, increased in relative abundance along the U concentration gradient, indicating that the corresponding microorganisms gained a selective advantage in sediments with elevated U concentrations.

The detection of *Geobacter* sp. GB1*, Geothrix* sp. GB2 and *Dyella* sp. GB3, is consistent with previous studies which have observed the abundance of close relatives in U contaminated aquifers (Green et al., 2012; Hemme et al., 2010; Zachara et al., 2013). The convergence of these trends suggest a broader pattern in the selective pressure exerted by U on microbial communities, a surprising finding given the divergent physiochemical features in groundwater systems and tropical billabong

sediments. Thus, U appears to exert a strong selective pressure in freshwater systems, driving microbial community convergence irrespective of other varied environmental conditions.

Interestingly, ecophysiological profiling of the six genome bins found these microorganisms undertook markedly different ecological roles. Data presented in Chapter 3 suggest that niche partitioning based on substrate utilisation and redox optima occurred within these communities. For example *Dyella* sp. GB3, Bacteroidetes sp. GB4 and Anaerolineae sp. GB5 appear to play roles in primary decomposition, while the limited catabolic potential of *Geobacter* sp. GB1*, Geothrix* sp. GB2 and the *Methanocella* spp. suggests that they use simple, labile carbon sources. Given that *Geobacter* sp. GB1*, Geothrix* sp. GB2 and the *Methanocella* spp. possess genes for anaerobic respiration and that the availability of these simple, labile carbon sources in anoxic sediment is low, this finding suggests a dependency on the metabolic activities of other microorganisms within this community, potentially *Dyella* sp. GB3, Bacteroidetes sp. GB4 and Anaerolineae sp. GB5. Additionally, in contrast with *Geobacter* sp. GB1*, Geothrix* sp. GB2 and the *Methanocella* spp., the genomes of *Dyella* sp. GB3 and Bacteroidetes sp. GB4 possessed aerobic respiration pathways, and thus these microorganisms may occupy separate redox-defined niches within the sediment matrix.

Both *Geobacter* sp. GB1 and *Geothrix* sp. GB2 had a genetic capacity for U(VI) reduction, indicating that these microorganisms have a metabolic specialisation which particularly equips them for survival in environments with high concentrations of U. This ability, however, is not broadly distributed among the genomes described in Chapter 3, suggesting that while some forms of U(VI) reduction are associated with a reduction in U toxicity (Cologgi et al., 2011), this is a positive side effect of the metabolic processes, and not a broad U tolerance strategy. In contrast, multiple genes assigned to the heavy metal efflux transporter subfamily, from the resistance nodulation division (HME-RND), were detected across these genomes. This finding is consistent with the findings of Hemme and colleagues, who found a similar trend for described genomes from a U contaminated aquifer (Hemme et al., 2010). Previous studies have determined that HME-RND genes are rare, often

occur on plasmids, and are associated with an enhanced tolerance of metals such as copper, zinc, cadmium and nickel (Nies, 2003). Together, these findings implicate the HME-RND transporter subfamily with a role in U tolerance.

6.3 SHOTGUN METAGENOMICS: OPPORTUNITIES AND SHORTCOMINGS

Chapters 2 and 3, use two different analysis strategies for the shotgun metagenomic data obtained in this study. In Chapter 2, sequenced reads were compared to a public database and the relative abundance of genes with known functions was compared along the U concentration gradient. This method circumvents the need for sequence assembly, which can be challenging for metagenomes of very complex microbial communities. In Chapter 3, reads from the highest U concentration were assembled into contigs which were then separated by their sequencing coverage and tetramer composition into genomes.

The ecophysiological profiling of assembled draft genomes demonstrates the remarkable utility of metagenomic sequencing. This method has allowed insights into the possible life-strategies, ecological roles and inter-species relationships of uncultured taxa. While follow-up experiments which aim to cultivate identified taxa are a worthy endeavour, these genomic findings may be used to guide such efforts (see section 6.7.3) and the interim insights afforded by genome ecophysiological profiling provides an important ecological context for the findings of metagenomic surveys. In Chapter 5, many taxa of interest belonged to phyla for which there are no described cultured representatives (e.g. Latescibacteria, Woesearchaeota and Microgenomates). The ability to determine ecologically meaningful information from uncultured organisms, thus informs future culture-based or metagenomic investigations.

Importantly, the genome assembly approach described in Chapter 3 was undertaken for all U treatments, but only the highest treatment had sufficient sequencing coverage for a significant amount of assembly, and subsequent genome identification. Despite having an average of 56.5

million reads per sample, the shotgun metagenomic datasets for all other treatments proved too diverse and complex for sufficient coverage, a reflection of the complexity and diversity of microbial communities in these treatments. In the highest U treatment, sufficient sampling depth was achieved because microbial community evenness and diversity were lowered by the high U dose. Similarly, successful shotgun metagenomic studies reporting the assembly of genomes typically investigate the less complex microbial communities in extreme environments, such as hypersaline soda lakes and acid mine drainage pipes (Hua et al., 2015; Vavourakis et al., 2016). For undisturbed sediments, which are a reservoir of microbial diversity (see Chapter 1), recent studies which successfully identify genomes from shotgun metagenomic sequencing, report large sequencing datasets of ~700 to 1,900 million reads (Baker et al., 2015; Castelle et al., 2013). The costs associated with this amount of sequencing, however, can be restrictive for many laboratories at this point in time and thus, this underscores a substantial limitation of shotgun metagenomic sequencing. Namely, that environments with highly diverse and even microbial communities are difficult to sequence at sufficient depth for genome-level analyses.

Given that genome assemblies could not be achieved in the remaining six U treatments, an alternative method was required. As such, a read-based analysis approach was undertaken in Chapter 2, and allowed comparisons to be carried out across the U concentration gradient that would have otherwise been impossible. This data offered support for the ecological relevance of taxonomic shifts observed in the 16S rRNA amplicon data, linking microbial community structure and functional composition. These findings may be used to guide future investigations, namely, *in situ* measurements of carbon and nitrogen flux (see section 6.7.1), and would not have been possible based solely on the assembled genome data presented in Chapter 3.

While both of the metagenomic analytical approaches outlined here provided novel, and important, insights into the microbial communities occupying U enriched sediments, both were, in part, limited by what is already known about microbial taxonomy, metabolism and ecology. Specifically,

metagenomic reads and assembled putative genes were searched against databases in order to identify their putative roles through homology. While novel metabolic pathways have been identified from metagenomes in the past, these studies are uncommon and require culture-based assays with *E. coli* fosmid libraries, a limitation of which, is that novel genes must belong to microorganisms with a close taxonomic relationship to *E. coli* (reviewed in Gilbert & Dupont 2011). Generally, insights from most metagenomic analyses are limited to genes which are similar to those in described organisms, however, relatively few microorganism have been described and, as demonstrated by this study, metagenomic investigations often yield novel sequences (Chapter 3). Thus, there is a pressing need to improve databases and metabolic models on which these metagenomic studies are informed (see section 6.7.3).

6.4 COPPER ALTERS THE TAXONOMIC AND FUNCTIONAL POTENTIAL OF SUBTROPICAL SEDIMENT MICROBIAL COMMUNITIES

6.4.1 Fungal communities

Fungi play an important ecological role in biomass turn-over and the movement of nutrients within the aquatic environment. Despite this, very few studies have been conducted on aquatic fungi, indeed, the current study is the first next-generation sequencing survey of freshwater sediment fungal communities. In addition, little is known about the effects of Cu on fungal communities, with studies investigating Cu toxicity typically involving axenic cultures. Thus, this study aimed to determine the fungal communities inhabiting freshwater ponds, surveying three distinct biomes; the pelagic, benthic and cellulose baits, and then examine the effects of Cu on these communities across a spiked Cu gradient.

Freshwater pelagic fungal communities were found to have a relatively high abundance of ascomycetes and basidiomycetes with yeast forms. This feature distinguished pelagic communities from their benthic and cellulose-attached counterparts, which had few yeast members. Benthic

communities were characterised by a comparatively high abundance of putatively mycorrhizal taxa from the basidiomycetes order Sebacinales. Taxa belonging to the same phyla and related to *Psathyrella*, were also relatively abundant, with *Psathyrella* spp. known to produce specialised aquatic fruiting bodies (Frank et al., 2010). Surprisingly, basidiomycetes represented a large portion of the benthic communities in these ponds, despite the phylum being considered largely non-aquatic (Shearer et al., 2007). Together, these findings suggest that a broader range of basiodomycetes taxa have specialised aquatic lifestyles or life stages. Future work, however, is required to establish these taxa as resident fungi, rather than immigrant taxa which are dispersed into the ponds without adaptions for this environment.

In contrast to pelagic and benthic communities, cellulose-baited communities showed a marked increase in putative nematophagous fungi and Chytridiomycota. In fact, three chytrid OTUs detected in the baits represented ~30% of these communities. This trend was perhaps the most striking for the fungal communities described, in part, because of the novelty of these chytrids. While physiological investigations of a handful of chytrids are documented in the literature (Haskins, 1946; Whiffen, 1941), it is difficult to extrapolate these findings with the novel chytrids detected here. Indeed, phylogenetic analyses of chytrid OTUs detected in the current study, demonstrate that much of this phylum's taxonomy is yet to be finalised. This knowledge gap is especially relevant to freshwater ecology, given the abundance of these chytrids as well as their putative roles in cellulose degradation and parasitism of aquatic biota (Gleason et al., 2008). Additionally, chytrids appeared to be particularly sensitive to sediment-bound Cu, with the phylum declining under concentrations of >45 $mgkg^{-1}$ of Cu, making these species putatively useful indicators of copper contamination.

6.4.2 Prokaryotic communities

Chapter 5 aimed to determine whether sediment copper effects the structure and function of microbial communities. This work demonstrated that, indeed sediment-bound Cu altered prokaryotic sediment communities at concentrations of >45 $mgkg^{-1}$ Cu. Additionally, Cu was found to

alter the sediment community's carbon utilisation, along with the prokaryotes colonising cellulosic material deposited on the surface of these sediments.

Fibrobacteres and Latescribacteria taxa were particularly sensitive to Cu and thus represent putative indicator taxa for Cu contamination in ecotoxicological studies. These taxa are ubiquitous members of sediments and soils and play a putative role in primary decomposition. Indeed, community level physiological profiling demonstrated that both the breadth and rate of carbon utilisation by sediment communities was impacted by Cu concentrations of >45 $mgkg^{-1}$. Some of these carbon sources included plant-derived sugars, mono aromatic compounds similar to those found in lignified plant material and amino acids, all of which are expected to be ecologically relevant sources of carbon and, in some cases nitrogen, to sediment microbes. A reduction in their utilisation by sediment communities may indicate a decline in biomass turn-over and nutrient cycling in sediments with elevated concentrations of Cu.

Interestingly, Cu also altered the prokaryotic taxa forming biofilms on the surfaces of cellulose baits. These communities were composed of both putative cellulolytic and non-cellulolytic members, including primary producers and thus, may be more accurately termed periphyton communities. The taxonomic composition of these periphyton communities shifted at >45 $mgkg^{-1}$, demonstrating a remarkable sensitivity to Cu, given that only the bottom half of the cellulose baits they occupied was in contact with sediment-bound Cu. It seems likely that copper-induced changes to sediment communities altered the pool of taxa for recruitment onto substratum. Periphyton biofilms account for a substantial portion of the food consumed by higher trophic levels, and provide additional ecosystem services such as primary production and oxygen evolution through photosynthesis, as well as pollutant removal and nitrogen fixation in freshwater systems (Liboriussen and Jeppesen, 2003; Vadeboncoeur and Steinman, 2002; van Dam et al., 2002). Thus, this compositional shift in periphyton communities may cause alterations to these processes. Additionally, if Cu disrupts the colonisation of cellulose baits by prokaryotes, it may have a similar effect on the colonisation of

other organic material deposited onto sediments. Early colonisers of organic matter play a substantiative role in establishing a microenvironment for decomposition (William Costerton, 1992), and thus, should this be the case, overall biomass turn-over could be altered by sediment-bound Cu.

The current study represents one of the few investigations into the effect of sediment-bound Cu concentration on freshwater prokaryotic communities. In many countries (i.e. Australia, New Zealand, Hong Kong and Norway), Cu sediment quality guideline values (SQGV) exceed 46 mg kg^{-1}, having been based on response data from macrobiota (Bakke et al., 2010; Burton, 2002; Simpson et al., 2013). The findings of this study suggest that current Cu SQGVs are inadequate for the preservation of microbial community biodiversity and function, with the ecological linkages between microbes and higher trophic levels suggesting that these may have broader ecological consequences. Thus, these findings warrant further investigation to determine whether current copper SQGVs are sufficient for protecting the overall structure and/ or function of freshwater ecosystems (see section 6.7).

6.5 SEDIMENT PROKARYOTIC COMMUNITIES APPEAR MORE SENSITIVE TO COPPER THAN URANIUM

Interestingly, prokaryotic communities in subtropical sediments were far more sensitive to Cu than tropical sediment communities were to U. For example, in tropical sediments, significant differences in prokaryotic community composition were observed at >650 mg kg^{-1} of U (Chapter 2), which is an order of magnitude greater than the 45 mg kg^{-1} of Cu which altered corresponding, temperate sediment, prokaryotic communities (Chapter 5). Even when considering the greater atomic mass of uranium compared with copper, these concentrations expressed as molar units are still four times higher for the uranium. Additionally, copper-induced changes to prokaryotic assemblages occurred at high taxonomic levels. For example, in total, 20 prokaryotic phyla showed differences in relative abundance when comparing total sediment communities in controls with one or more Cu

treatments (Figure 6.1). In contrast, only three phyla differed in response to U concentrations ranging from 60 to 1,500 mg kg^{-1}, and only nine phyla for the highest U treatment (>3000 mg kg^{-1} of U; Figure 6.1). Instead, taxa which declined in response to U concentration were often replaced by others from the same phyla. This poses the possibility that empty niches, and functional roles, are quickly filled by related taxa, and that these subtle taxonomic changes may not be functionally relevant.

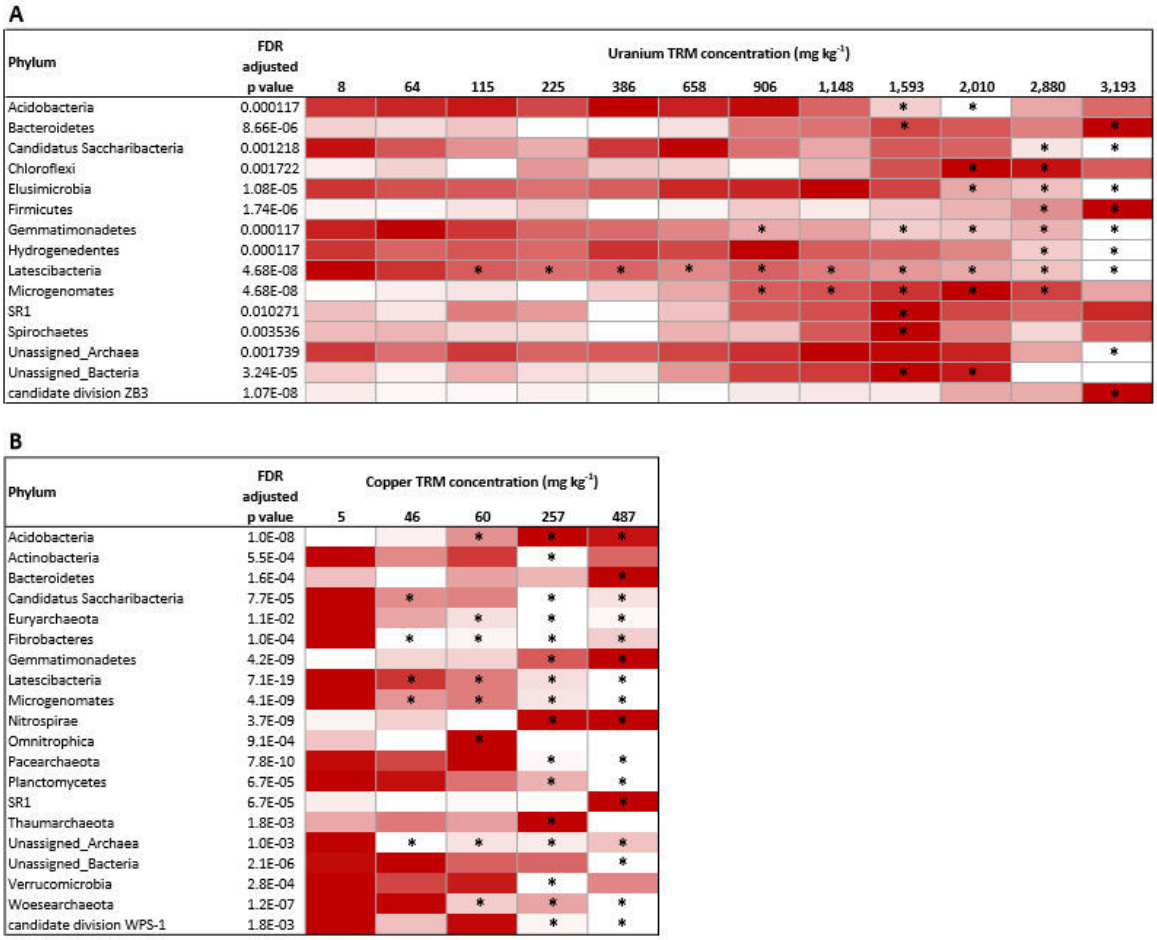

Figure 6.1. Prokaryotic phyla whose relative abundances were significantly different in control communities, compared with one or more metal treatments along A) a uranium gradient and B) a copper gradient. Relative abundances are coloured on a scale of white to red, with white indicating the lowest abundance for a particular phylum and a deep red indicating the highest relative abundance. Asterisks indicate treatments with a significant difference to controls.

One potential explanation for these different responses may be that Cu is an essential metal, while U is not. Thus, the molecular mechanisms for Cu uptake are present in all prokaryotic cells, but may not be ubiquitously present for U. Indeed, the combined rarity of Cu in most natural sediments, along with its essential role for life, may lead a number of microbes to specialise in high affinity scavenging of Cu. One example of this can be found in methanotrophs, which rely, in part, on the copper-dependant activities of particulate methane monooxygenase (pMMO) and thus, have high Cu demands (Balasubramanian et al., 2011). These microorganisms have been shown to release a high-affinity Cu chelator (methanobactin), which allows for the scavenging and uptake of Cu under low environmental Cu concentrations (Balasubramanian et al., 2011). Similar life-strategies may benefit a microorganism under broader ecological conditions, but be disadvantageous in less prevalent, copper-enriched environments. In comparison, few organisms utilise U and of those which do, some interact with U extracellularly. For example, *Geobacter* spp. export electrons outside the cell via specialised pili and thus, do not require U to cross the cell membrane in order to gain energy from its reduction (Cologgi et al., 2011).

Toxicity assays with U demonstrate the metal is able to cross prokaryote cell membranes in certain soluble forms (VanEngelen et al., 2010), however, the mechanism for transport is unknown. As discussed in Chapter 1, non-essential metals may be taken-up accidently by transporters involved in uptake of other elements. Should this be the case for U, it may be that non-specific U transport is less efficient than Cu transport by specialised, high affinity mechanisms. This speculation must be balanced with the observation that a specific Cu uptake system may be actively regulated when intracellular concentrations are high, whereas the regulation of a non-specific U uptake mechanism could not be mediated by intracellular U concentration.

It is also noteworthy, that between the Cu and U experiments, various other physiochemical parameters varied. Therefore, to confirm these differences, an experiment which applied U and Cu to parallel mesocosms, of the same sediment, deployed into the same environment would be

required. Further, there is a need to comprehensively compare the minimum inhibitory concentrations of a range of metals (including copper and uranium) on a suite of environmental microbial isolates. A literature review reveals that, to date, no direct comparisons between uranium and copper resistance have been made for cultured isolates - highlighting a substantial knowledge gap in the field. Two studies do, however, report copper sensitivity in isolates from uranium contaminated environments (Schmidt et al. 2009; Choudhary et al. 2012). These studies demonstrate that uranium tolerance is likely to involve different strategies to those employed for copper tolerance, but do not address whether uranium tolerance is more broadly exhibited in microorganisms than copper tolerance. The magnitude of difference between U and Cu concentrations eliciting a response in prokaryotic communities in this work is intriguing, and thus warrants further investigation.

6.6 COMMON TRENDS IN MICROBIAL RESPONSES TO URANIUM AND COPPER

6.6.1 Latescibacteria as an indicator taxa for elevated metal concentrations

Members of the Latescibacteria were sensitive to both U and Cu contamination in tropical and temperate sediments, respectively. This phyla showed a consistent and significant decline in relative abundance across both the metal concentration gradients tested, with significant declines at >45 mg kg^{-1} of Cu and >100 mg kg^{-1} of U (Figure 6.1). As discussed in Chapter 4, there are no cultured representatives of the Latescibacteria phylum, however, 16S rRNA sequences have been detected in a range of environmental samples including soils, marine and freshwater sediments along with pelagic communities (reviewed in Farag et al. 2017). Previous studies have indicated that relative abundances of Latescibacteria 16S rRNA reads are typically low (<5%) (Derakshani et al., 2001; Farag et al., 2017; Lentini et al., 2014; Wang et al., 2010), a finding which is consistent with the current study's results. Previous work by Bissett and colleagues, however, suggested that Latescibacteria may be a keystone taxa in saltmarshes, providing linkages between numerous other taxa (Bissett et al., 2013). Additionally, the high number of genes responsible for primary decomposition in the

pangenome of this phylum suggests that Latescibacteria play an important role in biomass turn-over (Farag et al., 2017). Thus, this taxon appears to be a metal-sensitive and ecologically relevant group of microorganisms.

6.6.2 Geobacteraceae and Holophagaceae as ubiquitous members of sediment assemblages exposed to high metal concentrations

Some members of the Geobacteraceae and Holophagaceae are known metal-reducers, deriving energy via the transfer of electrons to metal ions (Lovley et al., 1993; Mehta-Kolte and Bond, 2012). These microorganisms, therefore, are natural specialists of environments with elevated metal concentrations. Indeed, in Chapters 2 and 5, both taxa increased significantly in relative abundance under the highest Cu and U treatments tested (Figure 6.2). For Cu, this occurred at a TRM concentration of >400 mg kg^{-1} for both Geobacteraceae and Holophagaceae. For U, Geobacteraceae significantly increased at ≥2,800 mg kg^{-1}, while Holophagaceae relative abundances increased at ≥2,000 mg kg^{-1}. This trend mirrors the apparent differences in microbial sensitivity to Cu and U, suggesting that these are specialists in high metal environments.

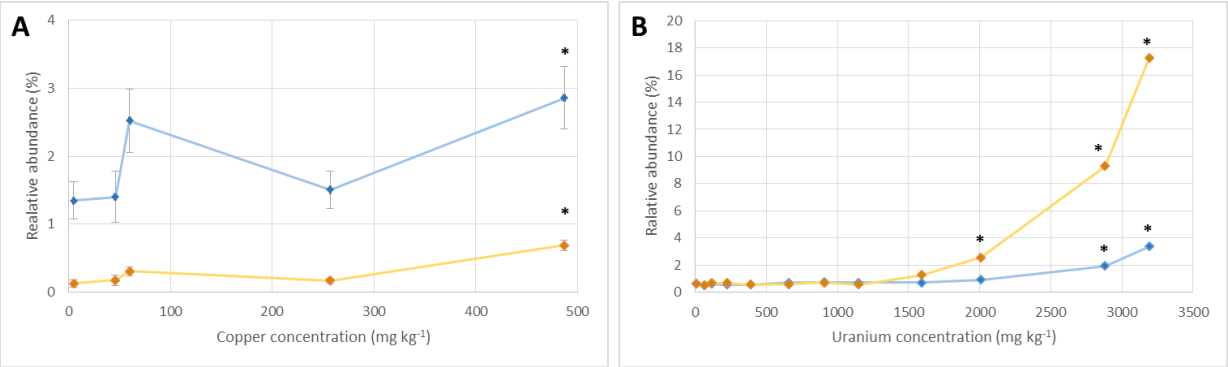

Figure 6.2 Average relative abundances of Geobacteraceae and Holophagaceae in sediments along a spiked concentration gradient of A) uranium and B) copper. Geobacteraceae are indicated by blue line (—♦—), Holophagaceae are indicated by yellow line (—♦—). Error bars indicate the standard error for averages. Asterisks indicate average relative abundances which are significantly different from those observed in controls.

Importantly, relatively little is known about the ecophysiology of Geobacteraceae and Holophagaceae taxa in natural settings, aside from their reduction of metals, and what little is known is derived from a small number of species. A broader understanding of the diversity in these

families and their ecological roles, would provide meaningful context for their significant, and consistent, increase in relative abundance under elevated metal concentrations. This information would also provide insights into whether these trends are broadly applicable to other sediments and metals. As stated in section 6.2, members of the Geobacteraceae appear reliant on the availability of simple, labile carbon sources, while both Geobacteraceae and Holophagaceae may rely on inter-species associations as a life-strategy in freshwater sediments. Thus, determining these controlling environmental factors, including the presence of other taxa, could inform future work as to whether these metal-reducers are present within a system.

6.6.3 Communities inhabiting freshwater systems with elevated sediment uranium and copper concentrations were diverse and contained a plethora of novel taxa

Across the community surveys performed within this body of work (Chapters 2, 4 & 5), care was taken to ensure that the OTUs identified were genuine, rather than PCR or sequencing artefacts. This was achieved by using a conservative quality control and OTU clustering pipeline (Usearch), a pipeline known to identify many fewer OTUs compared with other routinely used programs (Edgar, 2013). Further, additional quality controls were implemented as appropriate for each study. While it is impossible to validate each OTU individually, it is important to acknowledge that best practice was used, and these datasets are certainly more conservative than most. Regardless, a large amount of diversity was observed for microbial assemblages from tropical and subtropical systems (Table 6.1) and many of these were novel at a high taxonomic level (Figure 6.3).

Prokaryotic communities in freshwater sediments were highly diverse (Table 6.1). For example, in mesocosm controls, an average of ~7,400 OTUs were detected for tropical sediments (using a rarefaction cut-off of 35,000; Chapter 2), while in subtropical sediments an average of ~3,100 OTUs were detected (rarefaction cut-off of 5,000; Chapter 5). In both studies, OTUs were assigned to ≥40 different prokaryotic phyla groupings, showing an extensive phylogenetic breadth. While elevated U and Cu concentrations lowered prokaryote biodiversity, it remained high compared to other

environments (Table 6.1). In contrast, surveys of aquifer water communities which are extensively studied for responses to U, frequently report fewer OTUs, spanning many fewer prokaryotic phyla, when using similar methods to those described here (Eriksson et al., 2016; Hemme et al., 2015; Hong et al., 2013). This diversity represents both an opportunity for discovering new species and functions, and a methodological challenge with respect to metagenomic sequencing studies (see section 6.3)

Table 6.1. Average number of OTUs detected in all samples for Chapters 2, 4 and 5.

Prokaryotic community surveys			
Uranium study, 16S rRNA OTUs (Chapter 2)		**Copper study, 16S rRNA OTUs (Chapter 5)**	
Sample type	**Average number of OTUs per sample (± SE)**	**Sample type**	**Average number of OTUs per sample (± SE)**
Sediment		**Water**	
0 mg kg-1 U	7,412 ± 152	0 mg kg-1 Cu	724 ± 247
50 mg kg-1 U	7,106 ± 162	32 mg kg-1 Cu	591 ± 187
100 mg kg-1 U	7,110 ± 197	65 mg kg-1 Cu	1,110 ± 200
200 mg kg-1 U	7,344 ± 196	270 mg kg-1 Cu	1,325 ± 226
400 mg kg-1 U	6,849 ± 189	540 mg kg-1 Cu	937 ± 236
600 mg kg-1 U	7,042 ± 76		
800 mg kg-1 U	7,254 ± 309	**Sediment DNA**	
1,000 mg kg-1 U	7,236 ± 385	0 mg kg-1 Cu	3,095 ± 129
1,500 mg kg-1 U	7,408 ± 241	32 mg kg-1 Cu	3,037 ± 78
2,000 mg kg-1 U	7,387 ± 159	65 mg kg-1 Cu	2,831 ± 130
3,000 mg kg-1 U	7,399 ± 364	270 mg kg-1 Cu	2,244 ± 110
4,000 mg kg-1 U	6,757 ± 428	540 mg kg-1 Cu	2,451 ± 146
		Sediment RNA	
		0 mg kg-1 Cu	2,610 ± 248
		32 mg kg-1 Cu	2,520 ± 160
		65 mg kg-1 Cu	2,588 ± 207
		270 mg kg-1 Cu	2,371 ± 201.5
		540 mg kg-1 Cu	1,790 ± 172
		Bait	
		0 mg kg-1 Cu	1,337 ± 110
		32 mg kg-1 Cu	1,097 ± 75
		65 mg kg-1 Cu	1,394 ± 114
		270 mg kg-1 Cu	1,278 ± 70
		540 mg kg-1 Cu	1,229 ± 81

Fungal community survey	
Copper study, ITS1 OTUs (Chapter 4)	
Sample type	**Average number of OTUs per sample (± SE)**
Water	
0 mg kg-1 Cu	17 ± 12
32 mg kg-1 Cu	17 ± 9
65 mg kg-1 Cu	14 ± 8
270 mg kg-1 Cu	13 ± 9
540 mg kg-1 Cu	17 ± 9
Sediment DNA	
0 mg kg-1 Cu	31 ± 4
32 mg kg-1 Cu	31 ± 3
65 mg kg-1 Cu	26 ± 3
270 mg kg-1 Cu	22 ± 4
540 mg kg-1 Cu	27 ± 7
Bait	
0 mg kg-1 Cu	43 ± 7
32 mg kg-1 Cu	20 ± 8
65 mg kg-1 Cu	40 ± 7
270 mg kg-1 Cu	35 ± 8
540 mg kg-1 Cu	42 ± 14

The current study identified a number of the taxa which were novel at a high taxonomic level. In Chapter 4, an ITS1 survey of subtropical ponds, over one third of the OTUs detected could not be assigned below the order level, while in both 16S rRNA surveys, this number was greater than 50% (Figure 6.3). These values highlight the substantial novelty that remains undiscovered in freshwater

habitats. The abundance, and apparent responsiveness of some of these novel taxa to metal enrichments, suggests that these microorganisms are ecologically and ecotoxicologically relevant.

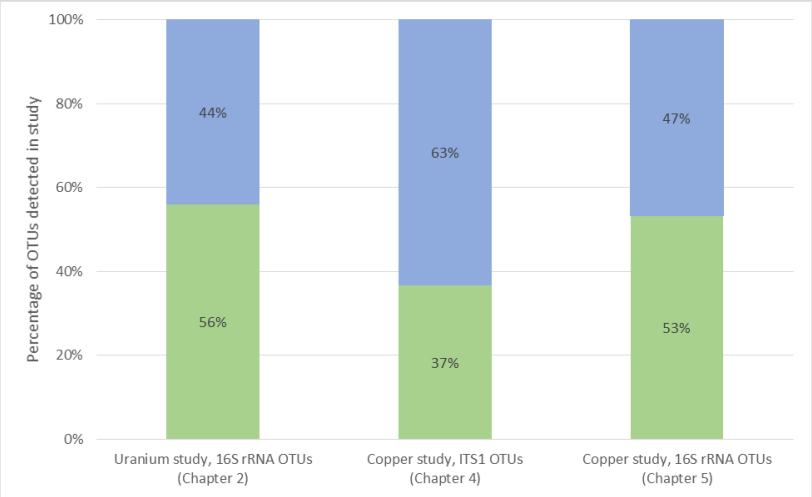

Figure 6.3 Percentage distribution of OTUs which could be taxonomically assigned below the order level (blue), and those which could not (green).

6.7 FUTURE DIRECTIONS

6.7.1 The effects of uranium on sediment microbial communities

The data presented in this study provide critical baseline information on microbial community responses to U. Having only been performed at a single time-point, and a single location, replication of this experiment across multiple locations (spatial variation) and at different time-points (temporal variation), would assist in determining the reproducibility and breadth of the trends observed here. Specifically, the region in which this study was carried out is subject to extreme weather fluctuations, with the wet season resulting in submerged sediments, while the dry season sediments are without overlying water. Such seasonal fluctuations represent a pulse-disturbance and undoubtedly effect prokaryotic communities inhabiting the sediments, as such, a relevant follow-up study would be to repeat this experiment during the dry season. Additionally, high U concentrations may be described as a press disturbance, a long-term selective pressure. The cumulative effects of combined seasonal pulse disturbances and this press disturbance may be significant, and thus

continued sampling over multiple years would be an intriguing long-term follow-up study for this work.

In addition, investigations into the effects of U at smaller scales would be a worthy aspirational goal for this research. Currently, most microscale investigations are limited to pelagic environments in which microbial community heterogeneity is driven by chemotaxis and mobility. The physical heterogeneity of sediments, however, is likely to result in a greater biological complexity, in addition to creating technical difficulties. For example, micro-sensors for the micro-scale measurement of pH, nutrients and redox have been successfully applied to liquid environments and biofilms (de Beer and Schramm, 1999; Kühl and Jørgensen, 1992). These micro-sensors, however, break when coming into contact with solid particles and are thus, difficult to use in sediments. In the interim, studies sectioning of sediments by macro-scale redox zones would be interesting and valuable. Such a method may simplify the communities being interrogated, improving the power of metagenomic sequencing. This approach was successfully demonstrated in a recent metagenomic survey which identified >80 genomes, many of which could be assigned to a specific redox zone (Baker et al., 2015).

Another key goal of microbial ecology, is the establishment of links between the genetic composition of a microbial community, and the function of that community. While microbial activities are known to drive global biogeochemical cycles (Falkowski et al., 2008), the communities engaged in these processes are thought to have a high degree of functional redundancy (Nannipieri et al., 2003; Yin et al., 2000). Thus, a change in the relative abundance of particular taxa, or indeed the relative abundance of certain functional genes, may not result in ecologically relevant changes to process rates. In Chapter 2 we observed a decline in the relative abundance of transporter genes associated with plant polysaccharide catabolism along the spiked U gradient. In Chapter 3, however, ecophysiological profiling of abundant genomes in the highest U spiked treatment, suggested that Bacteroidetes sp. GB4 was a specialist at plant polymer catabolism. These findings lead to a question

of whether a decline in the diversity or number of taxa engaged in a particular function results in ecologically relevant declines in that function, namely, plant biomass turnover in this example.

Future experiments which measure nutrient cycling rates, in parallel with microbial community profiling along perturbation gradients, would assist in establishing linkages between these two measures. For sediment-bound U concentrations specifically, the measurement of methane production, nitrogen fixation and plant polymer degradation would be highly relevant. Further, the use of stable-isotope probing (SIP) approaches may be used to link these nutrient transformations and fluxes to specific community members.

6.7.2 The effects of copper on sediment microbial communities

Chapters 4 and 5 showed that sediment fungal and prokaryotic communities were altered by very low concentrations of sediment-bound Cu (>45 mg kg^{-1} Cu). As this was the lowest Cu concentration tested, it is reasonable to assume that these microbial communities may also respond to lower concentrations of Cu. Thus, in order to establish whether the community threshold is <45 mg kg^{-1}, future studies should aim to extend the lower concentration range of this gradient, while including some concentrations from the current study for comparison. As indicated in the section above (6.7.1), the replication of these concentrations in other studies, and across different sites is required to assess whether the findings of this study are broadly reproducible. Additionally, variations in seasonality may effect these pond microbial communities, raising questions such as; are novel chytrids, and Latescribacteria, found in these ponds all year? If so, does seasonality effect their sensitivity to copper?

In Chapter 5, cellulose baits were used to recruit a subset of the microbial communities within freshwater mesocosms. While this method recruited a number of novel and interesting taxa, it proved non-specific for cellulolytic taxa. Cellulolytic microorganisms are an ecologically relevant functional group within microbial communities and correlations between these taxa and decomposition would be valuable in establishing links between communities and ecosystem

processes. The complexity of bait communities, along with the mass gain of many baits, however, prevented clear linkages from being identified in Chapter 5. This outcome suggests that the incubation period of ~3 months was too long for specificity and that sampling of the baits at earlier time points was needed.

Additionally, microbial communities forming on baits may have been restricted by the lack of nitrogen and other essential nutrients in the baits. Typically, detritus which contains cellulosic material will also have other substrates, for example DNA and proteins, which would act as a source of nitrogen. In Chapter 4, putative nematophagous fungi were particularly abundant on baits, with these organisms presumably supplementing the cellulosic carbon with nitrogen and phosphorus obtained through nematode predation. In a similar fashion, cellulolytic prokaryotes may have relied on nitrogen-fixation from co-occurring photosynthetic cyanobacteria as a source of nitrogen. Thus, the substrate used in this baiting method may have been incapable of supporting purely cellulolytic communities in freshwater mesocosms. Future experiments to investigate the effect of Cu on microbial decomposition would benefit from shorter incubation periods, in addition to more ecologically realistic substrates, for example leaf litter. Further, the use of inert substrates such as ceramic tiles, could be used to detect non-cellulolytic microbes involved in biofilm communities, with these communities used as subtractive controls for baited communities.

Alternative methods for decomposition rates include the application of fluorescently labelled polymers. In a study investigating enzymatic rates in marine sediments, small doses of these substrates were added to undisturbed cores, or core sections, and the fluorescence of pore waters after just one hour was able to identify differences in process rates temporally and spatially (Meyer-Reil, 1987). Another technique is to label the polymers with carbon isotopes (^{13}C or ^{14}C) and monitor the evolution of carbon dioxide isotopes (Brant et al., 2006; Kirk et al., 1975).

While microbial decomposition is an important ecological process, as outlined in Chapter 1, a number of other import ecosystem functions rely on microbial communities. The finding that low

concentrations of sediment-bound copper impact microbial communities may suggest that other microbially-driven ecosystem processes are impacted by environmental copper enrichment. Thus, future experiments which investigate the impact of copper on food webs, nitrogen cycling and carbon fixation would be important to assess the relevance of this work's findings on a broader range of ecosystem processes.

6.7.3 A need for greater cultured representation

Metagenomics has the power to investigate microbial communities in natural, undisturbed environments which are unlikely to be easily replicated in laboratories. This is extremely informative, providing insights into the taxonomic and functional diversity of microbial communities at a metagenomic scale, and in some circumstances, the taxonomy and functional potential of individuals within these communities. Almost all information inferred from this data, however, is based on previous culture-based characterisation of microorganisms. As discussed in section 6.6.3, much of the data obtained through metagenomics in the current study (both targeted amplicon sequencing and shotgun metagenomic sequencing) was associated with novel taxa which are only distantly related to culture representatives. This sets firm limitations on the interpretability of metagenomic data, and provides a good incentive for increasing culture-based efforts in future studies.

In addition to characterising novel genes, enzymes and pathways for further development of current databases, cultured microorganisms can be interrogated through manipulative experiments to test the findings of metagenomics-based surveys, further enhancing the impact of these surveys. In a demonstration of the complementary nature of these two approaches, metagenomic surveys may also inform culturing efforts. For example, the discovery that a dominant bacterium in acid mine drainage communities was the only community member with the genomic potential to fix nitrogen, led researchers to isolate *Leptospirillum ferrodiazotrophum* using nitrogen-free acidic medium (Tyson et al., 2005). Similarly, the partial genome of a bacterium detected in wallaby gut

microbiomes was used to develop a defined media on which it was subsequently isolated (Pope et al., 2011).

The genomic data presented in Chapter 3 provides information which may be used in genome-guided culturing efforts similar to these. For example, U could be used as a selective method, with *Dyella* GB3, Bacteroidetes GB4 and Anaerolineae GB5 grown on media with complex carbon sources, for example, peptides, cellulose and peptidoglycan, respectively. Anaerobic culturing is most likely needed in order to cultivate Anaerolineae GB5, *Geobacter* GB1, *Geothrix* GB2 and *Methanocella* spp., with simple carbon sources required by the later three.

Given the novelty of chytrids identified in Chapter 4, culturing efforts aimed at isolating and characterising these taxa would be useful in confirming their ecological roles. Given the enrichment of chytrids on cellulose baits in this study, a culturing method which first implements such a baiting method, before laboratory isolations, appears logical. Fortunately, chyrids can be readily isolated from cellulosic material (Whiffen, 1941), and could be inoculated onto media with cellulose as a sole carbon source in order to determine their cellulolytic capabilities.

Of relevance to both the U and Cu investigations undertaken in both these studies, as well as a broader understanding of microbial ecology and taxonomy, attempts to isolate a member of the Latescibacteria would be invaluable. Based on work by previous researchers, these organisms are likely to be oligotrophic primary decomposers and thus, slow-growing. Based on this information, a low nutrient medium with cellulose or peptides as sole carbon sources seems appropriate for the isolation of these taxa. The high through-put, microscale culturing method described by Rappe and colleagues to isolate the first SAR11 isolate may assist in isolating these low abundance taxa from sediments with high microbial biomass and diversity (Rappé et al., 2002). This method is assisted by fluorescent *in situ* hybridisation (FISH) for screening of microplate cultures, and the metagenomic data provided by the current study, along with other metagenomic surveys, may be used to design Latescibacteria-specific 16S rRNA probes for this method.

6.8 FINAL CONCLUSIONS

The current study presents the first metagenomic data from microbial communities inhabiting sediments along spiked U, and Cu, concentration gradients using *in situ* mesocosms. These data demonstrate that a range of taxonomic and functional changes occur in sediment microbial communities, and provide key information regarding threshold metal concentrations, metal-sensitive taxa, insights into the complex inter-species relationships and ecological roles of sediment microorganisms, and potential disruptions to these functions by elevated sediment-bound metal concentrations. These findings are of relevance to microbial ecologists, ecotoxicologists and international policy makers.

Broadly, microbial communities are responsive to elevated sediment-bound metal concentrations. These communities, however, appear to be more sensitive to Cu, which induced changes to community composition at higher taxonomic levels and at lower Cu concentrations, when compared to U. Members of the novel bacterial phylum Latescibacteria, and the fungal Chitridiomycota, appear to be particularly sensitive to elevated Cu concentrations, with Latescribacteria also sensitive to elevated U concentrations. Conversely, Geobacteraceae and Holophagaceae are typically the most abundant prokaryotes under high metal concentration conditions. Along both metal concentration gradients tested, increasing the metal concentration appeared to lower the functional potential of sediment microbial communities' with regards to the utilisation of complex carbon sources. This finding has implications for biomass turnover and nutrient cycling in contaminated environments, and emphasises an importance for future studies to incorporate carbon and nitrogen flux measurements.

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