MICROBIAL ECOLOGY OF THE SYDNEY BASIN TEMPERATE HIGHLAND PEAT SWAMPS ON SANDSTONE

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THESIS SUBMITTED FOR DEGREE OF DOCTOR OF PHILOSOPHY

25TH DECEMBER 2019

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ABSTRACT

The Temperate Highland Peat Swamps on Sandstone (THPSS) of the Sydney Basin are listed as endangered ecosystems, yet they continue to suffer habitat losses and degradation from anthropogenic pressures. Despite ongoing efforts to restore and better protect these swamps, they remain poorly understood, potentially hindering the effectiveness of management efforts. Vital to overall ecosystem function and the provision of services for human and environmental benefit is the microbial component of wetland/peatland ecosystems. Microbes are responsible biogeochemical cycling including the processes of carbon and nutrient cycling. Essentially, microbes control the bioavailability, export and sequestration of the elements fundamental for ecosystem function, thereby impacting peat formation and storage, primary production rates, greenhouse gas emissions and downstream water quality. Regardless of this importance, the microbial ecology of THPSS had not yet been studied.

The aim of this thesis is to characterise the structure and function of microbial communities in THPSS to support improved management for the preservation of these ecosystems. To address this aim, a series of studies were undertaken to reveal the structure of microbial communities and the environmental factors that influence them, with specific emphasis on two key disturbances: fire and urbanisation.

Results revealed both resilience and sensitivity of these systems. THPSS were resilient to a hazard reduction burn. Microbial communities and sediment properties in burnt swamps were similar to pre-burn conditions and control swamps one year after the fire. If hazard reduction burns prevent more intense and catastrophic burns, as they are intended to, they may be helpful in maintaining the integrity of THPSS microbial community. THPSS within urbanised catchments, however, were sensitive to the ongoing urban disturbance. Microbial community composition, gene expression and abundances differed between swamps affected and unaffected (or minimally affected) by urbanisation. Linked to catchment urbanisation was elevated pH, which was also identified to be a significant influence on microbial community composition. Employing strategies to maintain naturally acidic conditions in urbanised catchments may help maintain the function of microbial communities in THPSS. Taxonomic analysis of these differences revealed catchment urbanisation was altering microbial function and was resulting in a shift from oligotrophic to copiotrophic taxa. Ratios of taxa that represent these different trophic life strategies have been suggested as microbial indicators for ecological assessment, results from this thesis indicate this may be a useful tool for assessing THPSS ecosystem health, and restoration success.

Together, this body of work supports and adds to the understanding of THPSS and peatlands globally. In particular, it has highlights the effects of little studied consequences of urbanisation and fire disturbance to peatland microbial systems.

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: 25 December 2019

Nicole Christiansen

ACKNOWLEDGEMENTS

I could not have accomplished this thesis without the help or influence of several individuals that I will acknowledge here.

Firstly, I am grateful to my supervisors for giving me this opportunity and providing their guidance. I would like to thank my primary supervisor, Grant Hose, for your support and flexibility throughout my unconventional PhD expedition. I especially appreciated your inputs on the statistics and editing – I learned lots. Most of all, I truly valued your positive response (two times) to that whole having a baby thing. Thanks to my co-supervisor, Kirstie Fryirs, who also went along with it all and was very encouraging. Thank you for all your expert non-biological wisdom and the insightful editing that improved each draft.

Thanks to the volunteers who trundled through the some unforgiving terrain with me and hopefully gained an appreciation for these prickly but beautiful swamps. I would also like to acknowledge my fellow student researchers. Specifically, Lorraine, thank you for sharing your knowledge, ideas and encouragement, Kirsten, thank you for introducing me to the swamps and sharing your experiences and insights and Mike for helping me get started with DNA extractions and T-RFLP data analysis.

I am very appreciative of the support of MQ University and SIMS staff. I feel lucky to have had access to such great facilities and helpful people.

Thanks are due to my mother-in-law and parents for their wonderful childcare provided at critical moments. Without that help, I would still be working on this.

Really, none of this would not have been possible without the lifetime of love and influence from my family. I am exceptionally fortunate to have been surrounded by a family of loving, nurturing and hardworking people my whole life.

Huge thanks to my husband, who was integral to every aspect of the thesis, from volunteering in the field to helping in the lab and editing. You survived brutal run-ins with hakea and some serious manual labour, and once the soreness and bumps subsided, I am pretty sure you enjoyed it.

And finally, I must acknowledge my two boys who became a part of my life during this PhD endeavour. They did not, *in any way*, facilitate the completion of the thesis, however they have enriched my life immensely and I could not imagine this PhD journey without them. I dedicate this to them.

CHAPTER 1
GENERAL INTRODUCTION

INTRODUCTION

Globally, anthropogenic forces such as climate change, urbanisation and land use change are altering natural places and causing loss of biodiversity (Sanderson *et al.*, 2002, Dirzo *et al.*, 2014). As we lose biodiversity and function, the value of healthy ecosystems is realised (MEA, 2005). Functioning ecosystems provide tangible natural benefits, described as 'ecosystem services', such as clean water, flood control, food production and climate regulation (MEA, 2005). To maintain an ecosystem's ability to provide these services to society, management - protection and rehabilitation - is often required. Imperative to effective management is understanding the various components of these environments.

Peatlands are considered highly valuable ecosystems (de Groot *et al.*, 2012, Costanza *et al.*, 2014) and are a priority for restoration and protection (Zedler and Kercher, 2005, Clarkson *et al.*, 2017). The overarching aim of this thesis is to improve the understanding of endangered Temperate Highland Peat Swamps over Sandstone (THPSS) in eastern Australia by describing and examining the impact of disturbance on their microbial community to support protection and rehabilitation of these ecosystems.

BACKGROUND: PEATLANDS

Peatlands globally provide an array of valuable ecosystem services. They are intrinsically important for the habitat they provide and biodiversity they support (Minayeva *et al.*, 2008, IUCN, 2017). At a catchment scale, peatlands provide clean water and regulate flows, and so act as a natural purifier and flood control for downstream ecosystems and human communities (Wilson *et al.*, 2010, Acreman and Holden, 2013, Mitsch and Gosselink, 2015, IUCN, 2017). At a global scale, peatlands are one of the most significant stores of atmospheric carbon, storing an estimated 550 gigatonnes (Yu *et al.*, 2010). Covering less than 3% of Earth's terrestrial landscape, peatlands store twice as much carbon as Earth's forests, making them the most carbon dense terrestrial habitat (Parish *et al.*, 2008). Essentially peatlands are custodians of the climate that Earth has experienced since the Holocene (Parish *et al.*, 2008, Frolking *et al.*, 2010, Yu *et al.*, 2010).

Fundamental to long-term carbon sequestration and storage is a net imbalance where primary production exceeds decomposition (Clymo and Fogg, 1984). Waterlogged, anaerobic, nutrient poor and acidic conditions combined with recalcitrant organic materials of peatland vegetation inhibits decomposition and allows peat accumulation to occur (Rydin and Jeglum, 2006). The maintenance of appropriate conditions for peat accumulation and storage are intrinsically linked to climatic conditions (Parish *et al.*, 2008). For example, temperature and moisture, along with site topography and geology, affect hydrological conditions including the maintenance of a stable water table. Saturated sediments affect redox conditions that regulate decomposition rates. Climate conditions can also impact the lability of the organic materials of peatland vegetation also affecting peat storage potential (Weltzin *et al.*, 2003). Recalcitrant plant materials are slower to decompose and can affect the water chemistry resulting in acidic conditions that further restrict decomposition.

It is well established that human induced climate change is altering global temperature, evapotranspiration and precipitation norms (IPCC, 2014) under which current peatlands have been

sequestering carbon. While peatlands have existed and accumulated carbon for millions of years under different climate conditions (Parish *et al.*, 2008), a rapidly changing climate introduces great uncertainty as to the viability of current peatlands to continue to serve as carbon sinks and many may turn into net carbon sources (IUCN, 2017). Of additional concern, peatlands are sources of other significant greenhouse gases, namely methane (CH_4) (Lai, 2009, Couwenberg *et al.*, 2010, Bridgham *et al.*, 2013a) and nitrous oxide (N_2O) (Regina *et al.*, 1996, Liu *et al.*, 2019), which have far greater warming potential than carbon dioxide (CO_2) (IPCC, 2014). Anthropogenic stressors, a warming climate and changed precipitation regimes may increase the release of these greenhouse gasses. For example, degraded peatlands release greater nitrous oxide (Liu *et al.*, 2019) than undisturbed peatlands that tend to be nitrogen sinks. Disturbed peatlands release greater carbon dioxide emissions and methane emissions may decrease in drained situations (Couwenberg, 2009) or increase if wet conditions remain (Cowley *et al.* 2018) or are reinstated (Couwenberg, 2009). The release of potent greenhouse gases from degraded peatlands may create a positive feedback loop and exacerbate climate change (Palmer *et al.*, 2012)

MICROBIAL ECOLOGY OF PEATLANDS

Microbes are ultimately responsible for decomposition, nutrient and biogeochemical cycling that are fundamental to the storage of organic carbon and the purification of water as well as the release of greenhouse gasses in peatlands (Mitsch *et al.*, 2015). Despite the importance of microbial communities, there is still much to learn about their structure and function in peatlands (Andersen *et al.*, 2013).

General understanding of microbial communities in the environment has increased substantially in the last two decades with the advent of culture-independent molecular methods. Prior to this, knowledge was based on species that could be readily cultivated in a laboratory setting. Although several species had been identified in peatlands (Williams and Crawford, 1983), inherent bias of culturing techniques results in a poor representation of the total community. Peatlands in particular may benefit from new techniques as they are often dominated by oligotrophic and acidophilic microbes, such as *Acidobacteria* (Ausec *et al.*, 2009, Serkebaeva *et al.*, 2013), that are notoriously difficult to study with traditional cultivation methods (Ward *et al.*, 2009). Although non-culturing techniques sidesteps these biases, inferences of microbial ecological function from sequencing is presently based on a relatively small number of genome sequence and cultivation studies (Land *et al.* 2015) and so must be approached with caution at present. Given the overwhelming diversity of microbes, targeted and strategic analysis of ubiquitous taxa is needed to improve the understanding of microbial ecological function (Delgado-Baquerizo *et al.* 2018).

In peatlands, several bacteria phyla are commonly reported including *Proteobacteria*, *Acidobacteria, Planctomycetes, Bacteroides*, and *Actinobacteria* (Pankratov *et al.*, 2005, Chapter 5). Within these phyla are taxa with important roles and functional adaptions for life in peatlands. *Proteobacteria* and *Acidobacteria*, which are both large and diverse phyla, are often reported as being the dominant phyla (Ausec *et al.*, 2009, Tian *et al.*, 2019, Chapter 5). *Proteobacteria* includes members that are capable of a variety of functions that are important in peatlands, such as ammonia oxidation and methanotrophy

(Garrity, 2005). *Acidobacteria* are generally adapted to low pH and low resource conditions (Fierer *et al.*, 2007), although there are exceptions (Kielak *et al.*, 2016). Based on genome analysis of three *Acidobacteria*, within this large group are slow growers and members that are capable of reducing nitrate and nitrite, and utilising various carbon sources (Ward *et al.*, 2009). Members of *Planctomycetes* that are commonly found in boreal peatlands were shown to be slow decomposers and some may be capable of anaerobic ammonium oxidation (Ivanova *et al.*, 2017). Members of *Actinobacteria* have genes that infer the ability to degrade cellulose and lignin (Ausec *et al.*, 2011) and members of *Bacteroidetes* have been shown to degrade organic high molecular weight compounds (Martin *et al.*, 2012). Also significant to the microbial make up and function of peatlands are archaea (Yavitt *et al.*, 2012). Important archaea functions include ammonia oxidation under acidic and oligotrophic conditions (Sims *et al.*, 2012) and anaerobic methane generation (Zinder, 1993).

Microbial biota in peatlands is stratified as a result of energy and redox condition constraints deeper in the profile (Artz *et al.*, 2006, Andersen *et al.*, 2013). Efficient aerobic decomposition is restricted to the surface layer where oxygen is available along with more labile forms of carbon. Generally, microbial diversity declines with depth as conditions become less hospitable and require specialised adaptations to function (Morales *et al.*, 2006). In waterlogged conditions, such as in peatlands, oxygen is quickly depleted (Hargrave, 1972). In the absence of oxygen, NO_3^-, Mn^{4+}, Fe^{3+}, SO_4^{2-} and CO_2 sequentially serve as alternative terminal electron acceptors for heterotrophic microbial metabolism, which determine the communities and processes that can take place (Fenchel *et al.*, 1999).

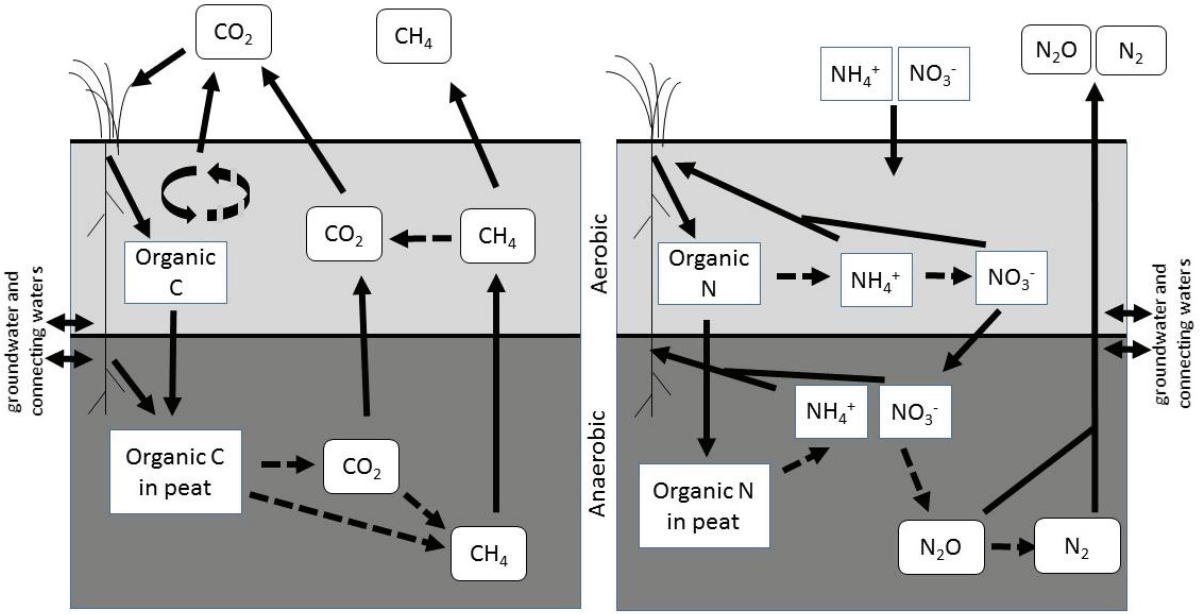


Figure 1.1 Simplified model of carbon and nitrogen cycling in peatlands. Dashed lines represent bacterial and archaeal mediated processes. Rounded rectangles represent gases. Adapted from (Rydin and Jeglum, 2006).

Two processes of ecological significance that occur in the anaerobic conditions of peatlands are denitrification and methanogenisis. These processes are important parts of the nitrogen and carbon cycle, respectively (Fig 1.1). Once oxygen has been depleted, if nitrate is available, there is the opportunity for denitrification to take place. Denitrification is the sequential reduction of nitrate (NO_3^-) to nitrogen gas (NO, N_2O or N_2) (Zumft, 1997) making peatlands effectively nitrogen sinks where the final product is returned to the atmosphere rather than released back into the aquatic environment (Yu *et al.* 2019). Methanogenisis is a decomposition process where formate (HCO_2^-) acetate ($CH_3CO_2^-$) or carbon dioxide (CO_2) are metabolised to produce methane gas (CH_4) by specialised archaea (Zinder, 1993, Conrad, 2007). Complete carbon and nitrogen cycling includes a number of microbial mediated steps in both aerobic and anaerobic conditions. Ammonia oxidation, mediated by ammonia oxidising archaea and bacteria in aerobic conditions is an important step in to supply nitrate for denitrification. Methane, from methanogenesis, can be consumed by bacterial methanotrophs that oxidise methane to carbon dioxide (Conrad, 2007). Up to 90 % of methane may be metabolised by methanotrophic bacteria (Segers, 1998).

Microbes are sensitive to environmental conditions (Urakawa and Bernhard, 2017) and communities can be altered by disturbance (Andersen *et al.*, 2013). In peatlands, determinants of microbial communities include vegetation and peatland type, water level, peat substrate quality and water chemistry (Andersen *et al.*, 2013). The pH has been shown to influence community composition across soil types (Fierer and Jackson, 2006, Hartman *et al.*, 2008, Chapter 4). Acidic conditions constrain the microbial community to species with adaptations for acidic environments (Fierer and Jackson, 2006). Similar patterns have been noted in peatlands. For example, more acidic *Sphagnum* bogs have lower microbial diversity (Lin *et al.*, 2012, Yavitt *et al.*, 2012) or lower activity (Preston *et al.*, 2012) than pH neutral *Carex* spp dominated fens. Nutrient availability can also affect microbial community and activity (Williams and Silcock, 1997). Increased microbial activity has implications for decomposition rates and therefore accumulation and storage of peat (Bragazza *et al.*, 2012). Higher nutrient conditions shift the ratio of archaea ammonia oxidisers to bacterial ammonia oxidisers (Sims *et al.*, 2012).

The importance of the microbial community to the function of ecosystems, combined with their sensitivity and responsiveness to environmental changes, makes microbes relevant and sensitive biological indicators (Merkley *et al.*, 2004, Sims *et al.*, 2013, Urakawa and Bernhard, 2017). The potential of microbial indicators for assessment of ecosystem health has been suggested and is gaining attention (Paerl *et al.*, 2003, Sims *et al.*, 2013, Urakawa and Bernhard, 2017). For example, microbial indicators have been used in peatlands to assess efficacy of restoration effort (Watts *et al.*, 2008, Reumer *et al.*, 2018).

AUSTRALIAN PEATLANDS

The majority of the world's peatland area is found in the boreal regions of North America and Russia, however peatlands also occur from the tropics to temperate regions (Rydin and Jeglum, 2006). With

exception of Tasmania, the prevailing climate of Australia does not support extensive peatlands, yet peatlands can be found in different regions (e.g. the wet tropics, alpine, coastal areas in the southeast and southwest, and even desert springs), however, they are generally rare and restricted (Pemberton, 2005). Like others around the world, the peatlands of Australia developed mainly during the Holocene (Fryirs *et al.*, 2014a) and contain dense stores of carbon (Hope and Nanson, 2015). Most Australian peatlands are composed of graminoid (Restionaceous and Cyperaceous) assemblages (Whinam *et al.*, 2003). *Sphagnum* peatlands are less common and confined to montane and alpine biomes (Whinam *et al.*, 2003).

TEMPERATE HIGHLAND PEAT SWAMPS ON SANDSTONE

Despite being rare across most of the Australia, peatlands occur in a relatively high density in the Sydney Basin Plateau (Fryirs *et al.*, 2018). Here, Temperate Highland Peat Swamps on Sandstone (THPSS), are a common feature of the low relief terrain above the escarpment (Fryirs *et al.*, 2018) (Fig 1.2). These peatlands are most similar in structure and function to northern hemisphere valley mire fens (Evans and Warburton, 2007). There are 3,208 identified THPSS, which cover an area of 10,100 ha, distributed over five regions of eastern Australia (Fig 1.2) (Fryirs *et al.*, 2018). THPSS occur on eastern sides where annual precipitation exceeds evapotranspiration (c.a. 1500 mm to 900 mm) (Young and Young, 1988) and at elevations between 600-1100 a.s.l. (Fryirs *et al.*, 2018). The area experiences temperate climate with inter-annual variation in rainfall due to El Niño-Southern Oscillation influences (Fryirs *et al.*, 2014a). The town of Katoomba, in the Blue Mountains, receives an average rainfall between 1100-1400 mm of rainfall each year with the greatest amounts received in the summer months (November to March). The mean maximum and minimum temperatures are 12°C and 23°C respectively in the summer and 3°C and 10°C in the winter (BOM, 2017). The THPSS form in elongated shallow valleys where sediment and organic material accumulate behind a drainage constriction or bedrock step (Fryirs *et al.*, 2014a). They are typically located in the headwaters of eastern draining rivers, many of which become part of the Sydney domestic water supply (Cowley *et al.*, 2018b). The swamps are contained within small catchment areas (c.a. 0.25 km^2) and have a moderate slope (c.a. 4.2%) (Fryirs *et al.*, 2014a, Fryirs *et al.*, 2018). They have formed since the last glacial maximum and basal sediments are typically <15,000 years old (Fryirs *et al.*, 2014a).

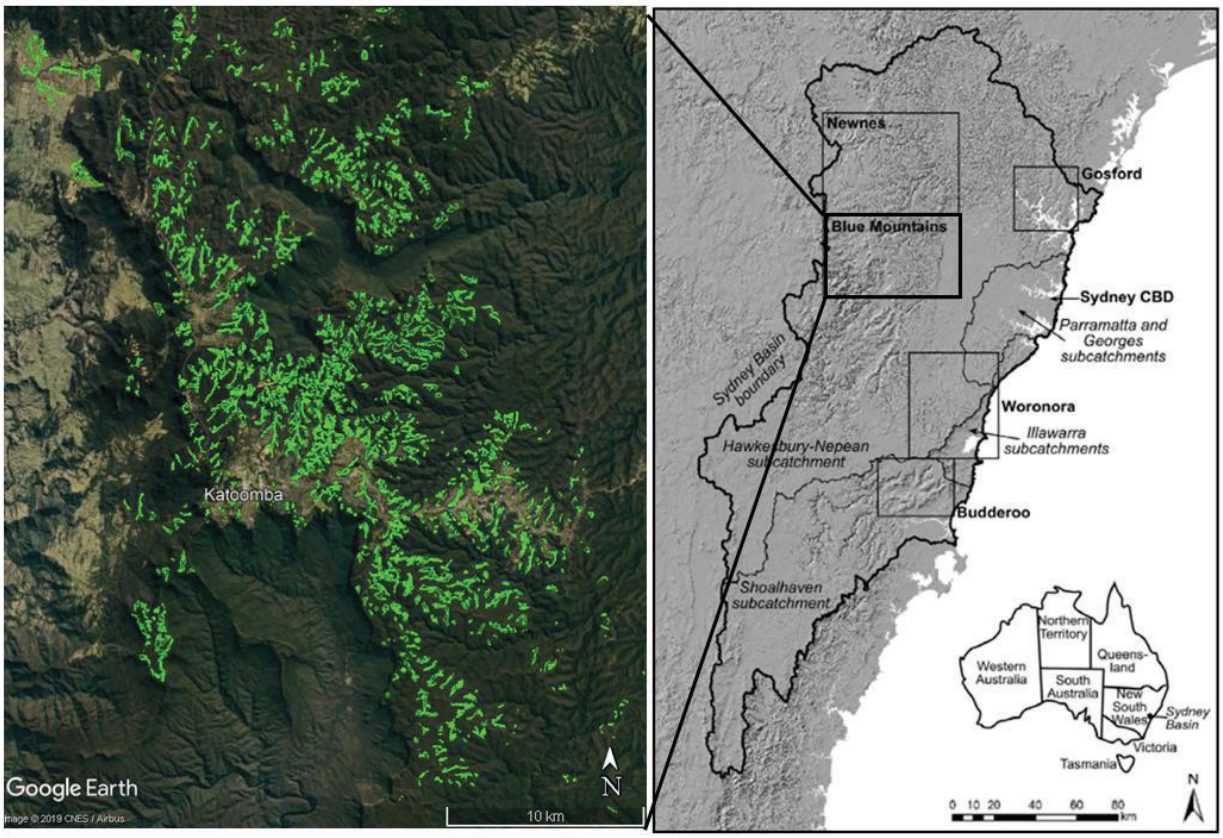


Figure 1.2 Areas of THPSS around the Sydney Basin. Map (right) shows the five areas of high density THPSS, modified from (Fryirs *et al.*, 2018). Satellite imagery (left) shows the Blue Mountains THPSS where studies for this thesis were undertaken. Green outlines indicate the location and extent of THPSS. KMZ overlay of Blue Mountains TPHSS are publicly available under Creative Commons Attribution 4.0 from *https://datasets.seed.nsw.gov.au*. Map Source: Google Earth (2018 Google DigitalGlobe CNES/Airbus.)

The studies for this thesis were undertaken in Blue Mountains, approximately 100 km west of Sydney, where there is a high density of THPSS (Fig 1.2). The Blue Mountains area is recognised for its outstanding natural value as a UNESCO World Heritage Area with areas protected as national parks. Several swamps form the headwaters to Sydney's drinking water reservoirs (Cowley *et al.*, 2018b). Despite these protections, many areas of the Blue Mountains are impacted by urbanisation (Fryirs *et al.*, 2016, Belmer *et al.*, 2018) and regularly experiences bushfires (Black and Mooney, 2006, Gorissen *et al.*, 2015).

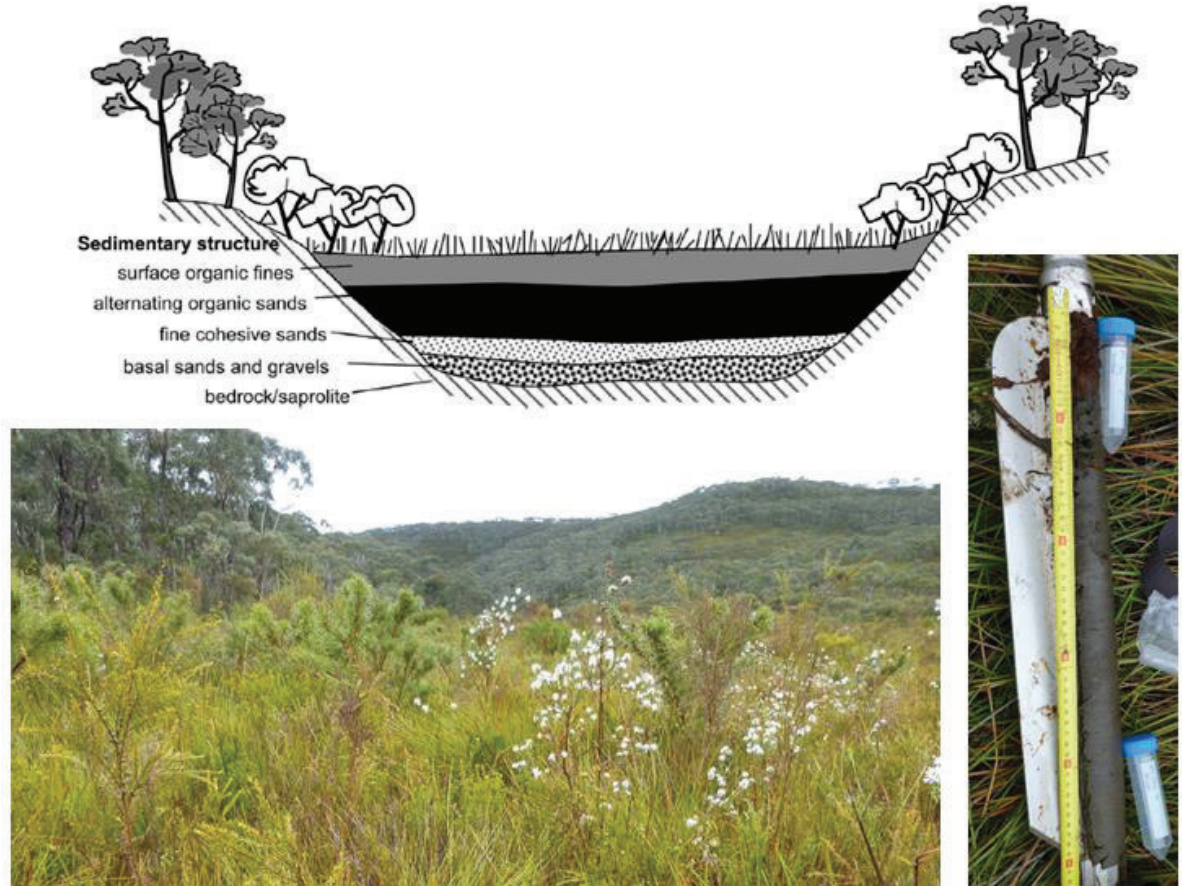


***Figure 1.3 THPSS structure*.** Illustration of THPSS sedimentological structure showing distinct sediment layers (upper), modified from (Fryirs *et al.*, 2014a). Typical THPSS vegetation structure showing sedge and shrub swamp area surrounded by eucalypt woodlands (lower left). Example sediment core showing top 50 cm profile and the transition from *surficial organic fines* to *alternating organic sands* (lower right).

The sedimentological structure of THPSS consists of distinct layers (Fig 1.3) and may be up to 4 m in depth (Fryirs *et al.*, 2014a, Cowley *et al.*, 2016). Overlying impermeable Hawkesbury Sandstone (Young and Young, 1988) are layers of *basal sands and gravel* and *fine cohesive sands* deposited prior to swamp formation (Fryirs *et al.*, 2014a). The bulk of sediment volume and peat stores is contained within a layer of *alternating organic sands*. Differing from northern boreal peat, THPSS peat is striated with mineral sand inputs from the catchment (Fryirs *et al.*, 2014a). Eastern Australia experiences significant inter-annual climate variation (cycles of high rainfall and drought) due to the influence of ENSO (Kirkup *et al.*, 1998, Black and Mooney, 2006). This variability precludes sustained peat accumulation, rather there are phases of peat formation when suitable moisture conditions occur (Fryirs *et al.*, 2014a). The surface layer is made up of *surficial organic fines*, which are composed of highly organic sediments and live and decaying plant materials (Fryirs *et al.*, 2014a, Cowley *et al.*, 2016).

THPSS rarely have standing water, instead the water is stored within the sediments and the water table is typically about 0.2 m below the sediment surface (Cowley *et al.*, 2018b), although capillary forces

within the highly organic sediments ensure that surface sediments are generally saturated (Hose *et al.*, 2014). Importantly, the swamps retain water and regulate downstream flows reducing flooding during heavy rainfall and supplying water during dry periods (Young and Young, 1988, Cowley *et al.*, 2018b). Groundwater is often a significant source of water for TPHSS, with up to 80% of swamp waters originating from groundwater sources (Cowley *et al.*, 2019).

The vegetation community of THPSS is made up of sedge and shrubs (*Carex* spp. *Lepidosperma limicolum*, *Dichelachne inaequalis*, *Poa labillardieri* var. *labillardieri*, *Epacris microphylla*, *Epacris paludosa*, *Grevillea acanthifolia* and *Hakea* spp.) (Keith and Myerscough, 1993), and is surrounded by drier sclerophyll woodlands (Keith and Myerscough, 1993, Hose *et al.*, 2014) (Fig 1.3). The dichotomy of moist and dry environments creates a heterogeneous mosaic of habitats that are a biodiversity hotspot. The swamps themselves support a number of threatened and endemic species (e.g. flora: *Dillwynia stipulifera*, *Boronia deanei* subsp*. Deanei*, fauna: giant dragonfly (*Petalura gigantean*) and Blue Mountains water skink (*Eulamprus leuraensis*)) (Benson and Baird, 2012).

As with other peatlands around the world (IUCN, 2017), a number of anthropogenic pressures have resulted in the destruction and degradation of some THPSS (Kohlhagen *et al.*, 2013). In recognition of their high ecological value, restricted range, and sensitivity to anthropogenic pressures, THPSS have been listed as endangered ecosystems in State (Threatened Species Conservation Act 1995) and Commonwealth (Environment Protection and Biodiversity Conservation Act 1999) legislation (Mooney and Martin, 2016). Despite this, THPSS continue to face an uncertain future. Mining activities have punctured aquifers resulting in swamp subsidence, dewatering and loss of function (Young and Young, 1988, Krogh, 2007, Gorissen *et al.*, 2017). Catchment urbanisation has altered the hydrology and geomorphic condition (Fryirs *et al.*, 2016), water chemistry (Belmer *et al.*, 2015) and biota of THPSS (Belmer *et al.*, 2018, Christiansen *et al.*, 2019, Hardwick 2019). Changes to the moisture regime and water chemistry are shown to affect the microbial community (Weltzin *et al.*, 2003, Hartman *et al.*, 2008, Sims *et al.*, 2012, Shrestha *et al.*, 2014). Swamps affected by urbanisation have higher carbon and methane exports (Cowley *et al.*, 2018a).

Climate change is expected to reduce the area with suitable conditions for TPHSS to occur (Keith *et al.*, 2014). Southeast Australia is expected to become hotter, more drought prone and see increase bushfire frequency and severity (CSIRO, 2015). The greater Sydney Basin is extremely bushfire prone (Black and Mooney, 2006) and THPSS regularly experience fire (Gorissen *et al.*, 2015, Gorissen *et al.*, 2018). To combat the threat of catastrophic bushfires, hazard reduction burns are common management strategy employed in the area and may also impact THPSS. Already, THPSS have formed in conditions at the limit of peat making ability (Fryirs *et al.*, 2014a), under increasing climate and anthropogenic pressures, they may become net carbon sources (Hope and Nanson, 2015).

In the face of anthropogenic pressures, restoring degraded THPSS to preserve their ecological value and function in the landscape is a high priority (Henson, 2010, Mooney and Martin, 2016, Clarkson *et al.*, 2017). In some THPSS, hard and soft engineering restoration structures have been installed (Freidman and Fryirs, 2014, Lane, 2016) to slow water flow through the swamp and help reinstate hydrological function. Successful rehabilitation requires thorough understanding of the ecosystem as a

whole. While the growing body of research on THPSS is improving our knowledge of these systems, many aspects of their ecology are yet to be explored.

GAPS IN KNOWLEDGE

Australian peatlands are generally overlooked in the literature (Rydin and Jeglum, 2006), and the THPSS are no exception. However, there have been recent efforts to improve our understanding of THPSS. In 2011, a THPSS Research Program was established after coal mining activities severely impacted three swamps on the Newnes Plateau, west of Sydney (Department of Environment and Energy, 2011). Although a regrettable and destructive incident, this led to an enforceable undertaking that funded a structured swamp research program, which has improved current understanding of THPSS (Mooney and Martin, 2016). This thesis is part of a greater project seeking to fill knowledge gaps in the geomorphic, hydrological and ecological function of these swamps (see Cowley, 2017, Hardwick, 2019).

For the body of work that forms this thesis, I focus on the microbial ecology of the TPHSS. To the best of my knowledge, there has not been any microbial ecology research undertaken in the THPSS prior to the work presented here. From research in wetlands and peatlands elsewhere, as outlined above, microbes are likely to be critical to peatland function for the role they play in decomposition, biogeochemical and nutrient cycling. They are likely to be fundamental to the significant ecosystem services of carbon storage and water purification. The main focus of the thesis is to explore how microbial communities are affected by the key disturbances of fire and urbanisation. More broadly, the effects of fire on wetlands, including peatlands, is poorly explored relative to terrestrial ecosystems and greater research is needed (Bixby *et al.*, 2015). As well, recent studies have shown microbial communities of waterways are effected by urban pressures (Ibekwe *et al.*, 2016, Wang *et al.*, 2016, Hosen *et al.*, 2017, Jani *et al.*, 2018, Roberto *et al.*, 2018, Wang *et al.*, 2018), however, few have focused on wetlands (Gilbert *et al.*, 2012, Gonzalez Mateu *et al.*, 2019) and I am unaware of studies on the effects of urbanisation on peatland microbial communities. Additionally, microbes are sensitive to environmental conditions and can act as indicators of ecosystem degradation (Sims *et al.*, 2013), making them potentially powerful tools in the assessment of THPSS condition.

The microbial community was analysed with molecular techniques, including terminal restriction fragment length polymorphism (T-RFLP), quantitative polymerase chain reaction (qPCR), and high-throughput amplicon sequencing (16S rRNA). Analyses were performed on sediment total DNA extractions, and tandem total RNA extractions. In support of the molecular data, sediment conditions were also analysed, to investigate what parameters were significant in explaining community differences. These include: electrical conductivity, pH, nitrate, ammonium, organic content and moisture content.

AIMS OF THESIS

The aim of this thesis is to increase overall understanding of THPSS by characterising the previously unexplored microbial component of the ecosystem. The overarching goal is to use this science to inform management that will monitor ecosystem health and preserve functioning THPSS. To address this aim, this thesis has a number of research objectives. These are:

1. Determine the structure of microbial communities and identify where ecologically important processes are occurring with emphasis on the processes of methane and nitrogen cycling.
2. Identify sediment properties that influence microbial communities.
3. Determine microbial community response to key disturbances that affect THPSS: urbanisation and fire.
4. Identify taxa associated with healthy and degraded systems and explore the potential of microbial indicators for THPSS ecosystem health.

The research to achieve these research objectives is described in a series of chapters as outlined below.

THESIS STRUCTURE

The thesis is comprised of six chapters and is structured with each chapter prepared in a format suitable for publication. The thesis includes a general introduction, four studies (Chapters 2-5) considering aspects of the microbial ecology of the THPSS and a final discussion chapter. Chapters 3-5 focus on the impacts of fire (Chapter 3) and urbanisation (Chapter 4 and 5) on microbial function in THPSS, thereby providing insights for management priorities and tools for monitoring and assessment (throughout and Chapter 6). As each of the study chapters has been prepared as a standalone work, there is some repetition in the background information presented. For the purposes of this thesis, 'microbial' includes bacteria and archaea and does not include fungi or protozoa.

Chapter 1 – General Introduction

This chapter provides a review of literature to identify knowledge gaps and provide context for thesis aims, objectives and hypotheses.

Chapter 2 – Heterogeneity of the conditions and microbial community of an upland swamp.

Hypothesis: THPSS microbial community would be stratified by depth, but be similar spatially across the swamp.

This is the first microbial ecology study of THPSS and served as a preliminary study of the microbial diversity and heterogeneity within a single swamp. It compared communities across depth profiles and a downstream spatial gradient. Community make up was stratified by depth. Community differences by

location revealed heterogeneity across the swamp. Most notably, the greatest differences were found at the location adjacent to stormwater outlet and previous rehabilitation efforts. This indicates a sensitivity of THPSS microbial community to disturbance and potential functional alteration of microbial processes.

Chapter 3 – Microbial communities of upland peat swamps were not different one year after a hazard reduction burn.

Hypothesis: The burn would result in changes to the microbial community due to altered sediment properties.

This study compares THPSS microbial community and functional gene abundances before and after a controlled hazard reduction burn. Sampling was undertaken one year prior and again one year after the burn event in both affected and nearby unaffected control swamps. Statistical differences were not found related to the burn suggesting that a hazard reduction burn did not have long lasting impacts to the microbial community.

Published in *International journal of wildland fire* (Christiansen *et al*., 2020)

Chapter 4 - The impact of urbanisation on community structure, gene abundance and transcription rates of microbes in upland swamps of Eastern Australia.

Hypothesis: catchment urbanisation would affect the microbial community and microbial function.

Following on from Chapter 2, where microbial communities and sediment properties were different near a stormwater drain, a broader scale investigation into microbial community differences between THPSS from urbanised catchments and those from catchments of intact native vegetation. Significant differences were found between the DNA and RNA communities between the catchment types. Gene and transcript abundances of bacteria 16S rRNA and archaea 16S rRNA were greater in urbanised surface sediments suggesting greater microbial activity in urbanised catchment swamps. The functional gene for methane consumption (*pmoA*) was found in greater abundances and transcripts of *pmoA* and the methanogen gene (*mcrA*) were more often detected in urbanised catchment swamps suggesting an altered methane cycling dynamic. Several of the microbial differences were correlated to pH. Results suggest that urbanisation has altered the microbial community and function of these THPSS. Although urbanisation effect on THPSS is the focus, this work is also significant on a broader scale as there are few comparative studies on the impact of urbanisation to peatlands microbes.

Published in *PLOS ONE* (Christiansen *et al.*, 2019)

Chapter 5 – Taxonomic differences of the bacterial community of urbanised upland swamps.

Hypothesis: taxonomic differences between urbanised catchment THPSS would indicate ecosystem degradation.

In this chapter high-throughput Illumina amplicon sequencing was employed to provide higher taxonomic resolution to the bacterial community differences found in the Chapter 4. Differences in taxon

proportional make up of reads between the catchment types indicate altered trophic status from oligotrophic to copiotrophic bacterial taxa. Taxa linked with pollution and degradation were associated with the urbanised catchment swamps.

Chapter 6 – General Discussion

This chapter reflects on thesis aims and objectives and the contribution of the thesis findings to the broader understanding of swamp management and conservation. The important primary findings from each study are highlighted with discussion of management strategies to protect natural microbial function of THPSS and the applicability of microbial indicators for ecosystem health assessment. Included is a supplementary discussion comparing molecular techniques used in this body of work and how they may be applicable for future ecosystem assessment of THPSS.

CHAPTER 2

HETEROGENEITY OF THE CONDITIONS AND MICROBIAL COMMUNITY OF AN UPLAND SWAMP

ABSTRACT

Peatlands systems are rare and poorly studied in Australia compared to their Northern Hemisphere counterparts. The Temperate Highland Peat Swamps on Sandstone (THPSS) of the Sydney Basin represent high ecological value and endangered peatland ecosystems. Unfortunately, many of these swamps have been destroyed or degraded. Although investment is being made to protect and restore these swamps, many aspects of their ecology remain uncharacterised, which may hinder the effectiveness of management actions. A notable knowledge gap is the microbial component of these systems. Microbes are the foundation of the key processes of carbon storage and nutrient cycling and are responsible for methane emissions. Thus, microbes are fundamental to overall ecosystem function and the provision of services for human and environmental benefit. To gain a preliminary understanding of the microbial ecology of THPSS, this study investigates the microbial community along a depth profile and across a swamp. Microbial communities were characterised using terminal restriction fragment length polymorphism (T-RFLP) fingerprinting techniques and the abundance of functional carbon (*pmoA*, *mcrA*) and nitrogen (archaea *amoA*) cycling genes using quantified using qPCR. Results revealed the microbial community, gene abundances and sediment conditions were stratified with depth. Bacteria and archaea 16S rRNA, archaea *amoA*, and *pmoA* genes decreased in abundance with depth. The pH, moisture and organic content were lower with depth. The sediment conditions and microbial communities were heterogeneous across the swamp. Most notably was a distinct community profile at the most upstream location adjacent to a stormwater outlet, suggesting ecosystem sensitivity to the disturbance of stormwater.

INTRODUCTION

Peatlands are highly valued for the large amount of terrestrial carbon that they store; for example, northern peatlands store approximately 30% of the global carbon pool in only about 3% of earth's surface (Gorham, 1991). Peatlands are also the largest natural source of the potent greenhouse gas methane (Bridgham *et al.*, 2013b). Degraded peatlands are prone to becoming net greenhouse gas sources rather than carbon sinks (Cowley *et al.*, 2018a). Maintaining peatlands in healthy ecological and hydrological condition is necessary for preserving their fundamental ecological value and carbon stores, and for reducing carbon emissions. Unfortunately, up to 25% of all peatlands have been destroyed and more have been degraded, so now peatlands are being prioritised for restoration and protection (Rydin and Jeglum, 2006, IUCN, 2017). However, successful peatland rehabilitation requires thorough understanding of the ecosystem as a whole, which in many regions is currently lacking.

In Australia, prevailing climatic conditions do not support extensive peatlands. Areas of peat accumulations are relatively rare and isolated to patches of suitable moisture and topographic conditions (Pemberton, 2005). One of the few places where peatlands do occur is along the Sydney

Basin Plateau (Fryirs *et al.*, 2018). Here, many peatlands, classified as Temperate Highland Peat Swamps on Sandstone (hereafter called swamps), have been destroyed or degraded due to anthropogenic activities (Kohlhagen *et al.*, 2013). Remaining swamps continue to be threatened by urbanisation, mining, groundwater exploitation and climate change (Department of Environment and Heritage, 2011, Benson and Baird, 2012, Keith *et al.*, 2014). In the Blue Mountains, where the highest concentration of these swamps are located (total of 1585 swamps) (Fryirs *et al.* 2018), a survey of over 450 sites found 57% of sites in moderate (45%) or poor (12%) geomorphic condition. Those in good condition were concentrated in protected areas (Fryirs *et al.* 2016). In recognition of the pressure they are under and their inherent value, swamps are listed as endangered ecosystems in State (Threatened Species Conservation Act 1995) and Commonwealth (Environment Protection and Biodiversity Conservation Act 1999) legislation, and restoration and protection is a high priority (Henson, 2010, Mooney and Martin, 2016).

Most of peatland research has been undertaken in the northern latitudes of North America, Europe and Asia (Rydin and Jeglum, 2006), where peatlands are most common. Many aspects of the Australian analogues have not yet been studied, potentially hindering the effectiveness of management actions (Fryirs *et al.*, 2014b). The swamps in the Sydney Basin are quite different in origin, structure and function compared to northern latitude peatlands (Fryirs *et al.*, 2014a). They formed during the Holocene in low relief valleys where eroded sediment and organic materials accumulated over time (Fryirs *et al.*, 2014a). Variable climatic conditions and highly mineralised sediments make these swamps marginally peat forming and so, even small changes to the ecosystem conditions may have implications for their ability to store and accumulate peat (Fryirs *et al.*, 2014a, Cowley *et al.*, 2018a). Additionally, the swamps have an important role in the health of the catchment. Aside from the carbon they store, they form the headwaters of Australia's largest domestic water supply (Cowley *et al.*, 2018b), are ecologically significant as habitat for endangered fauna (Gorissen *et al.*, 2015) and specialised flora communities (Keith and Myerscough, 1993).

Rehabilitation of degraded swamps is achieved through both hard and soft engineering solutions (Freidman and Fryirs, 2014, Lane, 2016). In some locations, soft engineering structures (e.g. coir logs) have been installed (c.a. 2008) to halt or reverse stream incision, mitigate the impact of stormwater drains and slow water movement through the swamps (Henson, 2010, Lane, 2016). It is assumed that reinstating hydrological function of degraded swamps will result in the biological components of the swamp naturally re-establishing (Fryirs and Brierley, 1998, Henson, 2010, Freidman and Fryirs, 2014). While the structure of a habitat is fundamental for supporting the biological components, the biotic recovery is complex and important to the overall ecosystem function (Urakawa and Bernhard, 2017). For example, the sediment microbial community regulates nutrient cycling and decomposition that are fundamental for long-term carbon storage (McLatchey and Reddy, 1998). Degraded swamps export more carbon, including methane, than those that are structurally intact (Cowley *et al.*, 2018a). A lag between restoration and the return of microbial function has been reported in a restored northern hemisphere sphagnum bog (Andersen *et al.*, 2006), and similar responses may be expected in Australian swamps although this remains untested. Baseline knowledge of the spatial and temporal dynamics of microbial communities in swamp sediments is critical for the management and

rehabilitation of swamps. Such knowledge is currently limited for these systems (Christiansen *et al.*, 2019).

The aim of this study is to explore in detail the spatial variability in the structure and function of microbial communities in an upland swamp, and relate observed patterns to environmental attributes. Focus was on the microbial communities of the primary sedimentary layers for peat formation and storage across a depth profile. We characterise the swamp microbial community using terminal restriction fragment length polymorphisim (T-RFLP), and quantify ecosystem function by targeting genes using quantitative PCR (qPCR). It was hypothesised that the distinct sediment layers would harbour differing conditions, microbial communities and gene abundances and these would be similar down the axis of the swamp.

METHODS

STUDY SITE

Wentworth Falls Lake Swamp (33°42'26.90S 150°21'45.50E) is a valley fill feature at 900 m above sea level (ASL) on the Blue Mountains Plateau. It is located approximately 80 km west of Sydney in the town of Wentworth Falls (Fig 2.1) and forms part of the headwaters of Jamison Creek that flows to Lake Burragorang within the Sydney water supply catchment (SCA, 2015). The swamp itself has an area of 0.06 km^2 with a catchment area of approximately 2.1 km^2. Within the catchment are parklands and native forests, as well as road and rail infrastructure, residential areas, and a golf course. The swamp receives stormwater from multiple drains. Despite these impacts, the swamp remains in relative good condition with limited channelisation (Fryirs *et al.*, 2016).

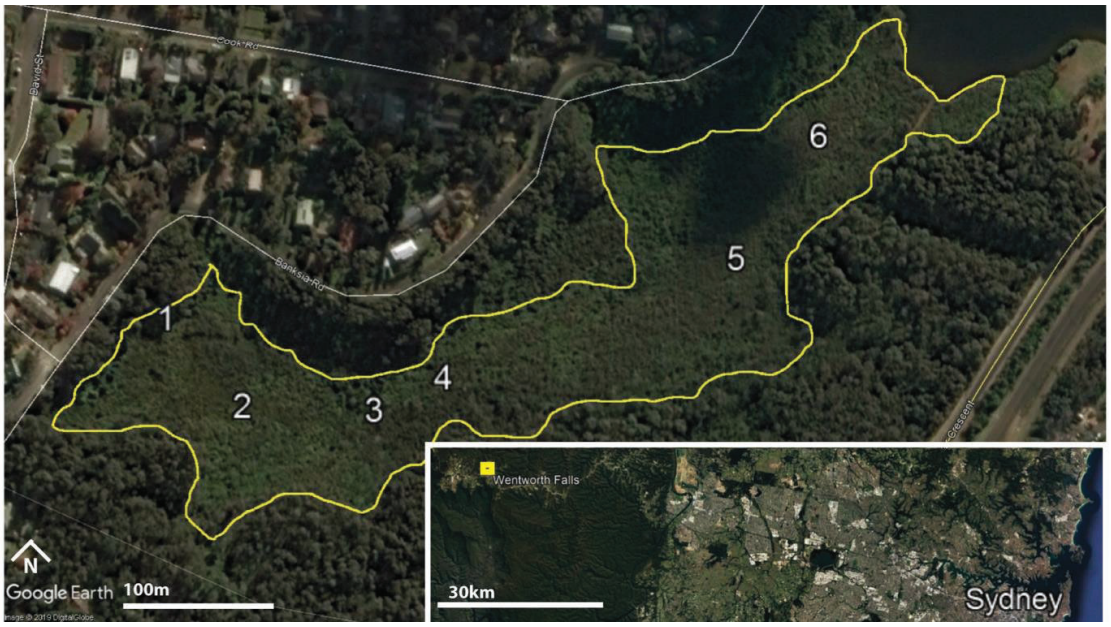


Figure 2.1 Map of Wentworth Falls Lake Swamp. Study swamp, outlined in yellow, showing sampling locations numbered 1-6 along a downstream gradient, location 1 being the furthest upstream. © Google Earth 2019.

The study site has a temperate climate. As recorded at the Katoomba weather station approximately 5 km to the west, mean maximum temperatures range from 23.3°C in January to 9.4°C in July, and mean minimum temperatures range from 2.6°C in July to 12.9°C in February with an average annual rainfall of 1400mm yr^{-1} (BOM, 2017).

The swamp does not have standing water, the water table for these swamps is on average about 20 cm below ground level, however the highly organic sediments are generally saturated (Hose *et al. 2014,* Cowley *et al. 2018b)*. Dense native vegetation covers the swamp including sedge, tea-tree, grevillea, banksia and emergent eucalypts (Keith and Myerscough, 1993). Some weeds have established immediately adjacent to areas where there has been disturbance from stormwater outlet points and restoration activities. Coir logs and sediment traps have been installed to reduce the hydrological impacts at the stormwater outlet points

Figure 2.2 Sampling at Wentworth Falls Lake Swamp. Upper left, core being collected using D-corer. Lower left, sediment samples being collected from core into sterile tubes for analysis. Middle, sampling equipment in typical vegetation of swamp. Upper right shows core taken at location 1 where weedy vegetation (e.g. *Ranunculus sp.)* was present. Lower right, core from location 3 with typical sedge vegetation

SEDIMENT SAMPLE COLLECTION AND ANALYSIS

Sediment samples were collected from six locations along a transect following the swamp axis (Fig 2.1). Location 1 was influenced by the stormwater outlet with weeds and coir logs were present. Generally, sampling locations were similar being along the central axis of the swamp with similar native vegetation communities. Location 1 was influenced by the stormwater outlet with weeds and coir logs were present. Locations 5 and 6 were furthest downstream and the sediment was wetter with taller sedge. The swamp ends in a reservoir which may contribute to the wet conditions at the downstream locations. At each

location, cores were extracted using a Russian D-corer. The cores were photographed and sediment samples were taken at 10 cm increments starting at 5 cm below the surface (Fig 2.2). At the time of sampling the sediment type was recorded based on characteristic distinct layers (Cowley *et al.* 2016). At all sampling locations the top layer of sediment was classified as *surficial organic fines* (SOF) comprised of live and decaying organic materials. Below was a layer of *alternating organic sands* (AOS). The AOS layer forms the majority of sediment fill of the swamps and is comprised of striations of sand and peat. At location 1 there was a layer of *contemporary sands* (CS) between the SOF and AOS. The layer of CS is a coarse sand with little to no organic materials that typically has formed as result of recent erosion event. (Fig 2.3). Since CS was only represented at a single location and in only two depths (e.g. two samples total) we have not compared the conditions of the CS layer to the SOF and AOS in Figure 2.4. The CS samples had low organic content and moisture content compared to the other sediment layers. The depth of the transition from SOF to AOS varied between the locations. The shallowest transition occurred 25/35 cm sampling depth at location 3 and the deepest transition occurred at 55/65 cm depth at location 4 (Fig 2.3).

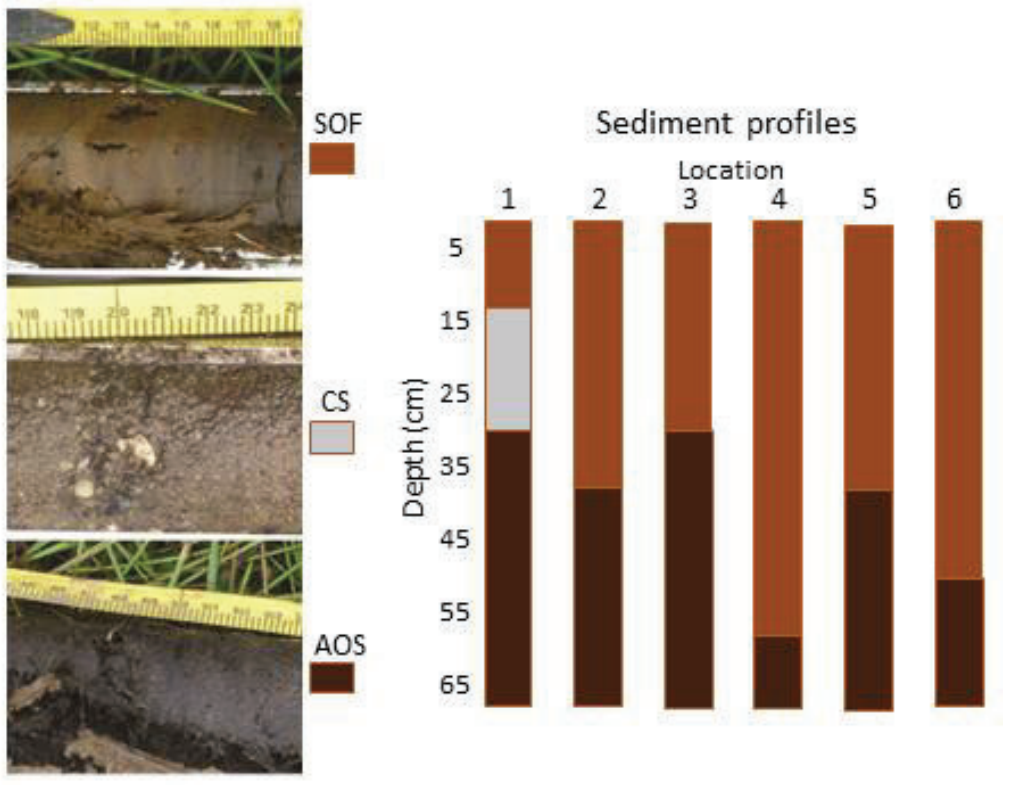


Figure 2.3 Sediment cores showing sediment types. Left: Top, *surficial organic fines* (SOF), found at the surface at each sampling location. Middle, *Contemporary sands*, associated with disturbance, found only at location 1 at intermediate depth between SOF and AOS. Bottom. Shows *alternating organic sands* (AOS) sandy organic layer, typically the thickest sediment layer and location of peat. Right: profile of sediment types found at each location colours indicate sediment type.

Sediment was collected directly into sterile tubes and immediately snap-frozen on dry ice. Samples for analysis were taken to a depth of 65 cm by where all cores had transitioned to AOS. For each location a single sample was taken at each depth. Upon return to the laboratory, samples for molecular and physico-chemical analysis were stored at -80° C and -20° C, respectively. Sediments were analysed for pH, soil moisture and organic content. The pH was measured in a 1:1 mass ratio sediment/deionised water slurry using a benchtop pH meter (Thermo Scientific™ Orion™ 3 star pH meter) (Hamman *et al.*, 2007). Soil moisture was measured gravimetrically after drying overnight and stable mass reached (105° C for >12 h) and total organic content was determined by loss on ignition (550° C for 5 h) (Heiri *et al.*, 2001). Soil moisture and organic content were calculated as a percentage of dry weight.

MOLECULAR ANALYSIS

EXTRACTION

DNA was extracted from sediment samples using the PowerSoil Total DNA Kit (MoBio) according to the manufacture's protocol. Purity and yield of DNA was quantified using a NanoDrop 2000 spectrophotometer (Thermo Scientific Inc.). Based on results of qPCR serial dilutions, the DNA was diluted with purified MilliQ water to avoid PCR inhibitors (1:10).

Table 2.1 The primers and conditions used for quantitative PCR.

Gene	Name	Function	Sequence 5' - 3'	Annealing (°C)	Reference
pmoA	A189gc	F primer	GGNGACTGGGACTTCTGG	62	(Holmes *et al.*, 1995)
	mb661	R primer	CCGGMGCAACGTCYTTAC		(Costello and Lidstrom, 1999)
AOA *amoA*	AamoAF	F primer	STAATGGTCTGGCTTAGACG	60	(Francis *et al.*, 2005)
	AamoAR	R primer	GCGGCCATCCATCTGTATGT		
mcrA	ML-F	F primer	GGTGGTGTMGGDTTCACMCARTA	60	(Steinberg and Regan, 2008)
	ME-2	R primer	TCATKGCRTAGTTDGGRTAGT		(Hales *et al.*, 1996)
16S rRNA Archaea	ARC787F	F primer	ATTAG ATACC CSBGT AGTCC	60	(Yu *et al.*, 2005)
	ARC915F	TaqMan*	AGGAA TTGGC GGGGG AGCAC		
	ARC1059R	R primer	GCCAT GCACC WCCTC T		
16S rRNA Bacteria	BAC338F	F primer	ACTCC TACGG GAGGC AG	60	(Yu *et al.*, 2005)
	BAC516F	TaqMan*	TGCCA GCAGC CGCGG TAATA C		
	BAC805R	R primer	GACTA CCAGG GTATC TAATC C		

* FAM and TAMRA labelled

GENE ABUNDANCE (QPCR)

Gene abundances were estimated using quantitative PCR (qPCR). A TaqMan qPCR assay was used to quantify the archaea and bacteria 16S rRNA gene using universal primer pairs (Table 2.1) and a

FAM-labelled probe with SensiFast Probe mix (Bioline) in 25 µL reactions. SYBR green qPCR assay was used to quantify the functional genes for methane production (*mcrA*), methane oxidation (*pmoA*) and AOA archaea ammonia monooxygenase (*amoA*) using universal primers (Table 2.1) in 8 µL reactions using SensiFast SYBR No-ROX (Bioline). All qPCR reactions were carried out on a BioRad CFX96 RT System C1000TM Thermal Cycler. PCR conditions were as follows: initial denaturation (94°C, 2 min) followed by 40 (TaqMan/SYBR analysis) cycles of denaturation (94°C, 5 sec) and hybridisation-elongation (annealing temperature, 45 sec). A subsequent melting temperature curve of the amplicon was performed in the SYBR green qPCR assays. The presence of targeted genes was confirmed with gel electrophoresis and analysis of the melting curve data. Calculations of amplicon copy numbers were based on serial dilutions of target genes cloned into the pCR4-TOPO vector (Thermo Scientific Inc.), standard curves all with $r^2 \geq 0.99$ and dynamic range of $1e^2 – 1e^8$ copies. For each qPCR run, negative controls were included to confirm the integrity of the results (Smith and Osborn, 2009).

TERMINAL RESTRICTION FRAGMENT LENGTH POLYMORPHISM (T-RFLP)

Terminal restriction length polymorphism (T-RFLP) analysis was used to compare community structure of bacteria and archaea between sampling locations and sediment depth profiles. The bacteria and archaea 16S rRNA gene was PCR amplified from DNA using the universal primer pairs (Table 2) using MyTaq polymerase (Bioline). PCR conditions were as follows: 94°C for 2 min then 35 cycles of 94°C for 15 sec, annealing temperature for 30 sec and 72°C for 2 min. PCR bacteria and archaea amplicons were digested with restriction enzymes (Table 2.2). Digested PCR products were analysed on ABI3730xl Genetic Analyser at the Australian Genome Research Facility with LIZ1200 size standard. Data were processed using GeneMapper Software and the online tool T-REX (Culman *et al.*, 2009). Peak noise was removed (Abdo *et al.*, 2006), peaks were aligned within two base pairs and peaks that occurred only once in the dataset were removed.

Table 2.2 PCR primers, conditions and references for terminal restriction length polymorphism (T-RFLP).

Gene	Name	Function	Sequence 5' - 3'	Annealing (°C)	Restriction enzyme(s)	Reference
16S rRNA Bacteria	27F	F primer*	AGAGTTTGATCCTGGCTCAG	54	HhaI	(Dunbar *et al.*, 2001)
	1492R	R primer	TACCTTGTTACGACTT			
16S rRNA Archaea	1Af	F primer*	TCYGKTTGATCCYGSCRGAG	53	HhaI, Sau96I	(Cadillo-Quiroz *et al.*, 2006)
	1100AR	R primer	TGGGTCTCGCTCGTTG			

* FAM labelled

STATISTICAL ANALYSIS

Sediment pH, moisture and organic content and gene abundances were compared by sediment type and depth. To test for differences between the sediment types a repeated measures analysis of

variance (ANOVA) was used. The repeated measures design accounts for potential non-independence of sediments at different depths from the same location. In these analyses, location was considered a random factor and sediment type a fixed factor. The relationship between the sediment conditions and gene abundances with depth in the sediment profile were assessed using analysis of co-variance (ANCOVA) to test for patterns with depth and if they were similar between locations. Assumptions of normality and homogeneity of variance were assessed visually using plots of residuals and Q-Q plots. Where necessary gene abundance and sediment pH/moisture/organic content were first log transformed to approximate normality. The assumption of sphericity was tested using Mauchley's test and the Greenhouse-Geisser correction used where necessary. The significance level (α) for all analyses was 0.05. The above analyses were conducted in Minitab v17 (Minitab Inc, PA, USA).

The T-RFLP community assemblage were analysed based on Bray-Curtis similarity index (Clarke, 2006). Relationships of the community assemblages were visualised using non-metric multidimensional scaling (NMDS). Differences in the community by sediment type and depth and in the sediment profile were compared using PERMANOVA emulating the repeated measures ANCOVA design described above. Prior to analysis, peak height data were standardised (by total sample peak area) and square root transformed (Clarke, 2006). These analyses were done using PRIMER & PERMANOVA + add on version 1.0.8 (PRIMER-E Ltd., Plymouth, UK). The significance level (α) for all analyses was 0.05.

RESULTS

SEDIMENT CONDITIONS

DEPTH PROFILE AND SEDIMENT TYPE

The pH, organic content and moisture content decreased with depth (all $p<0.001$). The depth and sediment type are inherently related due to the SOF being atop the AOS and as would be expected the results for sediment type were similar with the higher values being observed in the SOF. The mean (±SD) pH of the SOF (5.87 ± 0.23, n=23) was higher than the AOS sediment layer (5.40 ± 0.45, n=17, $p=0.04$). The SOF had higher mean organic content (17.90± 6.64%, n=23) than the AOS (8.31± 5.11%, n=17, $p=0.006$) and higher mean moisture content (SOF: 246.2 ± 156.7%, n=23) compared to the AOS layer (63.46 ± 37.89%, n=17, $p=0.002$) (Fig 2.4).

LOCATION

The pH had differences by location ($p=0.02$) with a significant interaction between sediment type and location (repeated measures ANOVA) and between depth and location (ANCOVA). Location 1 had the overall highest pH, and location 3 had the lowest. Location 2, 3, 5 and 6 showed declines in pH with depth while location 1 and 4 had more similar values though the profile (Fig 2.4). The moisture and organic content varied by location ($p<0.001$ for both), with an increasing trend downstream. There was a significant interaction between depth and location for moisture content ($p=0.009$). The furthest downstream sampling locations (location 5 and 6) had the highest moisture and organic content. Location 4, which had the deepest SOF sediment layer, had higher moisture and organic content deeper

in the profile (Fig 2.4). The moisture content at location 4 peaked at the 55 cm depth, differing from the other locations (Fig 2.4).

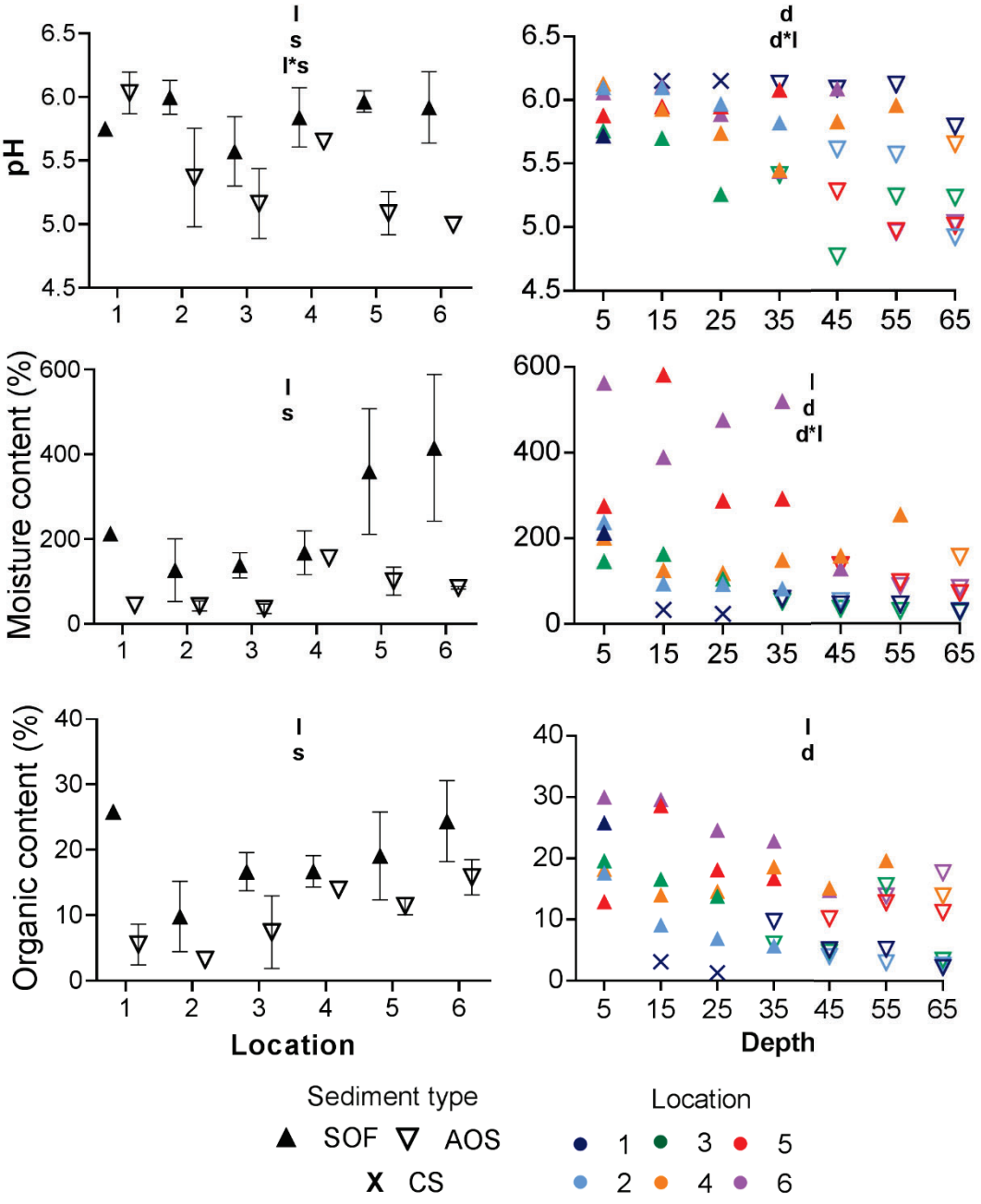


Figure 2.4 Sediment pH, organic content and moisture content for each sampling location by sediment type and depth. Column 1: Sediment types are *surficial organic fines* (SOF) and *alternating organic sands* (AOS). Statistical test by sediment type is repeated measures ANOVA, $p<0.05$ (SOF n=23, AOS n=17), 'l' indicates difference by location, 's' indicates difference between sediment types and 's*l' indicates significant interaction between sediment type and location. Column 2: Sediment indicators by depth, colours indicate location. Statistical test by ANCOVA, $p<0.05$ (location n=6, depth n=7) 'l' indicates difference by location, 'd' indicates difference by depth and 'd*l' indicates significant interaction between sediment type and location.

GENE ABUNDANCES (QPCR)

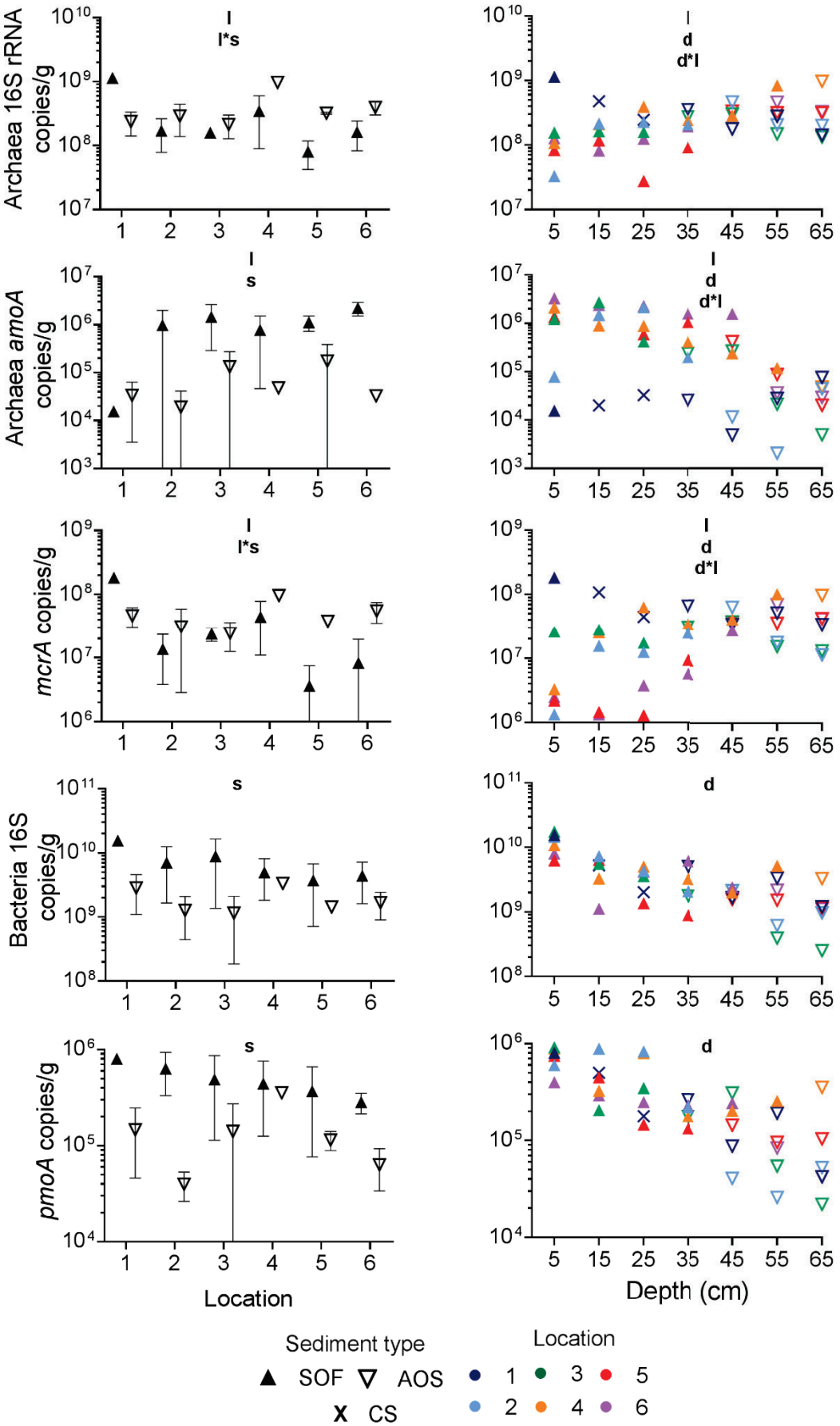
DEPTH PROFILE AND SEDIMENT TYPE

The bacteria 16S rRNA gene (p=0.013), methanotroph *pmoA* (p=0.011) and archaea *amoA* gene (p=0.007) were more abundant in the SOF layer than the AOS and showed a decline in abundance with depth (all p<0.001). The archaea 16S rRNA gene (p=0.002) and methanogen *mcrA* gene (p=0.001) varied by depth but did not show statistical differences by sediment type (Fig 2.4).

LOCATION

No significant differences of the bacteria 16S rRNA gene or *pmoA* gene abundances were found by location. The archaea 16S rRNA gene (p=0.007), methanogen *mcrA* (p=0.009) and archaea *amoA* (p=0.007) abundances did vary by location. These three genes also had an interaction of location and depth (archaea 16S rRNA p=0.007, *mcrA* p=0.01, archaea *amoA* p=0.028). The differences found by location appear to be driven by inverse relationship of depth profile to the gene or different abundance levels at location 1. The archaea *amoA* gene was the least abundant at location 1 while the *mcrA* gene was most abundant. Location 1 had approximately an order of magnitude greater abundance of archaea 16S rRNA at 5 cm sampling depth ($1.14e^9$ copies) compared with the other sampling locations ($3.29e^7$-$1.55e^8$ copies). At location 1, *mcrA* gene abundance was highest at the 5 cm sampling depth, whereas other location it was higher at greater depths. The archaea *amoA,* on the other hand, had relatively low abundances that were approximately one to two orders of less than at other locations, though the abundances increased to be of similar abundance to the other locations at the 55 and 65 cm (Fig 2.5).

Figure 2.5 Gene abundances by sediment type, depth and location (next page). Column 1: Sediment types are surficial organic fines (SOF) and alternating organic sands (AOS). Statistical test by sediment type is repeated measures ANOVA, p<0.05 (SOF n=23, AOS n=17), 'l' indicates a difference between locations, 's' indicates difference between sediment types and 's*l' indicates significant interaction between sediment type and location. Column 2: Sediment indicators by depth, colours indicate location. Depth where sediment became AOS is indicated by corresponding coloured triangle and for location 1 depth for which the sediment became CS indicated by x symbol. Statistical test by ANCOVA, p<0.05 (location n=6, depth n=7) 'd' indicates difference by depth and 'd*l' indicates significant interaction between sediment type and location.


Archaea 16S rRNA copies/g
l
l*s
l
d
d*l
Archaea amoA copies/g
s
mcrA copies/g
Bacteria 16S copies/g
pmoA copies/g
Location
Depth (cm)
Sediment type
SOF
AOS
CS
Location
1
2
3
4
5
6

Microbial community

Bacteria

Generally, with exception of location 1, the depth profiles of the bacterial community followed a pattern depicted on the NMDS spread from right to left and downward with depth (Fig 2.6). The bacterial communities of the SOF and those higher in the profile were generally more clustered together whereas the AOS and deeper samples communities were more dispersed (Fig 2.6). This suggests that the communities higher in the profile were more similar and the deeper communities were more variable. The NMDS depicts the microbial communities of location 1 clustered together and isolated from the communities at the other locations and a different pattern through depth profile. Reflecting this, the PERMANOVA showed differences between sampling locations ($p=0.001$) and an interaction between the sediment type and location ($p=0.001$). The PERMANOVA did not detect a significant difference in bacterial community composition between sediment types, but did by depth ($p=0.001$).

Archaea

Not all samples had adequate PCR amplification for T-RFLP analysis of archaea, so the dataset only has a subsample of the total samples collected. Generally, the deeper samples were better represented, with exception of location 1, adjacent to the stormwater drain, where the entire profile was sufficiently amplified. Results from the qPCR with the high levels of *mcrA* genes high in the profile may be a reason for better PCR amplification for location 1. The ordination of the archaeal community shows two separate groups. All of location 1 was clustered in the group on the left side. No statistical differences were found and this is maybe due to the poor representation of samples. The visualisation of the data shows there were generally two distinct community compositions (Fig 2.6) with all of the samples from location 1 were clustered on the left.

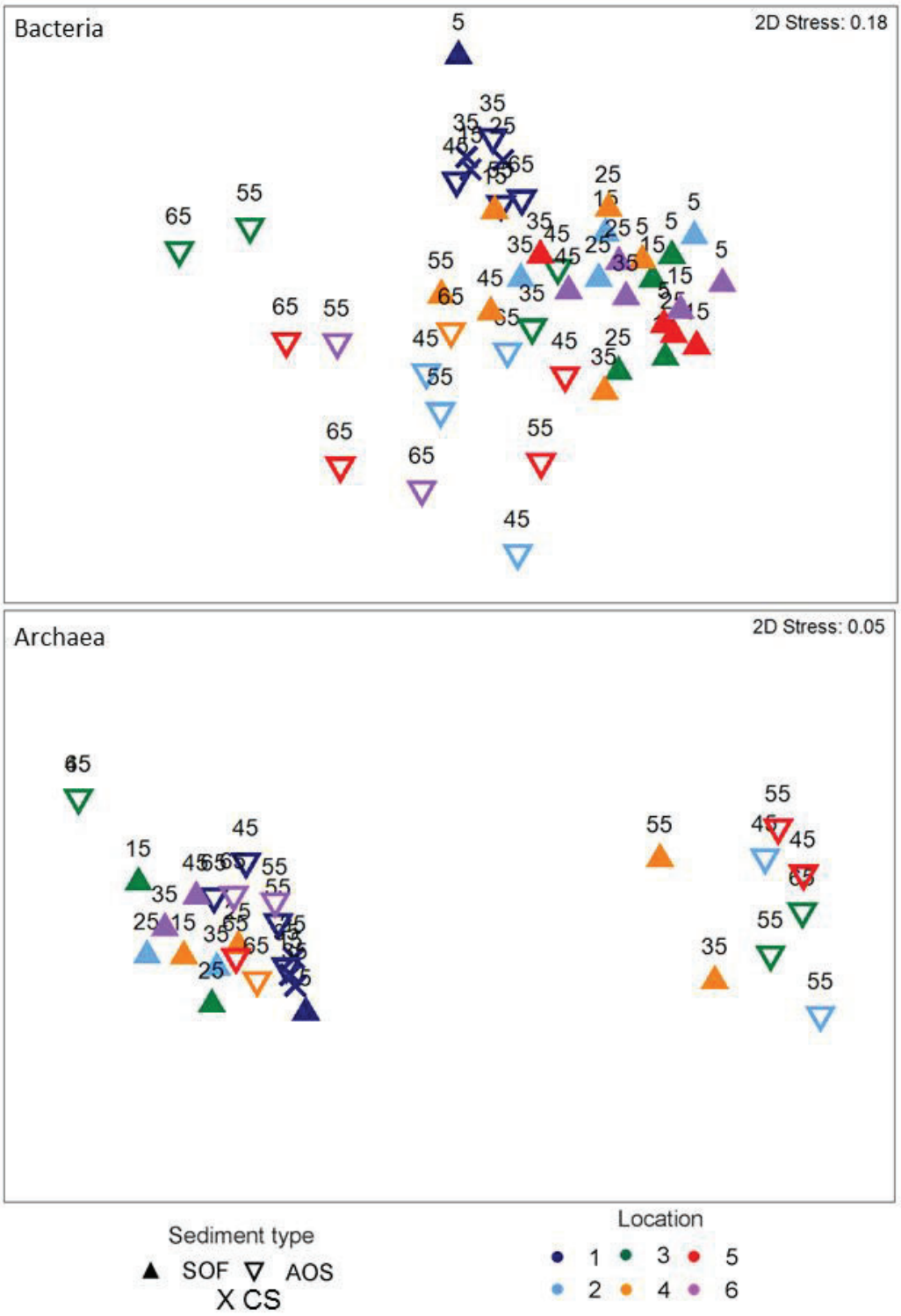


Figure 2.6 Microbial community NMDS. Visualisation of bacteria and archaea community using non-metric multidimensional scaling based on Bray-Curtis similarity matrix. Colours represent location in swamp as marked in Fig. 2.1, symbol represent sediment type and depth number indicates depth in cm.

DISCUSSION

Upland swamps provide landscape heterogeneity as an island of saturated wetland habitat in a sea of dry sclerophyll woodlands and forests. The results shown here suggest that heterogeneity occurs within an individual swamp, with both longitudinal and vertical changes in environmental conditions and microbial assemblages and functions. Changes in the sediment profile were associated with differences in the microbial structure and function. Our findings particularly highlight the sensitivity of swamp systems to activities and inputs from the surrounding semi-urban catchment. Location 1 has undergone restoration activity (c.a. 2008) with installed coir logs to buffer the effects of the stormwater inputs. Location 1 had a markedly different sediment profile and different microbial communities to the downstream parts of the swamp. Importantly, however, the impacts of the disturbance were localised, and consistent patterns with depth were evident in the downstream areas of the swamp.

In accordance with previous studies, the organic and moisture content of the sediments changed with depth and we found distinct sedimentary layers in a sequence expected for swamps of this type (Freidman and Fryirs, 2014, Fryirs *et al.*, 2014a, Cowley *et al.*, 2016). In the top 65 cm of the sediment profile there were generally two sedimentary layers, the SOF and AOS. Although the mineral content, variable climate and peat forming ability make these swamps quite different from those in the northern hemisphere (Fryirs *et al.*, 2014a), these primary sedimentary layers have similar functions and properties to the acrotelm and catotelm of northern latitude peatlands (Ingram, 1978, Cowley *et al.*, 2016). The uppermost layer, SOF, has higher organic and moisture content (Cowley *et al.*, 2016, Cowley *et al.*, 2018b) (Fig 2.4) compared to the AOS. The SOF is composed of live and decaying organic material, while the AOS is typically the thickest sedimentary layer and stores peat (Cowley *et al.*, 2016). We found the depth of the SOF to AOS transition in the sediment profile varied, but most locations (4 out of 6) was at or above 45 cm depths, and at all sites was above 65 cm. Our sampling did not extend beyond these depths however, the AOS layer at Wentworth Falls Lake may extend to over 200 cm deep in some areas, with fine cohesive sands (FCS) and/or basal sands and gravel (BSG) below, both of which are highly mineral with lower organic and moisture content (Fryirs *et al.*, 2014a).

In addition to the organic and moisture content differences between the layers and with depth, we unexpectedly found that the pH decreased with depth and was different between the sediment types. The SOF having higher (more neutral) pH than the AOS. Inverse to our results, peat profiles from sphagnum swamps have been reported to have a higher (more neutral) pH in deeper sediments (Steinmann and Shotyk, 1997, Cadillo-Quiroz *et al.*, 2006). Cadillo-Quiroz *et al.* (2006) ascribed the pattern to the wetland's vegetation history. Steinmann and Shotyk (1997) suggest the pH changed due to a shift from acid producing biogeochemical reactions to acid consuming reactions with depth due to increasingly reducing conditions. In the present case, we speculate the observed decrease of the pH with depth may be related to relative inputs from surface and groundwater sources in these swamps. Generally, the swamps of the Blue Mountains have significant proportion of waters derived from local, shallow groundwater sources (Cowley *et al.*, 2019). The groundwater contribution for Wentworth Falls

Lake swamp has not been specifically determined, but a nearby swamp in Wentworth Falls had about 80% of its waters derived from groundwater arising from the local Hawkesbury sandstone (Cowley *et al.*, 2019). Deeper sediments would more likely be influenced by the natural groundwater and/or the lake water of the reservoir, while sediments higher in the profile are more likely to be influenced by stormwater runoff with higher pH (Tippler *et al.*, 2014). Where there was the greatest influence of stormwater, location 1, higher pH was found throughout the depth profile.

Along the downstream gradient of the swamp, we found an increase in sediment moisture and organic content with increasing distance downstream (Fig 2.4). The natural swamp structure is a gently sloped valley (gradient 3-4%) of accreting sediment behind a bedrock step. (Freidman and Fryirs, 2014, Fryirs *et al.*, 2016, Cowley *et al.*, 2018b, Fryirs *et al.*, 2018). The bedrock step helps maintain the water table. With a sloped structure it is expected the water table would be closer to the surface at the downstream end explaining the moisture content results we found. In this particular swamp, the relationship is likely enhanced due to the swamp ending at a reservoir flooding a portion of what would have been the original swamp. It is logical that this would elevate the natural water table, especially in the sampling locations furthest downstream.

The bacterial communities associated with shallow sediments were clearly different from those in deeper (>45 cm) sediments (Fig 2.6) and the communities higher in the sediment profile were more similar (e.g. more closely clustered) than those deeper in the profile. Cadillo-Quiroz *et al.* (2006) observed a similar pattern where methanogenic communities were relatively more similar at the surface of different swamps than the communities deeper in the profile. This may be reflective greater heterogeneity at depth with varying influences of groundwater, stormwater, historical vegetation and decomposition. Even though the sediment types are related to depth, we surprisingly did not find statistical differences for the bacterial community assemblage by sediment type. Many species can be found throughout the depth profiles (Preston *et al.*, 2012), and may have impinged on the ability to detect more profile related statistical differences. The lack of difference specific to the sediment type may also be related to the position of the SOF transitioned to AOS which ranged from around 35 to 65 cm depth. Other physicochemical properties more directly related to depth, such as oxygen availability (Hargrave, 1972) and redox potential (Steinmann and Shotyk, 1997) may be more influential to the microbial communities than the properties specifically associated with the different sediment layers (organic and moisture content).

The gene abundance results support oxygen availability as an important determinant of microbial community make up. The gene abundance results for genes encoding for metabolic pathways requiring aerobic conditions, archaea ammonia oxidation (*amoA*) and methane oxidation (*pmoA*), declined with depth in in agreement with other findings (Höfferle *et al.*, 2010, Xu *et al.*, 2019, Zhou *et al.*, 2015). Inversely, we would have expected the *mcrA* gene, which requires anaerobic conditions for methanogenesis, to be more abundant at depth. However, we did not find a consistent relationship with depth or the sediment type, rather the pattern of *mcrA* versus depth varied by location. Location 1 had highest *mcrA* abundances at 5 cm depth while locations 5 and 6 (furthest downstream and the wettest), showed an increase through the depth profile to 55 cm (Fig 2.4). Studies in northern hemisphere

peatlands have found methanogenesis potential peaked below the water table, suggesting that reducing conditions as a determinant of methanogenesis (Sundh *et al.*, 1994, Cadillo-Quiroz *et al.*, 2006). However, rates of methanogenesis did not increase continually with depth, rather they peaked at intermediate depths. For example, Sundh *et al.* (1994) reported average methane production peaked at 12 cm below the water table and Cadillo-Quiroz *et al* (2006) found potential methanogenesis was higher at 10-20 cm depth than at 40 cm depth (Cadillo-Quiroz *et al.*, 2006). Although we are reporting gene abundances, which is different to direct production, gene abundance and production potential have been shown to be related (Petersen *et al.*, 2012).

The conditions and microbial differences at location 1, discussed above, seems to reflect the legacy of erosion and ongoing stormwater influences despite restoration efforts at that location. A layer of CS, which indicates previous erosion, sediment deposition and disturbance (Cowley *et al.*, 2016), was only present in location 1. Although there was a SOF layer above the CS, suggesting the reestablishment of organic surface sediments is occurring, the conversion of CS to functional AOS is likely to require a greater timeframe (Cowley *et al.*, 2016). AOS is primarily made up of peat with alternating sand layers (Fryirs *et al.*, 2014a). Critical to the formation and storage of peat is slow decomposition of organic materials which is generally mediated by anaerobic conditions and low pH (Mitsch and Gosselink, 2015). However, the pH at location 1 was higher than at other locations (Fig 2.4). The pH from naturally acidic catchments, such as these in the Blue Mountains swamps, can be elevated in stormwater runoff from an impervious surfaces and concrete infrastructure (Tippler *et al.*, 2014, Belmer *et al.*, 2015). The higher pH throughout the depth profile at location 1 is likely due to the localised stormwater inputs and may impinge on the sedimentological recovery at this location.

The return of microbial function in restored peatlands can also lag behind structural restoration and even the return of vegetation (Andersen *et al.*, 2006, Reumer *et al.*, 2018). In acknowledgement of the importance of microbial community and activity to the function of ecosystems, microbial indicators have been used to assess efficacy of restoration effort in peatlands (Watts *et al.*, 2008, Reumer *et al.*, 2018) as well as other ecosystems (Harris, 2003). The bacterial community at location 1 differed from that in the other locations in terms of composition and depth profile (Fig 2.6). The inverted pattern and overall higher abundances of *mcrA* (Fig 2.5) may be influenced by different substrate availability or the pH at location 1, both of which may be related to the stormwater input. Generally, methane production is greatest at circum-neutral pH (Wang *et al.*, 1993). The disturbance and runoff at location 1, and subsequent increase in sediment pH and nutrients may have negatively affected the archaea *amoA* abundances since *amoA*-mediated ammonia oxidation is generally favoured under acidic (Nicol *et al.*, 2008, Zhang *et al.*, 2012b) and oligotrophic conditions (Höfferle *et al.*, 2010, Sims *et al.*, 2013). Greater sampling and investigation are needed to confirm these observations.

Despite the significant role microbes play in carbon and nitrogen cycling in peatlands, the microbial ecology of the Sydney Basin swamps had not been explored. A survey of 24 bogs in north-eastern USA suggested there was little variation between peat bogs (Morales *et al.*, 2006). Greater investigation is needed to determine if these initial results that suggest spatial heterogeneity within a single swamp are found across the swamp type generally. These Australian swamps experience greater climatic

variability than northern hemisphere peatlands and more mineral substrate, which helps explain differences in their peat forming abilities (Fryirs *et al.*, 2014a). Variable climatic and sediment conditions also likely influence microbial diversity and heterogeneity. Clearly, further investigation into these swamps is required to improve our ecological understanding of them as they are distinct from their well-studied northern analogues. Specifically, understanding response to disturbance will be imperative to the ongoing protection and management of these endangered ecosystems. Urban encroachment, among other disturbances, continues to be a problem for these swamps (Kohlhagen *et al.*, 2013, Fryirs *et al.*, 2014b, Belmer *et al.*, 2018) and wetlands around the world (Zedler and Kercher, 2005). Studies that target the effect of anthropogenic disturbance on swamp microbial function are still needed (Chapters 3-5).

CHAPTER 3

MICROBIAL COMMUNITIES OF UPLAND PEAT SWAMPS WERE NOT DIFFERENT ONE YEAR AFTER A HAZARD REDUCTION BURN

Published as:

Christiansen, N. A., Fryirs, K. A., Green, T. J. and Hose, G. C. (2020) Microbial communities of upland peat swamps were not different 1 year after a hazard reduction burn', *The international journal of wildland fire.*

ABSTRACT

Fire in wetlands is poorly understood, yet hazard reduction burns are a common management practice and bushfires are becoming increasingly prevalent because of climate change. Fire may have long lasting implications to the microbial component of wetland ecosystems. Wetland microbes are significant for their role in carbon and nutrient cycling, so it is imperative to understand the effects of fire on wetland ecosystems, particularly on microbes that reside in sediment. Within the extremely fire prone Blue Mountains of south eastern Australia are endangered peat forming swamps that regularly experience both bushfires and hazard reduction burns. In a before-after-control-impact study we surveyed the sediment microbial community of these swamps to test the impact of hazard reduction burning on them. Along with sediment pH, moisture and organic content, we measured gene abundances including those relating to carbon cycling (qPCR of *pmoA*, *mcrA*, bacterial 16S rRNA and archaeal 16S rRNA), and bacteria community fingerprint (T-RFLP). One year after the hazard reduction burn there were no significant differences in the gene abundances or microbial community fingerprint that could be attributed to the fire, suggesting that the burn did not have long-term impact on these microbial communities.

INTRODUCTION

Fire is a widespread phenomenon, influencing ecosystem patterns and processes worldwide (Bowman *et al.*, 2009), and playing a key role in shaping the composition, structure and dynamics of many ecosystems (Bond and van Wilgen, 1996, Pausas and Keeley, 2009, Pausas and Parr, 2018). Natural fire regimes in many places have changed due to human activities either by directly altering the frequency and intensity of fires through fire exclusion or promotion practices, such as hazard reduction burning, or indirectly by climate change shifting the conditions and locations where fires occur (Woinarski *et al.*, 2004, Parks *et al.*, 2016, Williamson *et al.*, 2016). Even with standing water or saturated soils, wetland ecosystems can experience fires (Reardon *et al.*, 2005). However, there have been relatively few studies on the effects of fire in wetland ecosystems compared with terrestrial ecosystems (Kotze, 2013, Bixby *et al.*, 2015). Peat forming wetlands store more carbon than Earth's forests (Parish *et al.*, 2008) and wetlands have a key role in global nutrient cycling (Mitsch *et al.*, 2015). It is therefore imperative we improve our understanding of the impact of fire on these systems (Bixby *et al.*, 2015), particularly the microbial communities that mediate these processes (Andersen *et al.*, 2013, Medvedeff *et al.*, 2015).

How a microbial community will be affected by fire generally depends on a number of factors, such as the fire intensity, sediment characteristics and climatic conditions (Neary *et al.*, 1999). Immediately and in the short term, heat from the combustion reaction can kill microbes, reducing abundances or changing the makeup of the microbial community (Hamman *et al.*, 2007). Along with the intensity of the

fire, the moisture content, mineral and organic makeup of the soil will affect the heat transfer into the sediment profile, and whether, and to what depth this is lethal to the microbes (Klopatek *et al.*, 1990, Neary *et al.*, 1999, Choromanska and DeLuca, 2002, Penman and Towerton, 2008). Microbes may rapidly regenerate and re-establish with appropriate conditions. However, fire can alter the ongoing microbial sediment environment by changing nutrient levels, organic content, chemical properties and ambient temperature as a result of volatilisation, erosion, deposition and exposure to solar radiation due to vegetation loss (Neary *et al.*, 1999). Nutrients and carbon may be released for faster uptake by microbes, but also lost through combustion and erosion (Ward *et al.*, 2012). These changes to the sediment environment may alter the microbial community that re-establishes and potentially cause lasting changes to nutrient and carbon cycling.

The Blue Mountains, New South Wales, Australia, are notorious for extreme bushfires (Cunningham, 1984, Hammill and Tasker, 2010). Throughout the Holocene, fire – initiated by human activities and by natural events – has been an influence on this landscape. As a result, fire, along with climate conditions, has shaped the present ecological communities of the Blue Mountains (Black and Mooney, 2006). Climate change models suggest that south eastern Australia will experience harsher fire weather with more heatwaves, prolonged droughts and higher evaporation rates (Whetton, 2015). Because of the extreme risks of fires, hazard reduction burns are a common practice in the area to reduce standing fuel loads to protect ecosystems, human life and structures from extreme wild fires (Hammill and Tasker, 2010). Changes to fire regimes, including hazard reduction burns and natural fires, are likely to alter the biota, vegetation and functions of ecosystems.

Amongst the upland forests of the Blue Mountains are spatially and conditionally restricted endangered wetland peat swamps (Temperate Highland Peat Swamps on Sandstone, THPSS hereafter called swamps) that are vulnerable to fire and changing climatic conditions (Pemberton, 2005, Keith *et al.*, 2010, Keith *et al.*, 2014). The swamps are home to endangered and endemic species, and are important for overall catchment health as they feed into the domestic water supplies of Sydney, Australia (population >5 million) (Young and Young, 1988, Benson and Baird, 2012, Fryirs *et al.*, 2014b, Gorissen *et al.*, 2015, Cowley *et al.*, 2018b, Fryirs *et al.*, 2018, Gorissen *et al.*, 2018). Already, swamps within the Blue Mountains are regularly experiencing wild fires, as many as four times between 1967-2013 (Gorissen *et al.*, 2015) and a number of the swamps are near or within urbanised areas where hazard reduction burns are planned or already take place. Thus, it is important to understand the ecological repercussions of hazard reduction burns as a management practice and of fires in general for these swamps.

In this study, we undertook a before-after-control-impact study comparing the sediment microbial community of swamps in the Blue Mountains National Park to determine whether a hazard reduction burn had long-term (>1 year) measureable effects on the swamp microbial community. While hazard reduction burns are typically lower intensity and have reduced ecological repercussions than a bushfire (Certini, 2005, Dooley and Treseder, 2012, Brown *et al.*, 2014), we can consider such burns to also represent the conditions that would occur in a low intensity bushfire. We examined the total bacterial

community and specific functional genes of sediment microbes using molecular techniques (T-RFLP, qPCR). Sediment pH, moisture and organic content were also measured.

Because carbon cycling and storage is an important function of peat forming swamps and wetlands are also the greatest natural source of methane globally (Kirschke *et al.*, 2013), we were particularly interested in changes to methane cycling genes, the methyl coenzyme-M reductase gene (*mcrA*) associated with methane generation (*mcrA*) and methane monooxygenase gene (*pmoA*) associated with methane consumption (oxidation). Like many of their southern hemisphere analogues (Kotze, 2013), the swamps of the Blue Mountains have variable climatic conditions resulting in marginal peat formation (Fryirs *et al.*, 2014a). It has been shown that hydrological disturbance to the swamps results in greater fluvial carbon exports, in particular methane (Cowley *et al.*, 2018a).

We hypothesised that changes to sediment characteristics due to the fire would cause a shift in the microbial community. In particular, we expected the pH to increase post fire (Battle and Golladay, 2003, Danilova *et al.*, 2015, Bang-Andreasen *et al.*, 2017) with different pH levels influencing microbial communities and diversity (Hartman *et al.*, 2008). We also hypothesised that the abundance of methane cycling genes would be altered. Increased methane production has been observed after a fire (Levine *et al.*, 1990, Hogg *et al.*, 1992, Medvedeff *et al.*, 2015), as has an increase in methane oxidation activity (Danilova *et al.*, 2015).

METHODS

STUDY SITES

The study area (33°39'16.40"S 150°23'1.71E) is in the Blue Mountains National Park, approximately 100 km west of Sydney, Australia where low relief valley bottom swamps overlying sandstone are common (Fryirs *et al.*, 2018). The area has a temperate climate. The nearby town centre of Katoomba receives a mean annual rainfall of 1403 mm. Mean maximum temperatures range from 9.4°C in July to 23.3°C in January and mean minimum temperatures range from 2.6°C in July to 12.9°C in February (BOM, 2017).

A total of six swamps were sampled as part of this study. Three swamps within the area to be burned and three control swamps located nearby in areas not affected by the burn (Fig 3.1). The swamp sites were selected based on the anticipated extent of the planned hazard reduction burn and accessibility. All of the swamps were within naturally vegetated catchments with the only direct human impact being a gravel road along the ridge. The swamps were accessed from this road (Mount Hay Rd) that also served as a firebreak. The swamps are valley fills dominated by low sedge with typical native plant communities surrounded by eucalypt woodland (Keith and Myerscough, 1993). The geomorphic and sedimentological structures have been described previously (Freidman and Fryirs, 2014, Fryirs *et al.*, 2014a, Fryirs *et al.*, 2014b, Hose *et al.*, 2014, Cowley *et al.*, 2016, Chapter 1). Sediment is moist but not saturated at the surface, generally these swamps water table is about 20 cm below ground level (Cowley *et al*. 2018b).

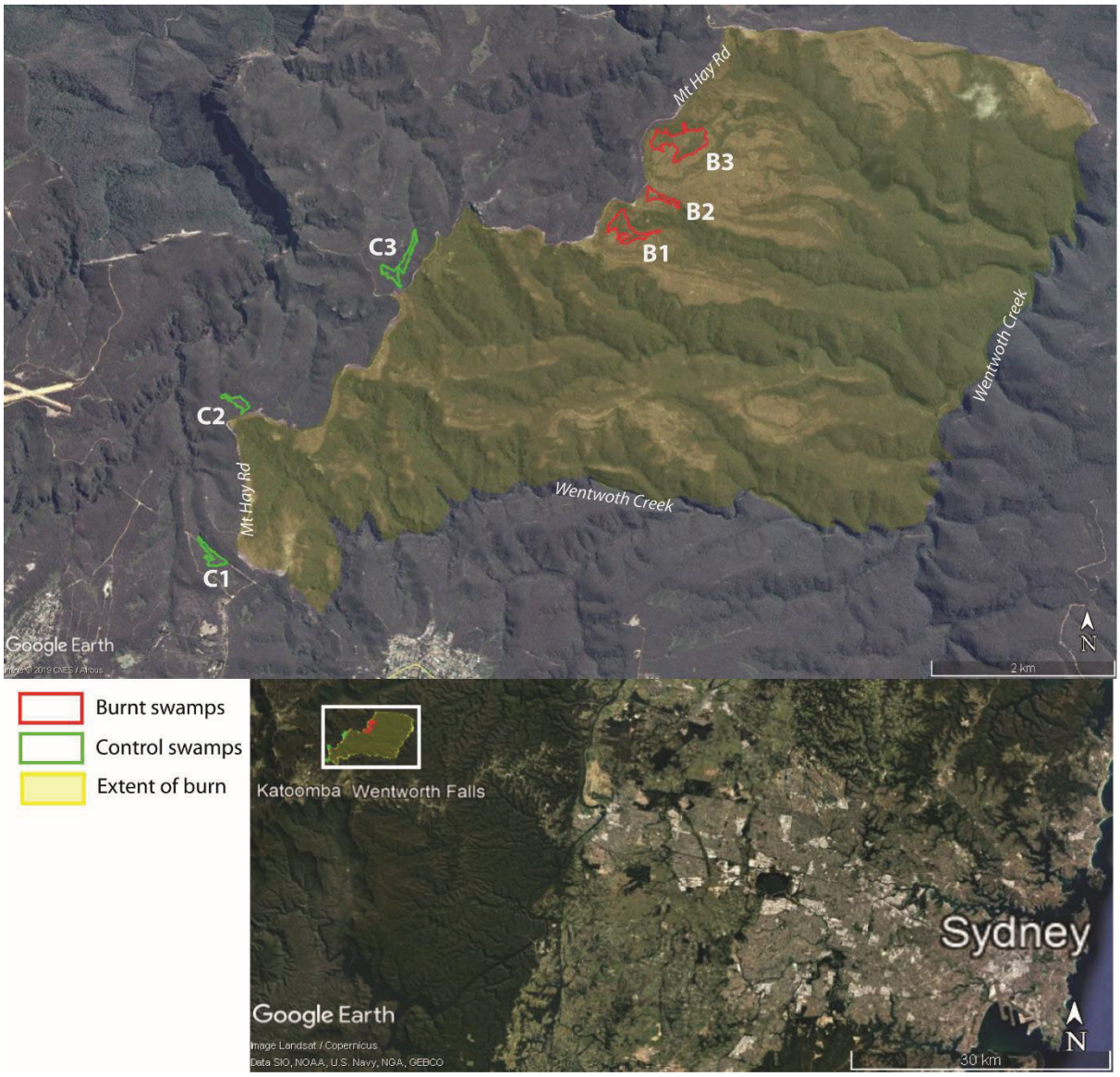


Figure 3.1. Map showing location of all swamps sampled. Aerial image taken after the fire and burnt areas are visible. Swamps outlined in red were burnt and swamps outlined in green were control - not affected by the hazard reduction burn. © Google Earth 2019.

HAZARD REDUCTION BURN

The hazard reduction burn was undertaken 5-6 May 2016 covering an area of approximately 3000 Ha, in an area 1 km north of the town of Wentworth Falls in the Blue Mountains National Park. The fire was contained within Mt Hay Road and Wentworth Creek (http://www.rfs.nsw.gov.au). Weather conditions at Katoomba on the days of the burn were warm (maximums 20°C and 22.3°C) and dry with no recorded rainfall between May 2 and 8 (BOM 2017). Satellite images show the extent of the fire (Figs 3.1 and 3.2).

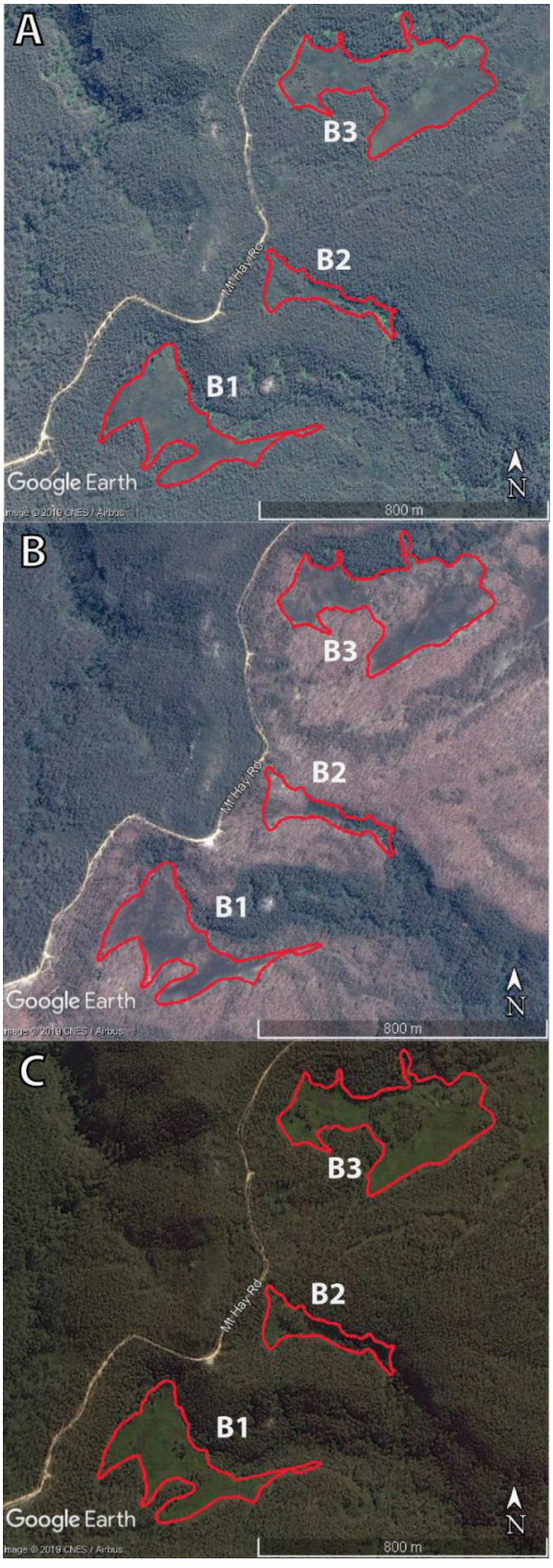


Figure 3.2. Area of hazard reduction burn and the affected swamps sampled before and after. Satellite images showing greater detail from (A) prior to fire (March 2014), (B) approximately 5 months after the fire (October 2016) (B), and (C) approximately 1 year after the burn (May 2017). Burnt swamps outlined in red. © Google Earth 2019.

SAMPLE COLLECTION

Sediment was collected from the swamps approximately one year prior to the burn (April 2015) and one year after the burn (April 2017). Each sample consisted of the top 2 cm of sediment and was collected from 5 random locations within each swamp that were similar and characteristic of the central swamp axis. Deeper sediments are less affected by disturbances such as fire (Penman and Towerton, 2008, Waldrop and Harden, 2008) and urbanisation (Christiansen *et al.*, 2019). The surface sediment layer of these swamps is composed of living and decomposing organic matter, fine silt and sand (Cowley *et al.*, 2016). Samples for molecular and chemical analysis were immediately snap-frozen upon collection using dry ice and stored at -80° C and -20°C, respectively.

Despite sampling at the same time of year to avoid any seasonal effects, leading up to the times of sampling the area experienced different rainfalls. In March 2015, rainfall at the nearest weather station was below average (110.6 mm) whereas in March 2017, rainfall exceeded the 95 percentile of the historic record (535.6 mm) (BOM 2017).

SEDIMENT PHYSICOCHEMICAL ANALYSES

Sediment was analysed for pH, moisture and organic content. The soil moisture and organic content was measured gravimetrically. Sediment was dried overnight (105° C for >12 h) to determine the moisture content. Then the total organic content was determined by loss on ignition (550° C for 5 h) (Heiri *et al.*, 2001). Soil moisture and organic content were calculated as a percentage of dry weight. This method of calculation can result in moisture content values higher than 100%, which indicates the water mass was greater than the dry sediment mass. This occurs commonly in wet, organic sediment with low bulk density (Cowley *et al.*, 2016). The pH was measured in a 1:1 mass ratio sediment/deionised water slurry using a pH meter (Orion 3 star pH meter).

SEDIMENT MOLECULAR ANALYSES

DNA was extracted from sediment samples using the PowerSoil DNA Isolation Kit (MoBio) according to the manufacture's protocol. Purity and yield of purified nucleic acids was quantified using a NanoDrop 2000 spectrophotometer (Thermo Scientific Inc.). DNA was diluted to a 1:10 ratio with sterile MilliQ water to reduce any effect of PCR inhibitors.

TERMINAL RESTRICTION FRAGMENT LENGTH POLYMORPHISM

Terminal restriction length polymorphism (T-RFLP) analysis was used to compare community structure of bacteria between treatments (control and burned) and sampling event (before - 2015, after - 2017). Although T-RFLP does not provide the same resolution as amplicon sequencing, particularly for rare taxa, and may underestimate diversity, it has been demonstrated that T-RFLP provides an equally

powerful tool for comparisons of diversity and community structure among samples or treatments such as we are testing here (Orcutt *et al.*, 2009, van Dorst *et al.*, 2014, Chapter 6). The bacterial 16S rRNA gene was PCR amplified from extracted DNA with MyTaq polymerase (Bioline) using universal primers (FAM-labelled 27F, 1492R) and digested with restriction enzymes HhaI (Dunbar *et al.*, 2001). PCR conditions were: 94°C for 2 min then 35 cycles of 94°C for 15 sec, annealing temperature of 54°C for 30 sec and 72°C for 2 min. Digested PCR products were analysed on ABI3730xl Genetic Analyser at the Australian Genome Research Facility with LIZ1200 size standard. Data were processed using GeneMapper Software and the online tool T-REX (Culman *et al.*, 2009). Peak noise was removed (Abdo *et al.*, 2006), peaks were aligned within 2 base pairs and peaks that occurred only once in the dataset were removed. Attempts were made to amplify archaea 16S rRNA gene for T-RFLP, however the majority of samples did not have a visible band on electrophoresis gel.

GENE ABUNDANCE (QPCR)

Gene abundances were estimated using quantitative PCR (qPCR). A TaqMan qPCR assay was used to quantify the archaea and bacteria 16S rRNA gene using universal primers (archaea: ARC787F, ARC915F (FAM TAMRA labelled probe), ARC1059R and BAC338F, bacteria: BAC516F (FAM TAMRA labelled probe) BAC805R) (Yu *et al.*, 2005) with SensiFast Probe mix (Bioline) in 25 µL reactions. SYBR green qPCR assay was used to quantify the functional genes for methane production (*mcrA*) (forward ML-F (Steinberg and Regan, 2008), reverse ME-2 (Hales *et al.*, 1996)) and methane oxidation (*pmoA*) (forward A189gc (Holmes *et al.*, 1995) reverse mb661 (Costello and Lidstrom, 1999)) using universal primers using SensiFast SYBR No-ROX (Bioline). All qPCR reactions were carried out on a BioRad CFX96 RT System C1000TM Thermal Cycler. PCR included the following steps, initial denaturation (94°C, 2 min) followed by 40 cycles of denaturation (94°C, 5 sec) and hybridisation-elongation (annealing temperature (16S rRNA bacteria and archaea 60°C *pmoA* 62°C, *mcrA* 60°C), 45 sec). The SYBR assay also included melting temperature curve of the amplicon. Presence of targeted genes was confirmed with gel electrophoresis and 100 bp DNA ladder. Calculations of amplicon copy numbers were based on serial dilutions of target genes cloned into the pCR4-TOPO vector (Thermo Scientific Inc.), standard curves all with $r^2 \geq 0.99$ and dynamic range of $1e^2 – 1e^8$ copies. For each qPCR run, negative controls were included to confirm the integrity of the results (Smith and Osborn, 2009).

STATISTICAL ANALYSIS

Sediment properties, gene abundances and Shannon's diversity index were compared using a 3-factor analysis of variance (ANOVA). In these analyses, sampling event (before and after the burn), treatment (burnt and unburnt control) were considered fixed factors and site was considered a random factor and nested within treatment type. Data were assessed for normality and homogeneity of variance using Q-Q plots and plots of residuals, respectively, and data were log transformed where necessary to meet these assumptions. Tukey's post hoc pair-wise comparisons were used to test for differences between

levels where there was a significant interaction between treatment and sampling event. The above analyses were conducted in Minitab (Minitab® version 17.3.1).

Assemblage data based on T-RFLP profiles were visualised using non-metric multidimensional scaling (NMDS). Relationships between assemblages and environmental variables were visualised using distance based redundancy analysis (dbRDA) (Legendre and Anderson, 1999) and distance based linear models (DistLM) using stepwise selection. Peak height data were standardised (by total sample peak area) and square root transformed (Clarke, 2006) prior to analysis. The abiotic data were normalised and checked for strong correlations with draftsman plots. Variables with a correlation coefficient greater than 0.9 were removed from subsequent analyses (Clarke, 1993).

The comparisons of community assemblages between burnt and unburnt controls by sampling event were done using PERMANOVA, with a 3-factor linear model, with factors as for the ANOVA described above. Multivariate analyses were done using PRIMER & PERMANOVA + add on version 1.0.8 (PRIMER-E Ltd.) using the Bray-Curtis similarity index. The significance level (α) for all analyses was 0.05. PRIMER was also used to calculate Shannon's diversity index.

RESULTS

SEDIMENT CONDITIONS

No measureable statistical differences were found for the sediment pH, moisture and organic content related to the burn (Fig 3.3). There were statistical differences between the sampling events (2015 compared with 2017, both treatments combined). The mean (±SD) pH was slightly but statistically significantly higher in 2017 (4.43 ±0.21) than in 2015 (4.32 ±0.16) p=0.05). The mean moisture content was significantly higher in 2017 (652.4% ±362.3) than 2015 (393.1% ±164.8) (p=0.008). Organic content was also significantly higher in 2017 (55.3% ±23.2) than 2015 (35.2% ±13.7) (p=0.001). The consistency of these differences across all sites, and the absence of a significant time x treatment interaction (p>0.05) indicate these differences were due to factors other than the fire.

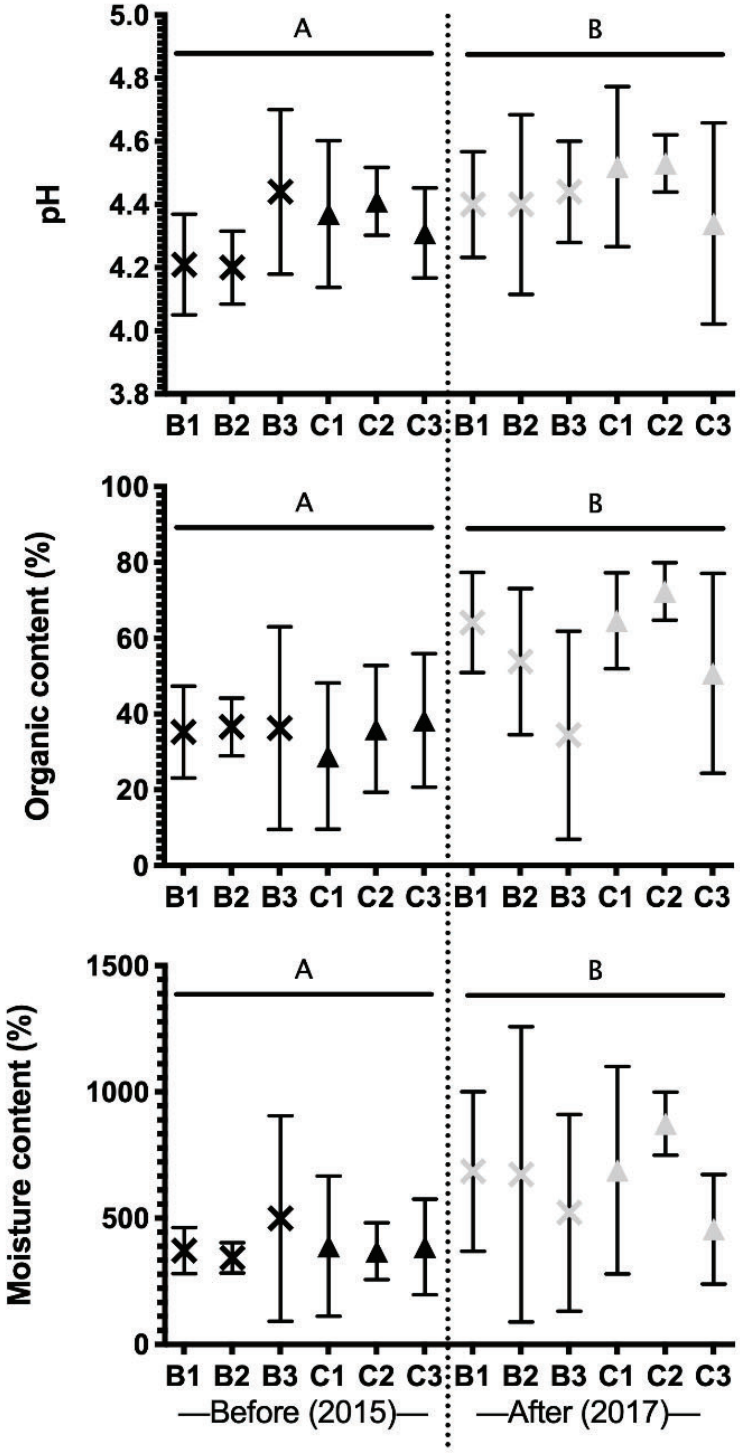


Figure 3.3 Sediment conditions in swamps. The 'x' symbols represent the swamps experiencing the hazard reduction burn and triangles are control swamps. Black symbols are average values from 2015 sampling, approximately 1 year prior to hazard reduction burn Grey symbols are from 2017, approximately 1 year after burn. Error bars are 95% CI; different capital letters indicate statistical differences between sampling events.

Table 3.1 Sediment conditions data. Presented are the mean and standard deviation (italics).

Date	Treatment	Swamp	pH	Moisture content (%)	Organic content (%)
Before (2015)	Control	C1	4.37	388.89	28.91
			0.19	*223.45*	*15.56*
		C2	4.41	369.46	36.06
			0.11	*112.54*	*16.71*
		C3	4.31	386.85	38.34
			0.14	*189.38*	*17.65*
	Burn	B1	4.21	371.87	35.25
			0.16	*91.77*	*12.11*
		B2	4.20	343.03	36.59
			0.12	*60.52*	*7.62*
		B3	4.44	498.98	36.29
			0.16	*255.52*	*16.80*
After (2017)	Control	C1	4.52	689.77	64.63
			0.25	*410.99*	*12.66*
		C2	4.53	874.74	72.38
			0.09	*124.85*	*7.56*
		C3	4.34	456.45	50.74
			0.32	*216.83*	*26.40*
	Burn	B1	4.40	685.11	64.15
			0.17	*315.41*	*13.19*
		B2	4.40	673.41	53.84
			0.28	*584.35*	*19.27*
		B3	4.44	521.37	34.45
			0.16	*389.65*	*27.46*

GENE ABUNDANCES

The number of copies of the archaea and bacteria 16S rRNA and the methanotroph (*pmoA*) did not vary significantly by sampling event or treatment. The abundance of methanogen (*mcrA*) gene copies did have a significant interaction between sampling event (time) and treatment (burnt) ($p=0.017$). The mean of the control swamps was greater at the second sampling (after the hazard reduction burn) (Fig 3.4). However, reviewing the results shows this relationship appears to be driven by control swamp 2, where the counts were much higher than the other swamps and we believe this statistical difference does not reflect a true change related to the hazard reduction burn or lack of the burn.

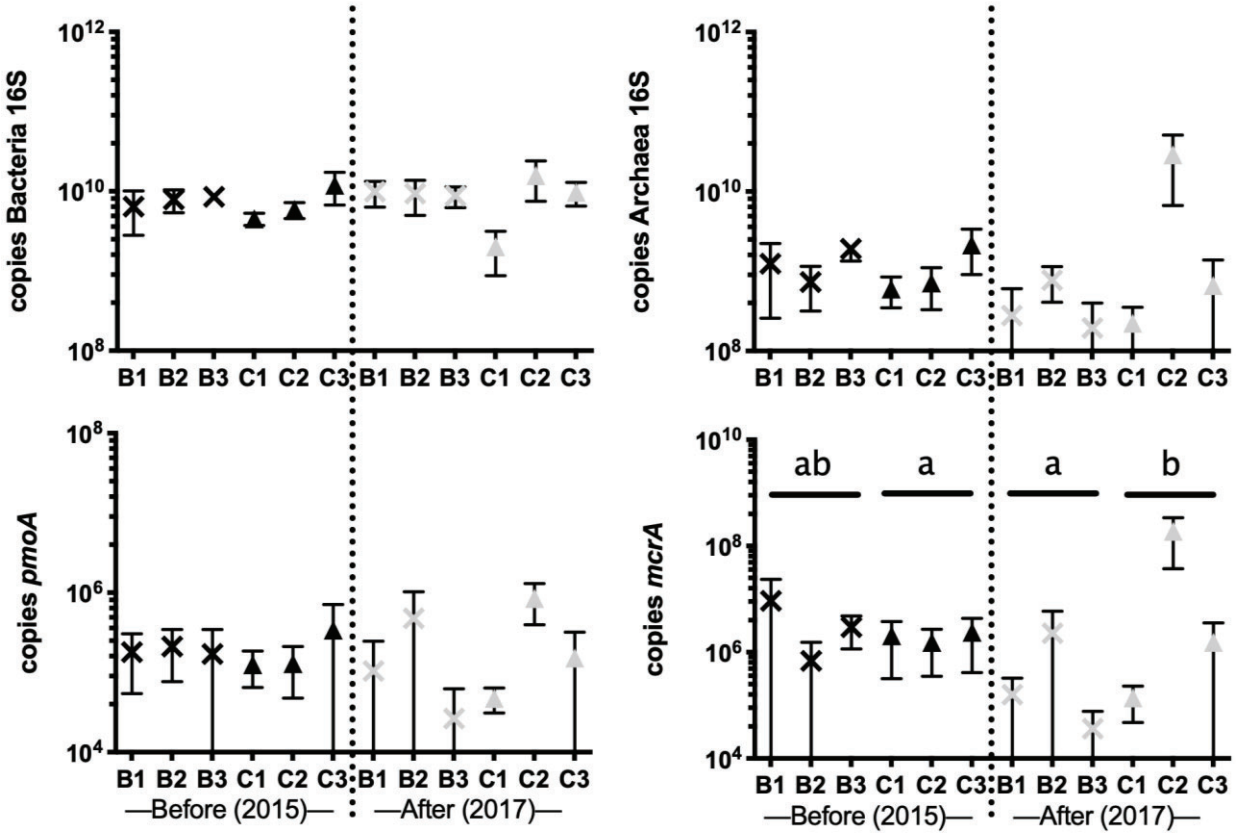


Figure 3.4 Gene abundances. The 'x' symbols represent the swamps experiencing the hazard reduction burn and triangles are control swamps. Black symbols are mean values from 2015 sampling, approximately 1 year prior to hazard reduction burn grey symbols are from 2017, approximately 1 year after burn. Units are copies of the gene per gram of sediment, error bars are 95% CI and different letters indicate statistical differences ($p<0.05$).

Table 3.2 Gene abundance data. Presented are the mean and standard deviation (italics).

Date	Treatment	Swamp	Bacteria 16S (copies/g)	Archaea 16S (copies/g)	*mcrA* (copies/g)	*pmoA* (copies/g)
Before (2015)	Control	C1	4.51E+09	5.99E+08	2.06E+06	1.25E+05
			8.96E+08	*2.86E+08*	*1.99E+06*	*6.89E+04*
		C2	5.89E+09	7.18E+08	1.53E+06	1.29E+05
			1.51E+09	*4.44E+08*	*1.35E+06*	*9.33E+04*
		C3	1.20E+10	2.15E+09	2.39E+06	3.40E+05
			6.04E+09	*1.41E+09*	*2.25E+06*	*4.21E+05*
	Burn	B1	6.45E+09	1.25E+09	9.27E+06	1.80E+05
			3.72E+09	*1.01E+09*	*1.46E+07*	*1.28E+05*
		B2	7.91E+09	7.35E+08	6.88E+05	2.12E+05
			2.56E+09	*4.25E+08*	*8.71E+05*	*1.38E+05*
		B3	8.54E+09	1.92E+09	2.98E+06	1.71E+05
			9.13E+08	*5.89E+08*	*1.86E+06*	*1.77E+05*
After (2017)	Control	C1	2.01E+09	2.23E+08	1.39E+05	4.74E+04
			1.16E+09	*1.32E+08*	*9.33E+04*	*1.67E+04*
		C2	1.58E+10	2.89E+10	1.89E+08	8.50E+05
			9.49E+09	*2.54E+10*	*1.73E+08*	*5.17E+05*
		C3	9.71E+09	6.63E+08	1.53E+06	1.52E+05
			3.26E+09	*7.42E+08*	*2.10E+06*	*1.71E+05*
	Burn	B1	9.85E+09	2.79E+08	1.59E+05	1.05E+05
			4.02E+09	*3.71E+08*	*1.91E+05*	*1.63E+05*
		B2	9.38E+09	7.76E+08	2.27E+06	4.75E+05
			5.02E+09	*4.19E+08*	*4.17E+06*	*6.30E+05*
		B3	8.80E+09	1.94E+08	3.67E+04	2.64E+04
			2.63E+09	*2.10E+08*	*4.11E+04*	*3.68E+04*

BACTERIAL COMMUNITY

From the T-RFLP analysis there were a total of 69 OTU peaks across the dataset. The number of peaks per sample ranged from 18 to 34 with a mean of 25.5 (SD±4.3) per sample. The average Shannon diversity index value was H'=2.4 (±0.2) and was not different by treatment type (burn/unburnt combined sampling times) ($p=0.516$) or by sampling event (before/after burn) ($p=0.740$), nor was there a significant interaction between sampling event and treatment ($p=0.052$).

The bacterial community fingerprint also did not show differences by treatment type (burn/unburnt combined sampling times) (p=0.074) or sampling event (before/after burn) (p=0.198), nor was there an interaction of time (before and after) and treatment (p=0.292). Visualised in the NMDS plot, the before and after of the burnt swamps are more closely clustered than the control (Fig 3.5) supporting the lack of differences found related to the fire.

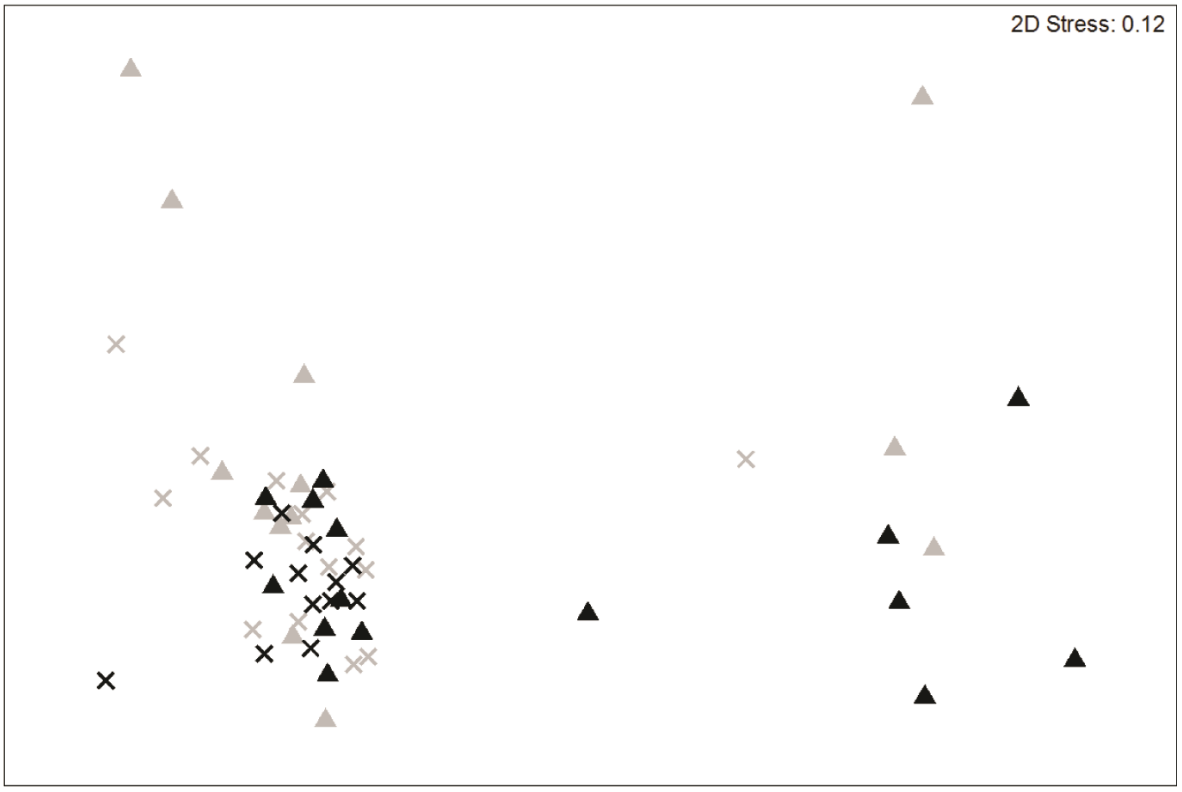


Figure 3.5. NMDS of bacterial community. Visualisation of the T-RFLP bacterial community data based on Bray-Curtis similarity matrix. Black symbols represent microbial community samples from before the burn (2015), the grey, after the burn (2017). Triangles are from control swamps that did not experience the hazard reduction burn and 'x' are from swamps that burned.

Correlations among environmental variables were all less than 0.9 so all were included in the DistLM. Moisture content and organic content were the most strongly correlated variables (r=0.88). DistLM sequential tests showed only the moisture content was significant in explaining the microbial community differences (Moisture content p=0.012; pH p=0.501; Organic content p=0.805) (Fig 3.6).

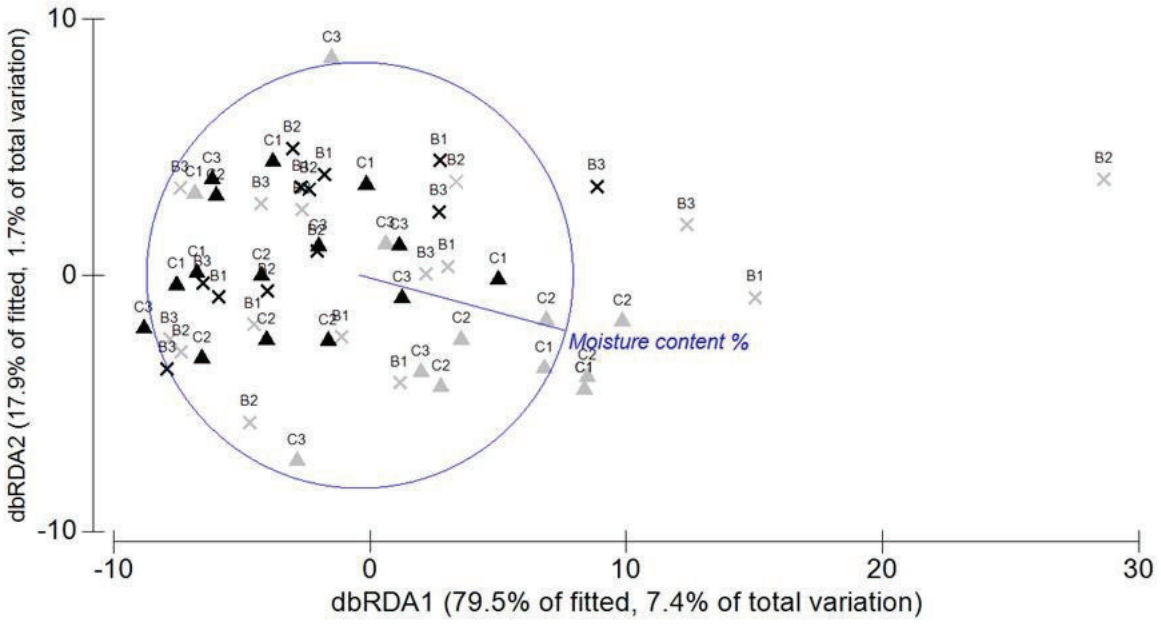


Figure 3.6. dbRDA of bacterial community. Visualisation of the T-RFLP bacterial community in relation to the sediment conditions data based on step-wise DistLM analysis. Only moisture content of the sediment was significant in explaining the variation. Black symbols represent microbial community samples from before the burn (2015), the grey, after the burn (2017). Triangles are from control swamps that did not experience the hazard reduction burn and 'x' are from swamps that burned

DISCUSSION

The effects of fires on wetlands ecosystems can be profound. Fire has the potential to shift a peat storing wetland ecosystem to shrub and grassland (Kettridge *et al.*, 2015) and altered conditions and microbial processes may persist for years after a fire (Belova *et al.*, 2014, Taş *et al.*, 2014, Danilova *et al.*, 2015). However, in upland swamps of the Sydney basin, we found no detectable shift in the swamp sediment bacterial community, methane cycling genes or measured sediment characteristics one year after a hazard reduction burn.

The severity of a burn will in part dictate the magnitude and duration of environmental and microbial changes (Watts *et al.*, 2015). Many of the changes are relatively short lived in surface fires compared to subsurface and peat consuming fires (Smith *et al.*, 2001). Hazard reduction burns are conducted to reduce standing biomass fuel by targeting only surface litter and vegetation (Agee and Skinner, 2005). They have less drastic impact on the soil profile (Certini, 2005, Penman and Towerton, 2008) and adjoining aquatic ecosystems (Arkle and Pilliod, 2010) than do wildfires. The sediment properties, particularly the moisture content, and the fire characteristics will influence the depth to which high temperatures penetrate into the soil profile and how lethal that is to microbes (Klopatek *et al.*, 1990,

Neary, 2005). Typically, in a surface fire, the heat transfer into the soil profile is minimal, only affecting the top 5-10 cm in most areas (Neary, 2005, Penman and Towerton, 2008). The hazard reduction burn of this study seemed to not cause significant subsurface impacts based on the rapid recovery of the vegetation. Within ten days the sedge had re-sprouted from charred stalks (Fig 3.7) indicating the subsurface rhizomes survived the burn. If temperatures lethal to microbes did not deeply penetrate the sediment or were not consistent across the substrate, bacterial could rapidly recolonise and may benefit from mild fire since they can withstand greater temperatures than can the fungal community, giving them a competitive advantage in a post fire environment (Bárcenas-Moreno *et al.*, 2011, Dooley and Treseder, 2012). It has even been noted that microbial biomass and metabolism in sediments were greater after a wetland fire, (Zhao *et al.*, 2012b). Those studies that found significant long-term differences in the microbial community also reported lasting changes to peat structure (carbon make up), water chemistry or vegetation community (Belova *et al.*, 2014, Taş *et al.*, 2014, Danilova *et al.*, 2015) suggestive of a more severe fire with significant ongoing repercussions.

Having sampled one year after the fire, we are likely to have missed short-lived changes to the microbial community resulting from the burn, however the focus of this study was to capture changes that would be of ongoing consequence to the microbial community. For example, post fire changes to microbial community structure or methane cycling gene abundances, which have been reported to occur 2-5 days after the fire (Levine *et al.*, 1990, Medvedeff *et al.*, 2013), would likely have resolved. Any changes to the microbial community that had returned to be similar to pre-fire conditions within a year, would have had a minimal impact relative to the typical inter-fire interval, which, for these Blue Mountains swamps' is between 6-35 years (Hammill and Tasker, 2010). A more severe fire or greater frequency of fire disturbance may not allow the system to recover as rapidly. For example, repeat controlled burning on ten year intervals seemed to influence carbon cycling between wetland vegetation and benthic microbial communities after 18 months since the most recent burn (Ward *et al.*, 2012). The swamps of this study had experienced 3 fires since 1967, the most recent being approximately 13 years prior to the hazard reduction burn of this study (Gorissen *et al.*, 2015). To place this in the regional context, some small areas of the Blue Mountains bushland have experienced as many as 7 bushfires within 40 years (1971-2009) (Hammill and Tasker, 2010).

Ongoing impacts to the microbial community would perhaps have been more likely if sediment environmental conditions had changed (Hartman *et al.*, 2008, Sims *et al.*, 2013). There are a number of conditions that are altered by fire disturbance, such as changes to soil temperature, moisture, and quantity and quality of organic matter and water chemistry, which can impact microbial processes (Andersen *et al.*, 2013). Deposited ash and burned materials increases pH, alkalinity, ammonium and phosphorous (Battle and Golladay, 2003). Although elevated pH has been recorded after seven years after wild fires (Taş *et al.*, 2014, Danilova *et al.*, 2015), the pH typically returns to pre-fire condition after a wet season (Ulery *et al.*, 1993). Indeed, the lack of difference in sediment pH between burnt and unburnt areas may be a consequence of the high rainfall (530 mm) rainfall in the month preceding the second sampling (BOM, 2017), which may have restored any changes to the pH to pre-fire levels in burnt sites.

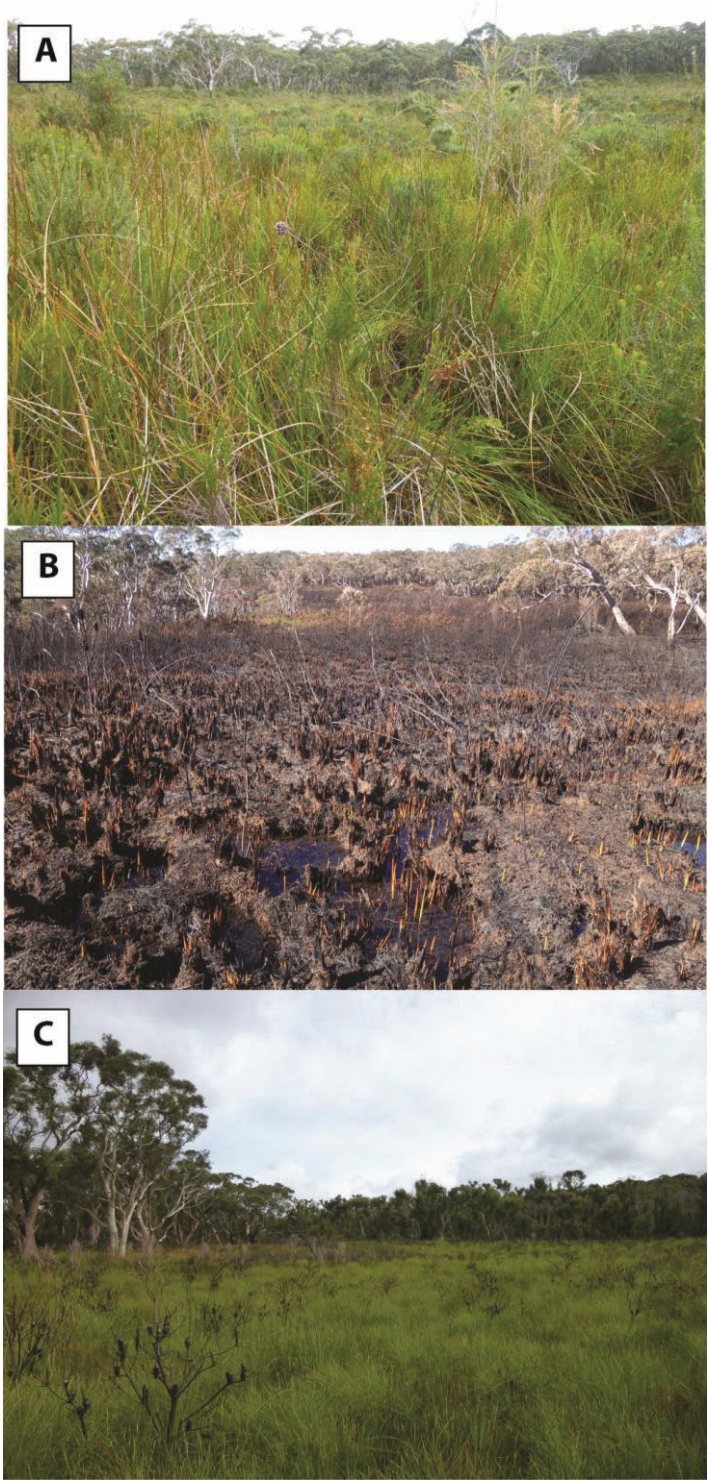


Figure 3.7. Swamp affected by hazard reduction burn. Photographs, top (A): taken at the first sampling in 2015, middle (B) approximately 10 days after the fire (photo credit: Ian Baird), and bottom (C) at the second sampling approximately 1 year after the burn in 2017. Note, 10 days after fire that sedge is re-sprouting, and 1 year after fire, sedge appears recovered, shrubby banksia had not, seedlings for shrubby species were observed at sampling.

Along with the hazard reduction seeming to have minimal subsurface impacts, the initial health of the swamps and catchment likely contributed to these swamps being resilient to the burn (Medvedeff *et al.*, 2013). Our sites were in undisturbed catchments and in good geomorphic and ecological condition - little or no channelisation or non-native vegetation at any of the sites. Undisturbed geomorphic condition, without channelisation, maintains a stable water table (Cowley *et al.*, 2018b), which is a critical factor for the recovery of peatland swamps after a fire (Kettridge *et al.*, 2015). It is also possible that there may be effects of the fire that had not yet manifested, however, we felt this was unlikely because sediment conditions are important in determining the microbial community (Sims *et al.*, 2013) and there were no post-fire differences in sediment conditions and the dominant sedge vegetation had also recovered, which would provide protection from exposure and erosion.

Unrelated to the burn, we found that the sediment conditions across all swamps were different between the two sampling events (Fig 3.3). This is likely due to markedly different rainfall conditions the area prior to each of the two sampling events (BOM, 2017). The wetter conditions in 2017 would explain the higher moisture content results found across all swamps. At the time of sampling, C2 was the wettest and least variable of the sites sampled (Fig 3.3), having areas of pooled surface water. Water logged sediments have typically low oxygen levels favouring anaerobic metabolic pathways, such as methane generation, which is facilitated by the functional gene *mcrA* (Fetzer and Conrad 1993).

Our results show that the fire had little impact on swamp microbial community structure and function over the time frame studied. Microbial metrics were more variable both within and between sites post fire, which we attribute to the effects of inter-annual climate variation and heterogeneity (Chapter 2) that would have affected all sites. The treatment level means show little difference over time and treatment, such that we are satisfied that our lack of statistically significant changes are not a consequence of low statistical power. Indeed, the magnitude of the changes in treatment means were in most cases within the range of variability (95% CIs) of individual swamps (Fig. 3.4). Our results suggest that other variables have a stronger influence on microbial community structure and function than does fire over the time frame of this study.

It is encouraging that there seems to be little long-term consequence of a hazard reduction burn on ecologically and geomorphically healthy swamps. In accordance with our results, another study of Blue Mountains swamps found the vegetation and endangered skinks populations had largely recovered within 15 months of a natural bushfire after an initial decline in vegetation coverage and skink abundance (Gorissen *et al.*, 2018). Gorissen *et al.* (2018) also reported that sediment moisture content was similar to unburnt control swamps, probably facilitating recovery, and rapid re-spouting of sedge, indicating limited subsurface smouldering. Swamps that have experienced alteration of water table or drainage may be more susceptible to fire and suffer more drastic or irreversible degradation (Page *et al.*, 2002, Benson and Baird, 2012, Kettridge *et al.*, 2015). Research is required to understand how swamps may fare with a more intense bushfire, or how degraded swamps, such as those with that have been channelised and are common across the Blue Mountains region (Fryirs *et al.*, 2016), respond to burns.

CHAPTER 4

THE IMPACT OF URBANISATION ON COMMUNITY STRUCTURE, GENE ABUNDANCE AND TRANSCRIPTION RATES OF MICROBES IN UPLAND SWAMPS OF EASTERN AUSTRALIA

Published as:

Christiansen, N. A., Fryirs, K. A., Green, T. J. and Hose, G. C. (2019) 'The impact of urbanisation on community structure, gene abundance and transcription rates of microbes in upland swamps of Eastern Australia', *PLOS ONE,* 14(3), pp. e0213275.

ABSTRACT

The Temperate Highland Peat Swamps on Sandstone of the Sydney Basin occur in the headwaters of Sydney's drinking water catchments and are listed as endangered ecosystems, yet they have suffered habitat losses and degradation due to human impacts such as urbanisation. Despite ongoing efforts to restore and better protect upland swamps, they remain poorly understood, potentially hindering the effectiveness of management efforts. Essential to overall ecosystem function and the provision of services for human and environmental benefit are the microbial component of wetland ecosystems. In the case of these swamps, the microbes, have not yet been studied. Here, we investigated differences in the microbial community of upland swamps in urbanised catchments compared to swamps from natural catchments in the Blue Mountains. A total of twelve swamps were sampled, six from within urbanised catchments and six within intact vegetation catchments, to compare sediment conditions and microbial community and genes expression and abundances. Catchment impervious area and number of stormwater drains entering a swamp, indicators for urbanisation, positively correlated with the pH and ammonium concentration of swamp sediment. Community analysis of the 16S rRNA gene (T-RFLP, qPCR) revealed the elevated pH of urbanised swamps coincided with changes to the abundance of bacteria and archaea. Furthermore, RT-qPCR revealed genes involved in carbon cycling (*mcrA* & *pmoA*) were more likely to be found in urbanised swamps. Taken together, our results indicate that urbanisation of the Blue Mountains is impacting the environmental services provided by the microbial community of upland swamps in the Sydney Basin.

INTRODUCTION

Wetlands, including swamps, bogs and fens, are important landscape features that serve to regulate water flow, improve water quality, provide wildlife habitat and store carbon (Mitsch and Gosselink, 2015). They can buffer the effects of activities in a catchment on downstream aquatic ecosystems and, consequently, are often prioritised for restoration and conservation (de Groot *et al.*, 2012, Costanza *et al.*, 2014).

Wetlands, classified as Temperate Highland Peat Swamps on Sandstone (THPSS), are a common feature of the low relief plateaus that surround the Sydney geological Basin, in South East Australia (Young and Young, 1988). The THPSS, hereafter called swamps, are ecologically important; they occur only within restricted ranges, harbour endangered species and unique plant communities (Mooney and Martin, 2016) and provide ecosystem services such as carbon storage and regulation of water quantity and quality (Cowley *et al.*, 2018a, Cowley *et al.*, 2018b). A defining feature of these systems is the peat-like sediments that arise from the accumulation of organic matter and slow microbial degradation in the anoxic, water logged soils.

Despite their inherent values, many swamps have been destroyed or degraded by human activities (Kohlhagen *et al.*, 2013) leading to the swamps being listed as a threatened ecological community under state and federal legislations (Wright *et al.*, 2011). Swamps located within the developed areas

of the Blue Mountains approximately 100 km west of Sydney NSW, have been particularly degraded due to urbanisation. Factors associated with urbanisation, such as increased impervious area in the catchment and construction of stormwater drains and bores, have altered surface water chemistry (Belmer *et al.*, 2015), water table depths, caused erosion and channelisation, and changted the sediment profiles of the swamps in the Blue Mountains (Fryirs *et al.*, 2016).

Restoration efforts have attempted to re-establish the hydrological processes within swamps by stopping or reversing erosion or buffering flashy stormwater flows (Freidman and Fryirs, 2014, Lane, 2016). While restoration of swamp hydrology and sedimentary structure is critical to restoring ecological functions (Freidman and Fryirs, 2014, Cowley *et al.*, 2016, Fryirs *et al.*, 2016), other symptoms of urbanisation, such as altered water chemistry, must also be addressed. High concentrations of nutrients (Walsh *et al.*, 2005) are a common side effect of urbanisation in surface waters. Higher pH values in runoff from naturally acidic catchments, such as the study area, have also been reported (Davies *et al.*, 2010, Tippler *et al.*, 2014). Nutrient concentration and pH levels have been shown to affect the microbial community structure in sediments generally (Hermans *et al.*, 2017). In wetlands, pH is a key determinant of microbial community (Hartman *et al.*, 2008). High nutrient levels in wetlands increase the microbial mediated release of organic matter (Grybos *et al.*, 2009) and provide more favourable conditions for greenhouse gas production (Wang *et al.*, 1993, Godin *et al.*, 2012, Palmer *et al.*, 2012). Since the microbial community is fundamental to carbon and nitrogen cycling, changes to these communities may be significant. Altered ecological function of these swamps may have implications for downstream water quality (Mitsch and Gosselink, 2015) and even shift wetlands from being carbon sinks to greenhouse gas sources (Mitsch *et al.*, 2013, Cowley *et al.*, 2018a).

In other environments, urbanisation causes shifts in the microbial community resulting in altered carbon and nitrogen cycling (Kaye *et al.*, 2005, Zhao *et al.*, 2012a, Wang *et al.*, 2018). Urban ecosystems (lawns) in arid and semiarid landscapes can have increased carbon cycling and altered carbon budgets and microbial biomass when compared to natural ecosystems and agricultural land uses (Kaye *et al.*, 2005). Urbanised lake surface sediments showed microbial community differences mainly due to increased nitrogen loads (Zhao *et al.*, 2012a). Microbial communities from the urban areas of China's Jialing River system were influenced by discharges from urban development with phosphorous, ammonia, iron and zinc concentrations being significant determinants of community structure (Wang *et al.*, 2018).

The aim of this study is to compare the microbial communities and functions of upland swamps in intact and urbanised catchments. Our hypothesis is that sediment characteristics will differ in swamps from urbanised and intact catchments, and these differences will relate to differences in the microbial communities. Using co-extracted environmental total DNA and RNA we employed the molecular techniques of T-RFLP community fingerprinting, to characterise the microbial communities. While T-RFLP does not offer the detailed resolution of amplicon sequencing, it allows a comparable assessment of microbial community structure relative to environmental variables with greater replication with time and funding constraints (van Dorst *et al.*, 2014, Chapter 6). Assessing the DNA and RNA provides insight into the presence, potential and activity of these genes and processes (Dedysh and Dunfield,

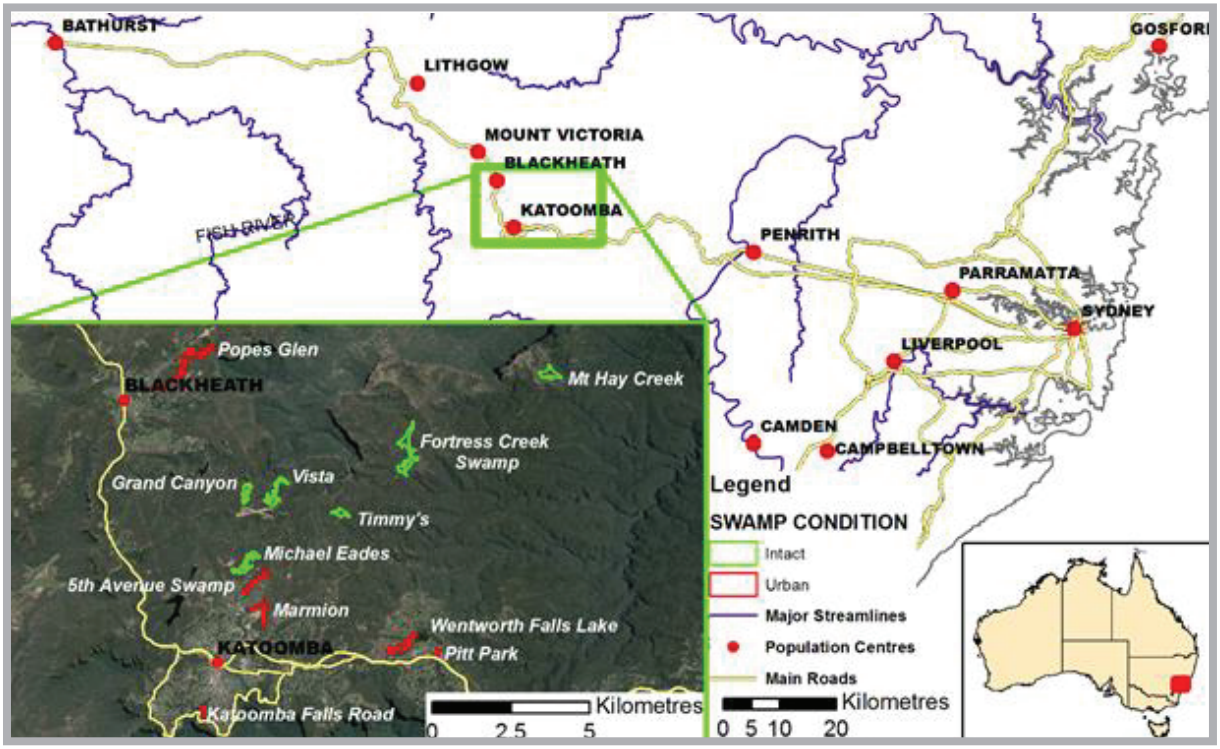
2011). DNA degrades slowly in the environment and so represents active, inactive and deceased organisms. RNA degrades rapidly, and so reflects only the genes and species that have been recently active at the time of sampling (Zhang *et al.*, 2014). We also measured gene abundances and transcription using quantitative PCR of the housekeeping 16S rRNA for bacteria and archaea to indicate the overall relative abundance and transcription/activity (Yu *et al.*, 2005), and functional genes related to microbial metabolic carbon/methane cycling (*mcrA* and *pmoA*) and nitrogen cycling (archaea *amoA*).

METHODS

STUDY SITES

The study area is approximately 100 km west of Sydney in the Blue Mountains (Fig 4.1). Within this area, low relief valley bottom swamps overlying sandstone (Temperate Highland Peat Swamps on Sandstone; THPSS) are common (Fryirs *et al.*, 2018). The town of Katoomba, located within the study area, receives a mean annual rainfall of 1403 mm. Mean maximum temperatures range from 9.4°C in July to 23.3°C in January and mean minimum temperatures range from 2.6°C in July to 12.9°C in February (BOM, 2017).

Twelve swamps were used in this study. Six 'reference' swamps that had intact catchments with natural vegetation, little to no impervious area (<15%), and no storm drains were compared to six swamps that had catchments with varying degrees of urbanisation (Fig 4.1, Table 4.1). The urbanised catchment swamps were located within the townships of Katoomba, Wentworth Falls or Blackheath. Based on remote sensing data (Fryirs *et al.*, 2016), the catchments of urban swamps have between 31.9% and 62.7% impervious area and between zero and eleven stormwater drains discharging into the swamp during and after rainfall events (Table 4.1). The intact catchment swamps had between zero and 13.1% impervious area (Table 4.1). Swamps were dominated by low sedge and surrounded by eucalypt woodland. All but one of the urban swamps were channelised (i.e. contained a continuous, incised channel) and some had non-native plant communities, while all of the intact catchment swamps were non-channelised valley fills with predominantly native plant communities (Keith and Myerscough, 1993). The geomorphic and sedimentological structure of the swamps have been described previously (Freidman and Fryirs, 2014, Fryirs *et al.*, 2014a, Fryirs *et al.*, 2014b, Hose *et al.*, 2014, Cowley *et al.*, 2016). In brief, the sediment profile has distinct layers with unique properties important to the structure and function of the swamps. The surface layer, classified as *surficial organic fines* (SOF), is comprised of living and decomposing organic matter and fine silt and sand. The SOF layer goes to a depth of approximately 10-40 cm. In some cases, below the SOF layer there is a *contemporary sand* (CS) layer. CS are usually associated with erosion and disturbance. Below the SOF (and CS if present) is an organic and mineralised sediment layer classified as *alternating organic sands* (AOS). The AOS layer is typically the thickest sedimentary unit, often extending over a metre in thickness, and the location of the greatest amount of stored carbon (Fryirs *et al.*, 2014a, Cowley *et al.*, 2016). Below the AOS layer are fine cohesive sands (FCS) and basal sands and gravel (BSG) that sit atop saprolite and the sandstone bedrock. The thickness of each layer can vary between swamps and within a swamp (Fryirs *et al.*, 2014a, Cowley *et al.*, 2016).


BATHURST
LITHGOW
MOUNT VICTORIA
BLACKHEATH
KATOOMBA
FISH RIVER
PENRITH
PARRAMATTA
SYDNEY
LIVERPOOL
CAMDEN
CAMPBELLTOWN
Popes Glen
Mt Hay Creek
Fortress Creek Swamp
Grand Canyon
Vista
Timmy's
Michael Eades
5th Avenue Swamp
Marmion
Wentworth Falls Lake
Pitt Park
Katoomba Falls Road
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SWAMP CONDITION
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Urban
Major Streamlines
Population Centres
Main Roads
Kilometres
0 2.5 5
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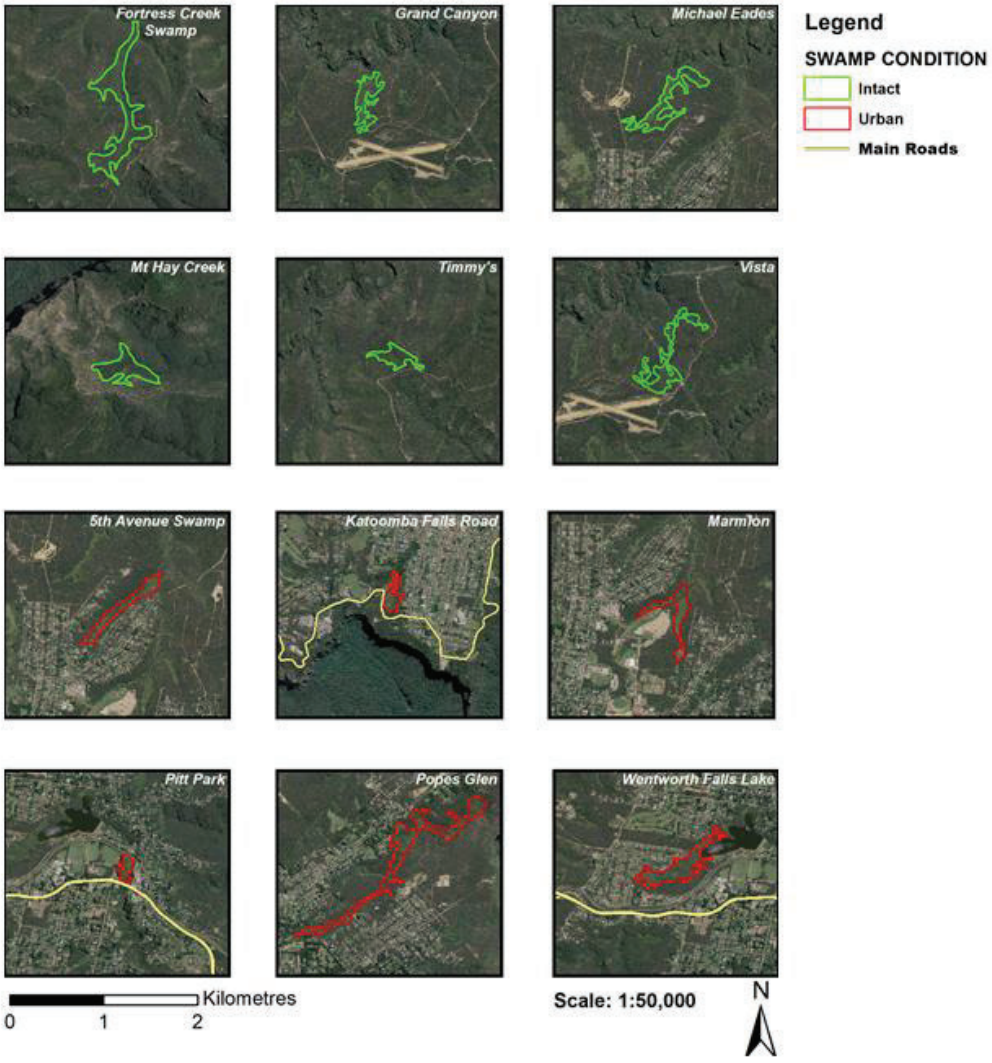
Fortress Creek Swamp
Grand Canyon
Michael Eades
Mt Hay Creek
Timmy's
Vista
5th Avenue Swamp
Katoomba Falls Road
Marmion
Pitt Park
Popes Glen
Wentworth Falls Lake
Legend
SWAMP CONDITION
Intact
Urban
Main Roads
Kilometres
0 1 2
Scale: 1:50,000
N

Figure 4.1 Study site locations (previous page). Red outlines are swamps in urbanised catchments and green outlines are swamps in intact catchments. Source: Basemaps produced with ArcGIS® software by Esri. Sources: Esri, DigitalGlobe, GeoEye, Earthstar Geographics, CNES/Airbus DS, USDA, USGS, AeroGRID, IGN, and the GIS User Community. Swamp outlines taken from the Macquarie University, K Fryirs & G Hose 2016, THPSS mapping layer. 6 maps showing the spatial distribution of THPSS were produced for the following areas: Blue Mountains - VIS_ID 4480 Budderoo - VIS_ID 4481 Gosford - VIS_ID 4482 Newnes - VIS_ID 4483 Woronora - VIS_ID 4484 Penrose - VIS_ID 4485. Creative Commons license at: https://data.nsw.gov.au/data/dataset/temperate-highland-peat-swamps-on-sandstone-thpss-vegetation-maps-vis-ids-4480-to-4485

Table 4.1 Summary of environmental conditions of sampled swamps.

Catchment type	Swamp	Latitude, Longitude	depth	Soil Moisture (%)	Organic Content (%)	Ammonium (mg/kg)	pH	EC (µS/cm)	Imperious Catchment (%)	Storm drains (#)	Elevation (m)	Channelised
Intact	Fortress Creek	S33.6551, E150.365	0	229.7 (151.7)	26.1 (13.4)	2.6 (0.6)	4.6 (0.2)	80.3 (5.9)	0	0	860	N
			50	42.6 (24.9)	6.2 (6)	0.9 (0.3)	4.7 (0.2)	35.8 (18.5)				
	Grand Canyon	S33.664, E150.319	0	259.4 (189.7)	21.5 (14.3)	4.3 (1.6)	5 (0.3)	99.5 (41.9)	4.9	0	962	N
			50	46.9 (24.2)	5.8 (4.7)	0.9 (0.4)	4.8 (0.5)	24 (21.2)				
	Michael Eade's Reserve	S33.6843, E150.319	0	302.6 (12.2)	46 (3.6)	12 (1)	4.5 (0.2)	86.6 (47.5)	13.1	0	944	N
			50	27.7 (18.1)	3.5 (1.8)	1.8 (0.3)	4.6 (0.2)	35.3 (20.7)				
	Mt Hay Creek	S33.6289, E150.405	0	521.5 (338.1)	42 (17.5)	3.2 (0.7)	4.7 (0.4)	68 (13)	0	0	782	N
			50	57.2 (42.3)	7.6 (5.4)	2 (1)	4.4 (0.1)	67.1 (22.4)				
	Timmy's	S33.6686, E150.347	0	311 (138.3)	34.8 (14.2)	1.9 (3.1)	4.6 (0.1)	87.9 (10.5)	0	0	924	N
			50	81.3 (48.4)	21.9 (16.1)	1.6 (0.5)	4.5 (0.2)	42.5 (15)				
	Vista	S33.665, E150.328	0	221.1 (55.8)	31.9 (4.5)	5.9 (1.6)	4.6 (0)	87.8 (40.3)	0	0	957	N
			50	44.6 (15.7)	9.2 (4.7)	0.9 (0.7)	4.7 (0)	41.5 (27)				
Urbanised	Fifth Avenue	S33.6865, E150.324	0	170.3 (112.6)	19.5 (10.6)	7.6 (2)	5.4 (0.8)	49.8 (25.1)	44.3	4	920	Y
			50	61.6 (53.9)	7.4 (10.2)	2.8 (2.2)	5.4 (0.2)	25.3 (23.3)				
	Katoomba Falls Rd	S33.7258, E150.307	0	307.3 (43.4)	35.1 (13.9)	15 (8.1)	5.6 (0.2)	102 (14.5)	51.9	2	948	Y
			50	62.9 (34.8)	10.8 (6.7)	3.6 (2.3)	5.4 (0.3)	21.2 (12.9)				
	Marmion Rd	S33.6956, E150.325	0	135.9 (101.1)	19.8 (13.5)	6.8 (3.4)	4.9 (0.5)	110.3 (47.7)	51.6	0	943	Y
			50	28 (4.6)	4.8 (0.8)	3 (3.5)	5.1 (0.5)	26.1 (13.8)				
	Pitt Park	S33.7087, E150.374	0	201.8 (20.1)	19.1 (0.5)	10.7 (3.1)	5.9 (0.2)	94.4 (18.7)	31.9	8	872	Y
			50	120.5 (91.7)	10.4 (7.9)	2.8 (0.3)	6.2 (0.2)	45.5 (28.2)				
	Popes Glen	S33.6336, E150.293	0	177.1 (117.4)	23.7 (16.3)	5.3 (3)	6 (0.1)	85.2 (72.3)	38.5	6	1024	Y
			50	31.4 (10)	3.6 (2.1)	2.7 (1.6)	5.8 (0.1)	30.3 (24.7)				
	Wentworth Falls Lake	S33.7077, E150.362	0	462.6 (19.4)	47 (8.8)	8.3 (2.7)	5.9 (0.2)	75.5 (41.4)	62.7	11	893	N
			50	61.1 (2.2)	6.3 (2.3)	4.6 (3)	5.3 (0.2)	23.8 (12.8)				

Sediment condition measures presented are averages (n=3), and numbers in parenthesis are standard deviation. Impervious catchment and storm drain data are from (Fryirs *et al.*, 2016).

SAMPLE COLLECTION

Sampling occurred at three locations along the central axis of each swamp using a Russian D-corer. Sediment samples were collected at the surface (top 1-2 cm) to target the SOF layer and at a depth of 50 cm to target the AOS layer or CS layer if present. The SOF layer is likely to have greater oxygen availability favouring aerobic microbial metabolic processes, and the AOS or CS at 50 cm depth is more mineralised and, being deeper in the sediment profile, is more likely to favour anaerobic microbial metabolic processes (Cowley *et al.*, 2018a). At the time of sampling, the sediment type and the vegetation community was noted and photographed. Of our 36 deep samples, 9 were CS and all but one of these were from urbanised catchment swamps. Sediment for molecular and chemical analysis was collected into separate tubes (2 ml Eppendorf™ for molecular and 50 ml Falcon™ centrifuge tubes for sediment analysis) and snap-frozen immediately using dry ice and stored at -80° C and -20° C, respectively.

SEDIMENT ANALYSIS

Sediments were analysed for electrical conductivity, pH, ammonium and nitrate concentrations as potential indicators of pollution and stormwater runoff (Walsh *et al.*, 2005, Belmer *et al.*, 2015) and soil moisture and organic content that we hypothesised would be potentially effected by altered water tables and erosion associated with urbanisation (Fryirs *et al.*, 2016). Electrical conductivity and pH was measured in a 1:1 mass ratio sediment/deionised water slurry using an electrical conductivity meter (Eutech Instruments Pte Ltd/Oakton Instruments Eutech Cyber scan CON 400) and pH meter (Thermo Scientific™ Orion™ 3 star pH meter). Ammonium and nitrate content was measured in sediment extract using APHA Standard Methods for the Examination of Water and Wastewater by the National Association of Testing Authority (NATA), accredited Sydney Analytical Laboratories, Seven Hills, NSW. Soil moisture was measured gravimetrically after drying overnight (105° C for >12 h) and total organic content was determined by loss on ignition (550° C for 5 hrs) (Heiri *et al.*, 2001). Soil moisture and organic content were calculated as a percentage of dry weight.

MOLECULAR ANALYSIS

EXTRACTION

RNA and DNA were co-extracted from sediment samples within one week of collection using the PowerSoil Total RNA Isolation Kit and DNA Elution Accessory Kit (MoBio) according to the manufacture's protocol. Genomic DNA was eliminated from purified total RNA using the Isolate II RNA mini Kit (Bioline) with on-column DNase digestion. Purity and yield of purified nucleic acids was quantified using a NanoDrop 2000 spectrophotometer (Thermo Scientific Inc.). For each sample, 100 µg of total RNA was converted into cDNA with Tetro cDNA Synthesis Kit (Bioline). DNA and cDNA was diluted with MilliQ water to avoid PCR inhibitors (1:30 cDNA and 1:150 DNA). Hereafter the RNA product of cDNA will be referred to as RNA or transcriptions in the text.

TERMINAL RESTRICTION LENGTH POLYMORPHISM (T-RFLP)

Terminal restriction length polymorphism (T-RFLP) analysis was used to compare community structure of bacteria and archaea between sites, catchment types and depth within the sediment profile. The bacteria and archaea 16S rRNA gene was PCR amplified from DNA and RNA samples using FAM-labelled universal primers (Table 4.2) and MyTaq polymerase (Bioline). PCR amplicons were digested with restriction enzymes (Table 4.2). PCR conditions were as follows: 94°C for 2 min then 35 cycles of 94°C for 15 sec, annealing temperature for 30 sec and 72°C for 2 min. Digested PCR products were analysed on ABI3730xl Genetic Analyser at the Australian Genome Research Facility with LIZ1200 size standard. Data were processed using GeneMapper Software and the online tool T-REX (Culman *et al.*, 2009). Peak noise was removed (Abdo *et al.*, 2006), peaks were aligned within two base pairs and peaks that occurred only once in the dataset were removed.

Table 4.2 PCR primers, conditions and references for terminal restriction length polymorphism (T-RFLP).

Gene	Name	Function	Sequence 5' - 3'	Annealing (°C)	Restriction enzyme(s)	Reference
16S rRNA Bacteria	27F	F primer*	AGAGTTTGATCCTGGCTCAG	54	HhaI	(Dunbar *et al.*, 2001)
	1492R	R primer	TACCTTGTTACGACTT			
16S rRNA Archaea	1Af	F primer*	TCYGKTTGATCCYGSCRGAG	53	HhaI, Sau96I	(Cadillo-Quiroz *et al.*, 2006)
	1100AR	R primer	TGGGTCTCGCTCGTTG			

* FAM labelled

GENE ABUNDANCE AND TRANSCRIPTION ABUNDANCE (QPCR)

Gene abundances (DNA) and transcription abundance (RNA) were estimated using quantitative PCR (qPCR). A TaqMan qPCR assay was used to quantify the archaea and bacteria 16S rRNA gene using universal primer pairs (Table 4.3) and a FAM-labelled probe with SensiFast Probe mix (Bioline) in 25 µL reactions. SYBR green qPCR assay was used to quantify the functional genes for methane production (*mcrA*), methane oxidation (*pmoA*) and AOA archaea ammonia monooxygenase (amoA) using universal primers (Table 4.3) in 8 µL reactions using SensiFast SYBR No-ROX (Bioline). All qPCR reactions were carried out on a BioRad CFX96 RT System C1000TM Thermal Cycler. PCR conditions were as follows: initial denaturation (94°C, 2 min) followed by 40 (TaqMan/SYBR analysis) cycles of denaturation (94°C, 5 sec) and hybridisation-elongation (annealing temperature, 45 sec). A subsequent melting temperature curve of the amplicon was performed in the SYBR green qPCR assays.

Table 4.3 The primers, conditions used for the quantitative PCR.

Gene	Name	Function	Sequence 5' - 3'	Annealing (°C)	Reference
pmoA	A189gc	F primer	GGNGACTGGGACTTCTGG	62	(Holmes *et al.*, 1995)
	mb661	R primer	CCGGMGCAACGTCYTTAC		(Costello and Lidstrom, 1999)
AOA *amoA*	AamoAF	F primer	STAATGGTCTGGCTTAGACG	60	(Francis *et al.*, 2005)
	AamoAR	R primer	GCGGCCATCCATCTGTATGT		
mcrA	ML-F	F primer	GGTGGTGTMGGDTTCACMCARTA	60	(Steinberg and Regan, 2008)
	ME-2	R primer	TCATKGCRTAGTTDGGRTAGT		(Hales *et al.*, 1996)
16S rRNA Archaea	ARC787F	F primer	ATTAG ATACC CSBGT AGTCC	60	(Yu *et al.*, 2005)
	ARC915F	TaqMan*	AGGAA TTGGC GGGGG AGCAC		
	ARC1059R	R primer	GCCAT GCACC WCCTC T		
16S rRNA Bacteria	BAC338F	F primer	ACTCC TACGG GAGGC AG	60	(Yu *et al.*, 2005)
	BAC516F	TaqMan*	TGCCA GCAGC CGCGG TAATA C		
	BAC805R	R primer	GACTA CCAGG GTATC TAATC C		

* FAM and TAMRA labelled

The presence of targeted genes was confirmed with electrophoresis gel and analysis of the melting curve data. Calculations of amplicon copy numbers were based on serial dilutions of target genes cloned into the pCR4-TOPO vector (Thermo Scientific Inc.), standard curves all with $r^2 \geq 0.99$ and dynamic range of $1e^2$ – $1e^8$ copies. For each qPCR run, negative controls were included to confirm the integrity of the results (Smith and Osborn, 2009).

STATISTICAL ANALYSIS

Swamps were considered the replicates, so multiple values collected per swamp were averaged to provide a single value. Sediment and catchment properties and gene abundances were compared using a 3-factor repeated measures analysis of variance (ANOVA). In these analyses, catchment type (between-subject factor) and depth (within-subject factor) were considered fixed factors and site was considered a random factor and nested within catchment type. Data were assessed for normality using Q-Q plots and log transformed where necessary to meet this assumption. Sphericity was tested using Mauchley's Test and the Geisser-Greenhouse Adjustments used where the assumption was not met. Tukey's post hoc pair-wise comparisons were used to test for differences between levels where there was a significant interaction.

Gene abundance data were log transformed to approximate normality. Relationships between the sediment variables, gene abundances and measures of urbanisation (impervious catchment and stormwater drains entering swamp) were tested using Pearson correlation analysis. The above analyses were conducted in NCSS version 10.0.7.

Assemblage data based on T-RFLP profiles were visualised using non-metric multidimensional scaling (NMDS). Relationships between assemblages and environmental variables were visualised using distance based redundancy analysis (dbRDA (Legendre and Anderson, 1999)) and distance based linear models (DistLM) using stepwise selection. Distance-based redundancy analysis is a constrained ordination method

similar to Redundancy Analysis, which allows the use of non-euclidean distance measures, here, Bray-Curtis. Peak height data were standardised (by total sample peak area) and square root transformed (Clarke, 2006) prior to analysis. The abiotic data were normalised and checked for strong correlations with draftsman plots. Variables with a correlation coefficient greater than 0.9 were removed from subsequent analyses (Clarke, 1993).

The comparison of assemblages between catchment types and depths was done using PERMANOVA, with a 3-factor linear model replicating that of the repeated measures ANOVA described above. Multivariate analyses were done using PRIMER & PERMANOVA + add on version 1.0.8 (PRIMER-E Ltd.) using the Bray-Curtis similarity index. The significance level (α) for all analyses was 0.05 and was adjusted, where necessary, to account for multiple comparisons using the Holm-Bonferroni method (Holm, 1979).

RESULTS

SEDIMENT CHARACTERISTICS

Sediment pH varied by catchment type ($p<0.001$) with a significantly higher pH in urbanised catchments (mean 5.6 stdev $\pm$0.5) than in intact catchments (mean 4.6 stdev $\pm$0.3). There was no difference in soil pH with depth nor was the interaction significant ($p>0.05$). Soil ammonium concentrations were significantly ($p=0.003$) higher in the swamps of urbanised catchments (Table 4.1, Table 4.4 Fig 4.2) compared to swamps with intact catchments, and also higher in surface sediments than in deep sediments ($p<0.001$), whereas the depth x catchment interaction was not significant.

Table 4.4 Summary of sediment conditions by catchment type.

Catchment type	Sample depth	Soil Moisture (%)	Organic Content (%)	Ammonium (mg/kg)	pH	EC (µS/cm)
Intact	0	308 (184)	33.7 (13.6)	4.98 (3.75)	4.67 (0.26)	85.0 (28.2)
	50	50.1 (31.3)	9.05 (9.07)	1.35 (0.67)	4.61 (0.29)	41.0 (22.3)
urbanised	0	243 (134)	27.4 (14.6)	8.93 (4.82)	5.60 (0.53)	86.2 (40.5)
	50	60.9 (49.6)	7.20 (5.79)	3.25 (2.11)	5.53 (0.44)	28.7 (18.9)

Sediment condition measures presented are mean (n=18), and numbers in parenthesis are standard deviation.

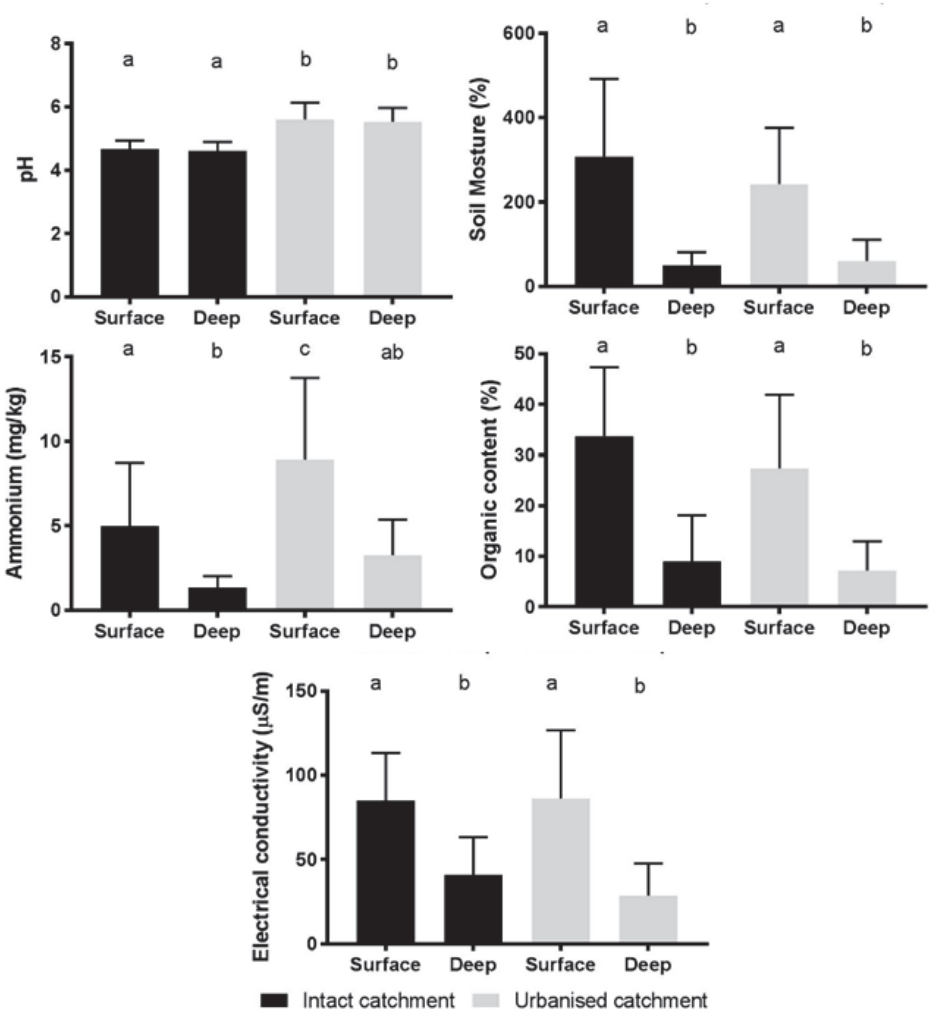

Figure 4.2. Sediment parameters by catchment type and depth. Mean (± Std dev) sediment quality parameters from surface (1-2 cm) and 50 cm depth in swamps with urbanised and intact catchments (n=6). Different letters indicate statistical differences ($p<0.05$).

The remaining abiotic variables, organic content, soil moisture content, and electrical conductivity did not vary by catchment type ($p=0.31$), but all varied by depth. There were no significant depth x catchment type interactions ($p=0.29$). The organic content, moisture content and electrical conductivity were all significantly ($p<0.001$) higher in the surface than deep sediment (Table 4.1, Table 4.4, Fig 4.2). Nitrate concentrations were below the method detection limit of 0.1 mg/kg, with three exceptions, two 0.1 mg/kg (Wentworth Falls Lake a surface and a deep sample) and one 0.2 mg/kg (Grand Canyon surface sample). Because nitrate was detected in so few samples and only in very low concentrations it was not included in subsequent analyses.

Soil pH was significantly correlated with catchment impervious area and the number of stormwater drains entering the swamp, in both shallow and deep soils (Table 4.5). Ammonium concentrations in deep soils were correlated with catchment impervious area and the number of stormwater drains, but not in shallow soils (Table 4.5). Other sediment properties were not significantly correlated with either the measure of urbanisation (Table 4.5).

Table 4.5 Pearson correlation coefficient for significant relationships between sediment characteristics and measures of urbanisation and gene and transcription abundances.

				DNA				RNA	
		Impervious catchment area (%)	Number of storm water drains	Bac 16S	Arc 16S	*pmoA*	*mcrA*	Bac 16S	Arc 16S
Surface	pH	0.75	0.87	0.81	0.77	0.77	ns*	0.82	0.83
	EC (µS/cm)	ns	ns	ns	ns	ns	ns	ns	ns
	Ammonium (mg/kg)	ns	ns	ns	ns	ns	ns	ns	ns
	Soil Moisture (%)	ns	ns	ns	ns	ns	ns	ns	ns
	Organic Content (%)	ns	ns	ns	ns	ns	ns*	ns	ns
Deep	pH	0.77	0.74	ns	ns	ns	ns	ns	ns
	EC (µS/cm)	ns	ns	ns	ns	ns	ns	ns	ns
	Ammonium (mg/kg)	0.78	0.75	ns	ns*	ns	ns	ns	ns
	Soil Moisture (%)	ns	ns	ns	ns	ns	ns	0.74	ns
	Organic Content (%)	ns	ns	ns	ns	ns	ns	ns	ns

Pearson's coefficients for correlations between sediment properties in swamps and indicators of catchment urbanisation and gene abundance. EC = electrical conductivity. ns = non-significant (p>0.007) following Holm-Bonferroni Correction *Pearson correlation coefficient significant prior to Holm-Bonferroni Correction (α=0.05).

Among the sediment properties, sediment moisture and organic content were strongly correlated (r=0.91). Consequently, soil organic content was excluded from subsequent multivariate analyses (see below). Correlations among other soil variables were <0.8.

MICROBIAL COMMUNITY ANALYSIS (T-RFLP)

BACTERIAL COMMUNITY (DNA)

The bacterial community composition (DNA) differed significantly by catchment type (PERMANOVA, p=0.001, r^2 = 0.12) and depth (p=0.001, r^2 = 0.18) and but their interaction was not significant (p=0.218, r^2 = 0.04). This difference is evident as clear separation of surface and deep samples of urbanised and intact swamps in the NMDS ordination (Fig 4.3A).

When tested alone, all variables were significantly correlated with microbial community structure (r^2 =0.12-0.17, S1 Table), but stepwise selection identified only electrical conductivity and pH as explaining a significant and unique proportion of the variation in the bacterial community composition (DistLM, r^2 = 0.35). The addition of other variables did not increase significantly the variation explained by the stepwise model. As suggested by the analysis of the abiotic variables above, pH was strongly correlated with catchment type whereas electrical conductivity was correlated with sample depth.

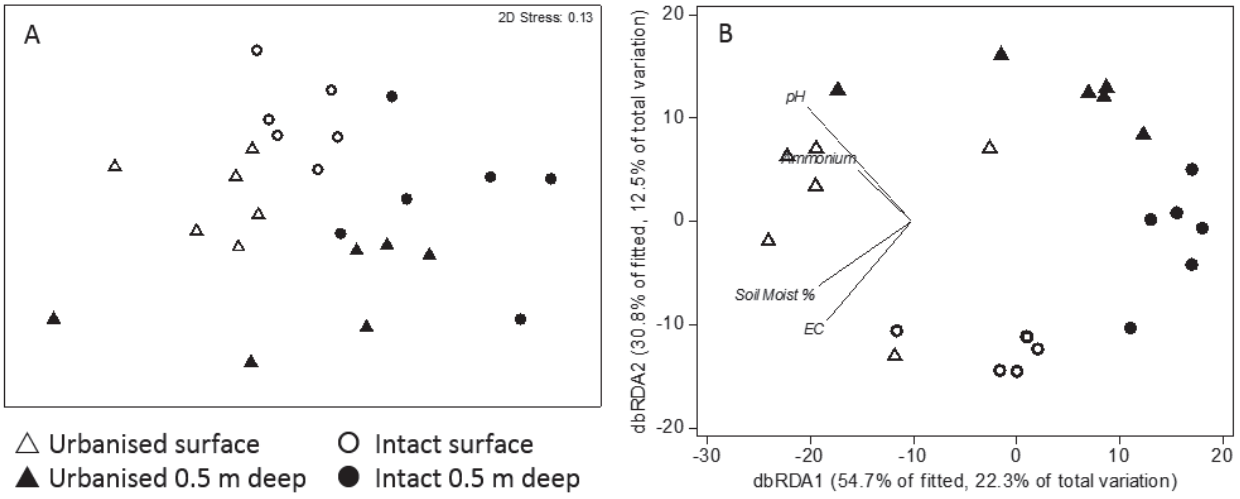

Figure 4.3 NMDS plot of bacteria DNA community. Communities from intact catchment swamps are indicated by circles and those from urbanised catchments marked triangles. Open symbols represent surface samples and closed symbols represent samples from 50 cm depth. Soil Moist % = soil moisture as % dry weight, EC = electrical conductivity (µS/cm) and ammonium = ammonium concentration (mg/kg).

TRANSCRIBING BACTERIAL COMMUNITY (RNA)

The metabolically active bacterial community (RNA) differed significantly between depths (p=0.009, r^2 = 0.10), but this separation was not clear in the NMDS ordination (Fig 4.4A). There was no significant difference in assemblages between catchment types (p=0.526, r^2 = 0.04), nor was the catchment type x depth interaction significant (p=0.781, r^2 = 0.02). The difference in assemblages with depth is evident in figure 4.4B, with deep samples to the left of the figure and shallow samples to the right. This separation is correlated is with differences in soil moisture, as indicated by the vector in that same direction.

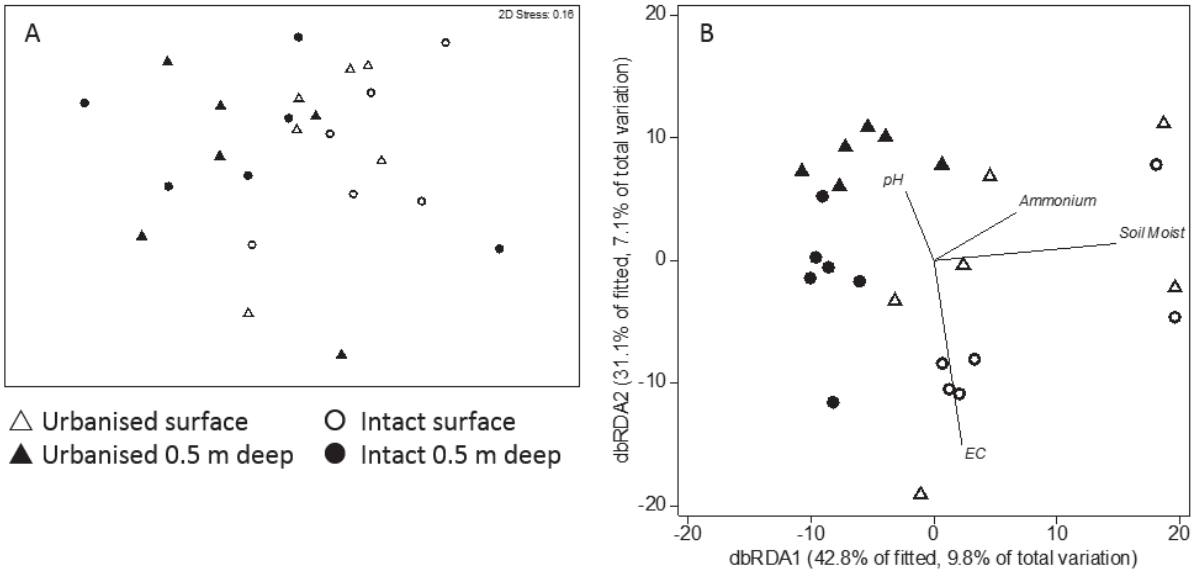

Figure 4.4 NMDS and dbRDA ordination plots of bacteria RNA community. Communities from intact catchment swamps are indicated by circles and those from urbanised catchments marked triangles. Open symbols represent surface samples and closed symbols represent samples from 50 cm depth. Soil Moist % = soil moisture as % dry weight, EC = electrical conductivity (µS/cm) and ammonium = ammonium concentration (mg/kg).

When tested alone, soil moisture and electrical conductivity were significantly correlated with microbial community structure (r^2 =0.09, 0.08, respectively, p≤0.025, S1 Table), while ammonium and pH were not (p>0.06). Stepwise selection identified only soil moisture as explaining a significant and unique proportion of the variation in the bacterial community composition (DistLM, r^2 = 0.09) and the addition of electrical conductivity, pH and ammonium did not increase significantly the variation explained by the model (S1 Table).

ARCHAEAL COMMUNITY (DNA)

A number of the samples did not PCR amplify the archaea 16S rRNA gene sufficiently for T-RFLP analysis (S2 Table). As a consequence, we only had T-RFLP profiles for surface soils from 2 of the 6 intact sites and no depth sample from VS (intact swamp). Despite the limited sampling of intact sites, the archaeal communities from intact and urbanised catchment sites were significantly different (p=0.045, r^2 = 0.10) although depth (p=0.619, r^2 = 0.03) and the interaction term (p=0.343, r^2 = 0.06) in the PERMANOVA were not significant.

Differences in archaeal community structure were not clearly evident in the NMDS ordination (Fig 4.5A) but a separation is evident in figure 4.5B, in which samples from intact and urbanised sites separate along the X axis, which is correlated most strongly with ammonium and soil moisture. Similar separations of samples by catchment type was evident in the NMDS and dbRDA ordinations when only deep samples were analysed (data not shown). The archaeal community structure was not significantly correlated with any of the environmental variables (DistLM, p>0.05).

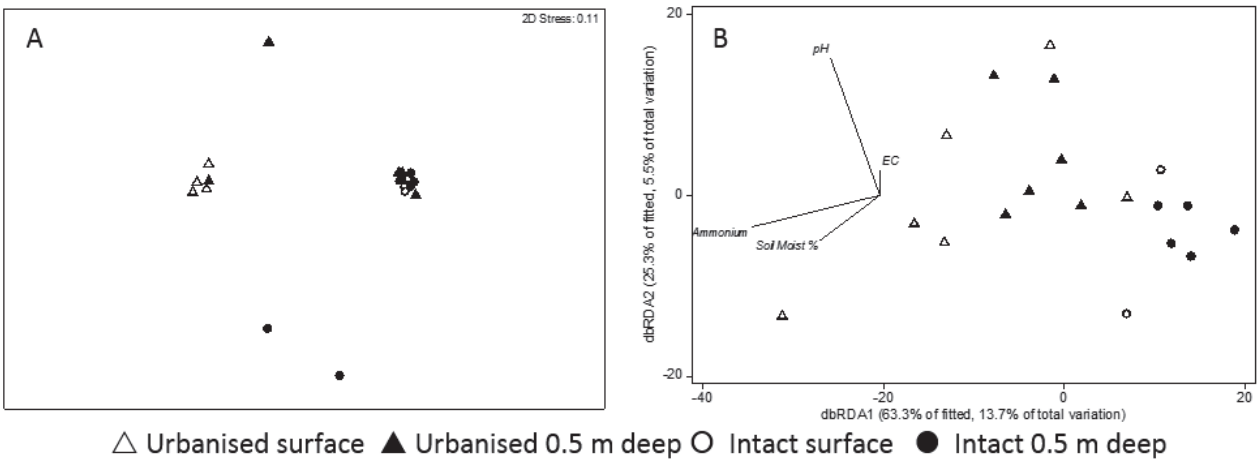

Figure 4.5 NMDS and dbRDA ordination plots of archaeal DNA community. Communities from intact catchment swamps are indicated by circles and those from urbanised catchments marked triangles. Open symbols represent surface samples and closed symbols represent samples from 50 cm depth. Soil Moist % = soil moisture as % dry weight, EC = electrical conductivity (µS/cm) and ammonium = ammonium concentration (mg/kg).

TRANSCRIBING ARCHAEAL COMMUNITY (RNA)

Only seven surface and seven deep samples amplified and provided T-RFLP profiles (S2 Table). Only one of the surface samples and three of the deep samples were from an intact catchment swamps, which did not represent the variation across our sites, so these data were not analysed further.

GENE ABUNDANCES QPCR

GENE ABUNDANCE (DNA)

The bacterial 16S rRNA gene was significantly more abundant in the urbanised catchment swamps than the intact catchment swamps ($p=0.004$) and more abundant in the surface samples than the deep samples ($p<0.001$), but the interaction was not significant ($p=0.100$). The archaea 16S rRNA gene abundance varied by both catchment type ($p=0.003$) and depth ($p=0.020$) and the interactions between these factors was also significant ($p=0.044$), indicating that the variation with depth was not consistent across catchment types. The pair-wise comparisons showed significantly lower abundance of the archaea 16S rRNA gene in the intact catchment surface samples than in the urbanised catchment samples and intact catchment deep samples (Table 4.6, Fig 4.6).

Table 4.6 Gene and transcription abundances summary by catchment and depth.

Catchment type	Sample depth	RNA Bacteria 16S rRNA	RNA Archaea 16S rRNA	DNA Bacteria 16S rRNA	DNA Archaea 16S rRNA	DNA *mcrA*	DNA *pmoA*
Intact	0	2.36E+9 (4.12E+9)	1.28E+6 (2.66E+6)	1.78E+9 (1.33E+9)	2.08E+7 (5.78E+7)	3.38E+6 (8.76E+6)	2.02E+5 (1.70E+5)
	50	1.24E+7 (1.88E+8)	6.86E+6 (2.04E+7)	6.12E+8 (6.63E+8)	8.33E+7 (8.91E+7)	1.39E+7 (1.55E+7)	2.81E+5 (2.27E+5)
urbanised	0	6.64E+10 (1.20E+11)	5.04E+7 (8.76E+7)	1.04E+10 (1.12E+10)	1.39E+8 (1.47E+8)	1.93E+7 (2.81E+7)	2.04E+6 (2.60E+6)
	50	4.56E+8 (7.68E+8)	9.86E+6 (1.80E+7)	7.78E+8 (1.07E+9)	1.72E+8 (2.03E+8)	1.85E+7 (2.90E+7)	3.25E+5 (6.10E+5)

Summary of qPCR results summary as mean number of copies per gram of sediment. Numbers in parenthesis are standard deviation.

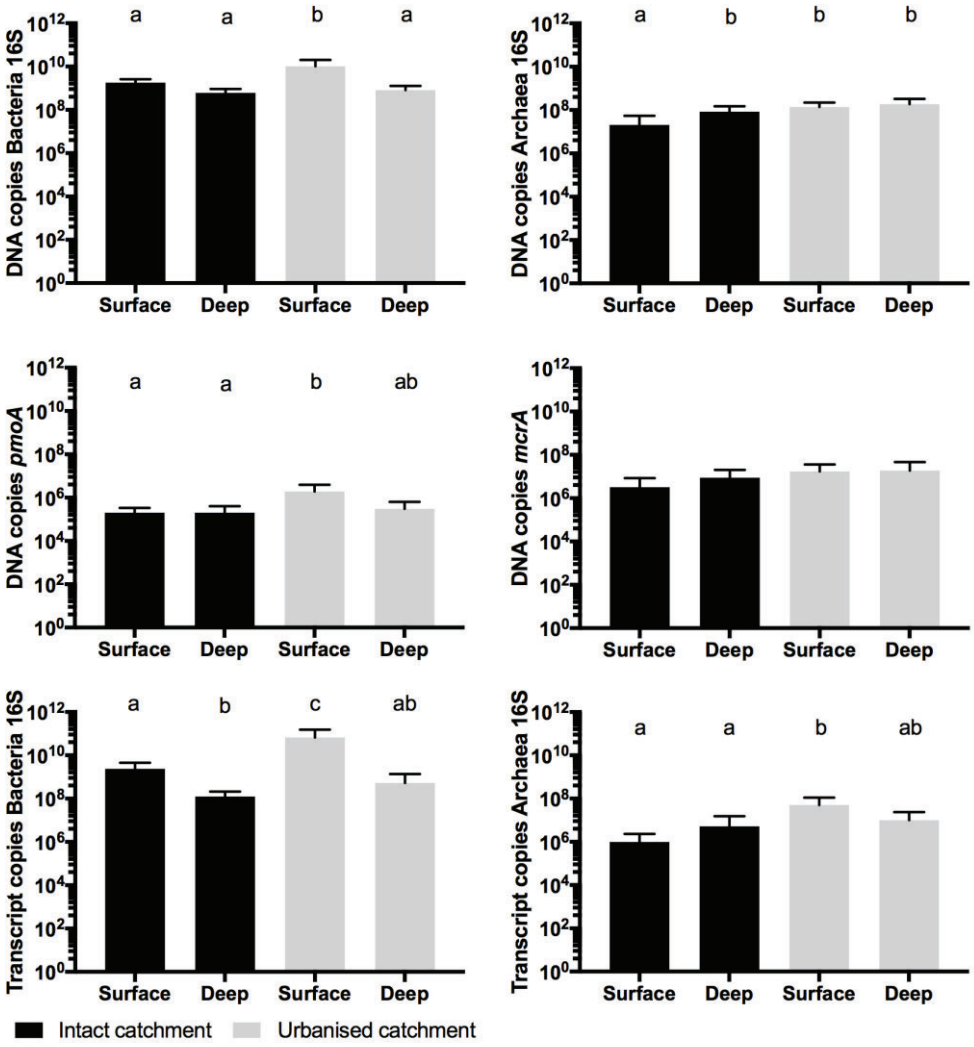

Figure 4.6 Gene and transcription abundances by catchment and depth. Mean (± Std dev) number of DNA/RNA gene copies in sediment collected from the surface and at depth (50 cm) in swamps with urbanised and intact catchments. (n=6).

The abundance of the *pmoA* genes was significantly lower (p=0.037) in samples from intact swamps than in those from urbanised swamps, while there was no difference in gene abundance with depth (p=0.126), nor was the interaction significant (p=0.086, Fig 4.6). The abundance of *mcrA* genes did not vary between catchment types (p=0.088) or depth (p=0.957), nor was the interaction significant (p=0.802). The ammonia oxidizing archaea *amoA* gene had the lowest detection rate only being detected in 15 out of 71 samples. Since so few samples had detections we did not compare abundances, however it is worth noting that 12 (80%) of the archaea *amoA* detections were from urbanised swamps.

GENE TRANSCRIPTION ABUNDANCE (RNA)

The bacteria 16S rRNA gene transcription was significantly more abundant in swamps with urbanised catchments than in those with intact catchments (p=0.016), and in surface compared to deep samples (p=<0.001). The interaction of these factors was not significant (p=0.101, Fig 4.4). Archaea 16S rRNA gene

transcription abundances were also greater in urban than in intact catchment swamps (p=0.016, Fig 4.4) but did not differ with depth (p=0.366). The interaction of depth and catchment type was also not significant (p=0.139).

We were not able to detect the functional gene transcription numbers in most of the samples, which suggests low or no activity of these genes. Although we were not able to compare transcription abundances, there were patterns in the relative frequency of detections. For example, the methanogen *mcrA* gene transcription was detected at 50% of the intact catchment swamps and in all but one of the urbanised catchment swamps, however, the reasons for this heterogeneity are unclear. In both catchment types, *mcrA* was more frequently detected in the deeper samples (unpooled samples: intact 6/18, urbanised 10/16) than the surface samples (unpooled samples: intact 3/18, urbanised 7/18). The *pmoA* gene transcription was also more frequently detected in the samples from urban catchment swamps (6/6 surface and 4/6 deep) than in the samples from intact catchment swamps (4/6 surface and 0/6 deep).

SEDIMENT CHARACTERISTICS AND COMMUNITY STRUCTURE

BACTERIAL COMMUNITY (DNA)

The influences of the abiotic variables on the microbial community (DNA) modelled by DistLM indicated that pH had the greatest influence at both depths. In the surface sediments, pH was the only variable to explain a significant proportion (22%) of the variation in community structure (Fig 4.4). In the deep sediments, pH (12.9%), electrical conductivity (8.9%) and organic content (1.6%) were significant ($p<0.05$) in explaining the variation in community structure.

BACTERIAL TRANSCRIPTION COMMUNITY (RNA)

In the surface sediments, pH was the only variable to explain a significant proportion of the variation in the transcribing community (8.7%). In the deeper samples none of the environmental variables were significant in explaining the variation.

ARCHAEAL COMMUNITY (DNA)

Environmental variables did not explain a significant proportion of the variation in community structure in either the surface or the deep sediments. In part, this may be due to the low number of samples that had enough PCR product for analysis (11 surface samples and 21 deep samples each out of 36 total samples). The number of RNA samples were 7 for each the surface and deep communities, out of a total of 36 of each.

SEDIMENT CHARACTERISTICS AND GENE ABUNDANCE AND TRANSCRIPTION

There was a significant positive correlation ($p<0.05$) between the pH and the abundance of each of the genes except *mcrA* measured in the surface sediment (Table 4.5) however, no other genes or variables in the surface sediments were significantly correlated ($p>0.05$). In the deep sediments, the abundance of the archaea 16S RNA were negatively correlated with both sediment organic content and moisture. No other genes or variables in the deep sediments were significantly correlated.

AOA *amoA* (ammonia oxidising archaea) was only detected in 21% of samples and therefore was not included in the correlation analysis. It is worth noting, however, that sediments in which AOA *amoA* was detected had above average pH values (5.51 versus 5.1) and ammonium (7 versus 4.6 mg/kg) concentrations.

DISCUSSION

The upland swamps in the World Heritage-listed Blue Mountains appear to be affected by catchment urbanisation. Our results suggest that urbanisation is impacting the microbial community and may be impacting the subsequent capacity for delivering ecosystem services, as well as changing water and sediment quality. Increases in the pH of stream water are a common consequence of catchment urbanisation (Davies *et al.*, 2010), and, not surprisingly, pH was strongly and positively correlated to the number of stormwater drains entering the swamp and the impervious catchment area in this study (Table 4.5). The sediment pH was on average about 1 pH unit (ten times H^+ ion concentration) higher in urbanised catchment swamps than in intact catchment swamps (4.6 ±0.27 vs. 5.7±0.48), which agrees with previous studies of swamp surface (Belmer *et al.*, 2015, Lane, 2016, Belmer *et al.*, 2018) and pore waters (Cowley *et al.*, 2018a). An increase in pH is not surprising considering naturally acidic waters, such as in these catchments, cause dissolution of calcium, bicarbonate and potassium ions from concrete from urban infrastructure and storm drains, thereby increasing the alkalinity and pH of waters (Davies *et al.*, 2010, Tippler *et al.*, 2014). Nutrient levels typically increase within urbanised catchments (Walsh *et al.*, 2005), and we also found that ammonium levels were correlated to the measures of urbanisation. Although we had measureable levels of ammonium, nitrate was generally below detection limits. This suggests that the nitrate in the swamps, which are naturally nutrient poor (Keith and Myerscough, 1993, Keith *et al.*, 2006), is being lost through leaching, microbial cycling and denitrification, or by plant uptake (Johnston, 1991).

The elevated pH that is associated with an urbanised catchment, may affect how microbial assemblages cycle and store carbon. Less acidic/more neutral pH (Bergman *et al.*, 1999) and increased nutrients (Clymo *et al.*, 1998, Bragazza *et al.*, 2006), have both been linked to higher carbon mineralisation in peatlands. Globally, peatlands store approximately a third of global carbon and are also responsible for the greatest natural source of methane emissions (Clymo *et al.*, 1998, Turunen *et al.*, 2002, Limpens *et al.*, 2008). Small perturbations of the natural state of peatlands can shift these ecosystems from carbon sinks to net greenhouse gas sources (Andersen *et al.*, 2013, Mitsch *et al.*, 2013). In general, these east coast Australian peat forming swamps differ from high latitude northern hemisphere peatlands, in that they experience significant inter-annual climatic variation (Pemberton, 2005) resulting in variable hydrology (Fryirs *et al.*, 2014b) and marginal, localised and varied peat formation (Fryirs *et al.*, 2014a). Carbon cycling, both accretion and storage, in peatland swamps is controlled by complex interactions between the plants, soil conditions and the microbes present (Updegraff *et al.*, 1995, Blodau, 2002). The upland swamps of eastern Australia are most similar to valley mire fens and tend to be naturally nutrient poor, groundwater and rainwater fed, dominated by sedges and mixed species (Keith and Myerscough, 1993, Fryirs *et al.*, 2014b, Hose *et al.*, 2014) and have a natural pH between 4 and 5 (Fig 4.2) (Keith and Myerscough, 1993, Keith *et al.*, 2006, Lane, 2016, Cowley *et al.*, 2018a). Sedge dominant fens tend to mineralise carbon, produce more methane and cycle nutrients more rapidly than sphagnum dominated peat swamps (Updegraff *et al.*, 1995). The study swamps are marginally peat forming (Fryirs *et al.*, 2014a) and so, altered conditions may have profound impacts on the microbial communities and carbon cycling and storage in these swamps.

Acidic conditions in sediments restrict the species of bacteria present to those adapted to metabolising in acidic conditions (Fierer and Jackson, 2006). Indeed, we found that the transcription and abundance of the16S rRNA genes positively correlated with pH (Table 4.5). This is also shown by catchment type. The

more pH-neutral urbanised catchment swamps had increased transcription of the bacteria and archaea16S rRNA gene compared with the more acidic intact catchment swamps (Fig 4.4). Using the 16S rRNA gene as a general estimate for relative bacteria and archaea abundance and activity (Kembel *et al.*, 2012), suggests surface sediments in urbanised catchment swamps had more archaea and the archaea and bacteria were more active generally than in intact catchment swamps. The potential implication of greater microbial activity may be increased organic material breakdown and carbon export (Imberger *et al.*, 2008), which could result in decreased peat storage and formation in the urbanised catchment swamps.

The higher pH in swamps with urbanised catchments could also increase methane production. Although acid adapted methanogens exist, it is at circum-neutral pH that methane production is optimised (Dunfield *et al.*, 1993, Wang *et al.*, 1993) and the greatest diversity of methanogens can be found (Yavitt *et al.*, 2012). We did not find significant differences in the abundance of the methanogen gene (*mcrA)* or methanotroph gene (*pmoA*) by catchment type, however *pmoA* did have strong positive relationships with increases in pH (Table 4.5), and *mcrA* was significant at $p<0.05$, but not when the correction was applied. Others have found a relationship between pH and methanogens elsewhere (Yavitt *et al.*, 2012). We were unable to statistically test the abundances of the transcription copies for *mcrA* and *pmoA* against sediment conditions or by catchment type, however the positive detection rates by catchment type suggest that *mcrA* and *pmoA* are more likely to be transcribed in the urbanised catchment swamps than in swamps with intact catchments. Transcription for the methanogen *mcrA* gene was detected about twice as frequently in urbanised catchment swamps compared with intact catchment swamps (50% vs. 28% detection rate). The *pmoA* gene was only detected in a single sample from an intact catchment swamp while it was detected in 11 urbanised catchment swamps samples. Transcription of the *pmoA* is likely to mean a reduction in the net methane production, but it does also indicate that methane is available for metabolic processes and is being produced (Blodau, 2002).

Swamps in the Blue Mountains that have become channelised tend to be those most affected by urbanisation (Fryirs *et al.*, 2016). These swamps export up to 18 times more carbon than swamps that are not channelised (Cowley *et al.*, 2018a). Further, carbon dioxide and methane emissions account for 0.1% and 0.001% of the total carbon export in non-channelised swamps, respectively, whereas carbon dioxide and methane make up 19% and 0.06% of the carbon exported from channelised swamps (Cowley *et al.*, 2018a). Although much of the increase in carbon export is attributed to the geomorphological changes that occur with swamp channelisation (Clymo *et al.*, 1998, Cowley *et al.*, 2018a), it is ultimately a combination of physical, microbial and chemical factors, and feedbacks between them that results in greater carbon export (Cowley *et al.*, 2018a).

The bacterial community analysis further supports the hypothesis that changes in pH associated with urbanisation are affecting the microbial function of these swamps, particularly in the surface sediment. Although we detected community differences by catchment type and that increased pH is a likely consequence of urbanisation (as discussed above), the pH was a stronger influence on community structure than was the catchment-scale changes in imperviousness. Other wetland studies have found that pH is a strong driver of bacterial community composition and diversity over large scales (Hartman *et al.*, 2008, Lauber *et al.*, 2009). Indeed, pH was a stronger determinant for microbial community than the geographic location, wetland type, nutrient or soil carbon levels, which is also consistent with previous studies (Hartman *et al.*, 2008, Hermans *et al.*, 2017).

Understanding how urbanisation affects the microbial community is imperative to reducing impacts to swamps affected by urbanisation. Our results suggest that a more natural, acidic sediment pH (pH < 5) is important for maintaining the microbial community and swamp function. As pH increased, so did the abundance of archaea and bacteria16S rRNA gene, *mcrA* and *pmoA*, which may contribute to the augmented carbon cycling and methane production that has been observed in these swamps (Cowley *et al.*, 2018a). Given the link between elevated pH and stormwater infrastructure and impervious area, it is essential to continue to reduce these impacts. In addition to current strategies to minimise flash stormwater flows, using materials not prone to dissolution by naturally acidic waters may help maintain more natural pH levels (Tippler *et al.*, 2014).

CHAPTER 5

TAXONOMIC DIFFERENCES OF THE BACTERIAL COMMUNITY OF URBANISED UPLAND SWAMPS

ABSTRACT

Using Illumina high-throughput 16S rRNA DNA amplicon sequencing we characterised the sediment bacterial communities of high conservation value wetlands, Temperate Highland Peat Swamps on Sandstone (THPSS), located in the Sydney Basin, Australia. Like many wetlands around the world, a number of these swamps have been impacted by urban development. Included in this study were swamps from catchments with natural, intact vegetation and swamps from catchments with urbanisation. We compared the bacterial community in swamps at two depths, surface and 50 cm, and by catchment type, and related community structure to sediment physicochemical properties (pH, moisture content, organic content, electrical conductivity and ammonium). Significant differences were found in the bacterial community structure by catchment type and by depth. Differences were apparent at the phyla level, with several phyla varying in relative proportion between intact and urbanised catchment types. *Acidobacteria* were more common in intact catchment swamps while *Nitrospirae* and *Bacteroidetes* were more common in urbanised catchment swamps, suggesting of a shift from oligotrophic to copiotrophic conditions. Of the sediment properties, pH, which was higher in the urbanised catchment swamps, had the greatest correlation with bacterial community structure. As the first study describing the bacterial community structure of THPSS using high-throughput sequencing, we reveal greater resolution of the bacterial community structure and indicate that urbanisation of the Blue Mountains is impacting the microbial ecology of these ecosystems.

INTRODUCTION

Wetlands have the potential to mitigate some of the most pressing and widespread anthropogenic disturbances - surface water pollution and carbon emissions (Mitsch and Gosselink, 2015). Yet, wetlands globally are threatened by a multitude of pressures (Asselen *et al.*, 2013). Urbanisation is one major threatening process that can affect how wetlands function and deliver ecosystem services including biogeochemical cycling mediated by microbes. It is well known that impervious catchment area negatively affects the ecology of receiving waters (Arnold and Gibbons, 1996) by altering the hydrology, augmenting nutrient and pollutant concentrations and changing physicochemical conditions (Walsh *et al.*, 2005, Tippler *et al.*, 2014). Endangered wetlands classified as Temperate Highland Peat Swamps on Sandstone (THPSS), here after called swamps, around the Sydney Basin in South Eastern Australia have undergone habitat loss and degradation since European settlement and urbanisation (Kohlhagen *et al.*, 2013). These swamps, particularly in the developed areas of the Blue Mountains, continue to be under pressure from the effects of urbanisation (Belmer *et al.*, 2015, Fryirs *et al.*, 2016, Lane, 2016, Belmer *et al.*, 2018). It has been shown that impervious catchment area and stormwater drains entering these swamps have negatively impacted the hydrological and geomorphic condition (Fryirs *et al.*, 2016). Swamps in urbanised catchments have altered macroinvertebrate communities (Belmer *et al.*, 2018, Hardwick, 2019) and chemical properties (Belmer *et al.*, 2015). Microbial communities are sensitive and responsive to changing environmental conditions and shifts in microbial community composition have the potential to alter ecosystem function (Urakawa and Bernhard, 2017).

Surprisingly, there have been few comparative studies investigating the impacts of urbanisation on wetland microbial communities. The literature has emphasised microbial communities and functions of northern

hemisphere peatlands (Golovchenko *et al.*, 2007, Ausec *et al.*, 2009, Serkebaeva *et al.*, 2013), and focused on specific processes such as nitrogen (Wray and Bayley, 2007, Petersen *et al.*, 2012, Sims *et al.*, 2012, Correa-Galeote *et al.*, 2013, Seo *et al.*, 2014, Shrestha *et al.*, 2014, Song *et al.*, 2014) or methane cycling (Sun *et al.*, 2012, Yavitt *et al.*, 2012, Yun *et al.*, 2012, Bridgham *et al.*, 2013b, Seo *et al.*, 2014). Recently, a number of studies have reported impacts from urbanisation on microbial community composition in the water column and or sediments of rivers and streams (Ibekwe *et al.*, 2016, Wang *et al.*, 2016, Hosen *et al.*, 2017, Jani *et al.*, 2018, Roberto *et al.*, 2018, Wang *et al.*, 2018). Studies that have focussed on microbial communities in urban wetlands, have found that urbanisation affects the microbial community (Gonzalez Mateu *et al.* 2019) but there is little difference between created and remnant wetlands in urban areas even with different contaminant inputs (Gilbert *et al.* 2012). Christiansen e*t al.* (2019) (Chapter 4) recently reported differences in surface sediments of the Sydney Basin swamps from urbanised catchments compared to those with natural intact vegetation catchments based on community fingerprint analysis (T-RFLP) and found differences correlated to pH, which was higher (more neutral) in urban swamps.

To determine which taxa are responsible for differences between urbanised catchment and unimpacted swamps, and to increase our understanding of how community functions differ we have amplicon sequenced the 16S rRNA bacteria gene from environmental DNA samples with the Illumina Mi-seq platform. Samples came from twelve swamps, six from urbanised catchments, and six from naturally vegetated catchments. The aim of this study was to describe the bacterial community of these high conservation value swamps, identify key taxa that were different between the swamps from urbanised and naturally vegetated catchments, and what environmental variables influence the taxa of bacterial communities.

METHODS

STUDY SITES

The study area is located in the Blue Mountains approximately 100 km west of Sydney (Fig 5.1). Here, situated atop the Sydney Basin plateau, are low-relief, valley-bottom swamps overlying sandstone (Temperate Highland Peat Swamps on Sandstone; THPSS) (Fryirs *et al.*, 2018). The area experiences a temperate climate with mean maximum temperatures between 9.4°C (July) to 23.3°C (January) and mean minimum temperatures between 2.6°C (July) to 12.9°C (February), with mean annual rainfall of 1400 mm (BOM, 2017). Further description of typical vegetation communities is available in Keith and Myerscough (1993). Geomorphic and sedimentological structure of the swamps have been detailed in several studies (Freidman and Fryirs, 2014, Fryirs *et al.*, 2014a, Fryirs *et al.*, 2014b, Hose *et al.*, 2014, Cowley *et al.*, 2016).

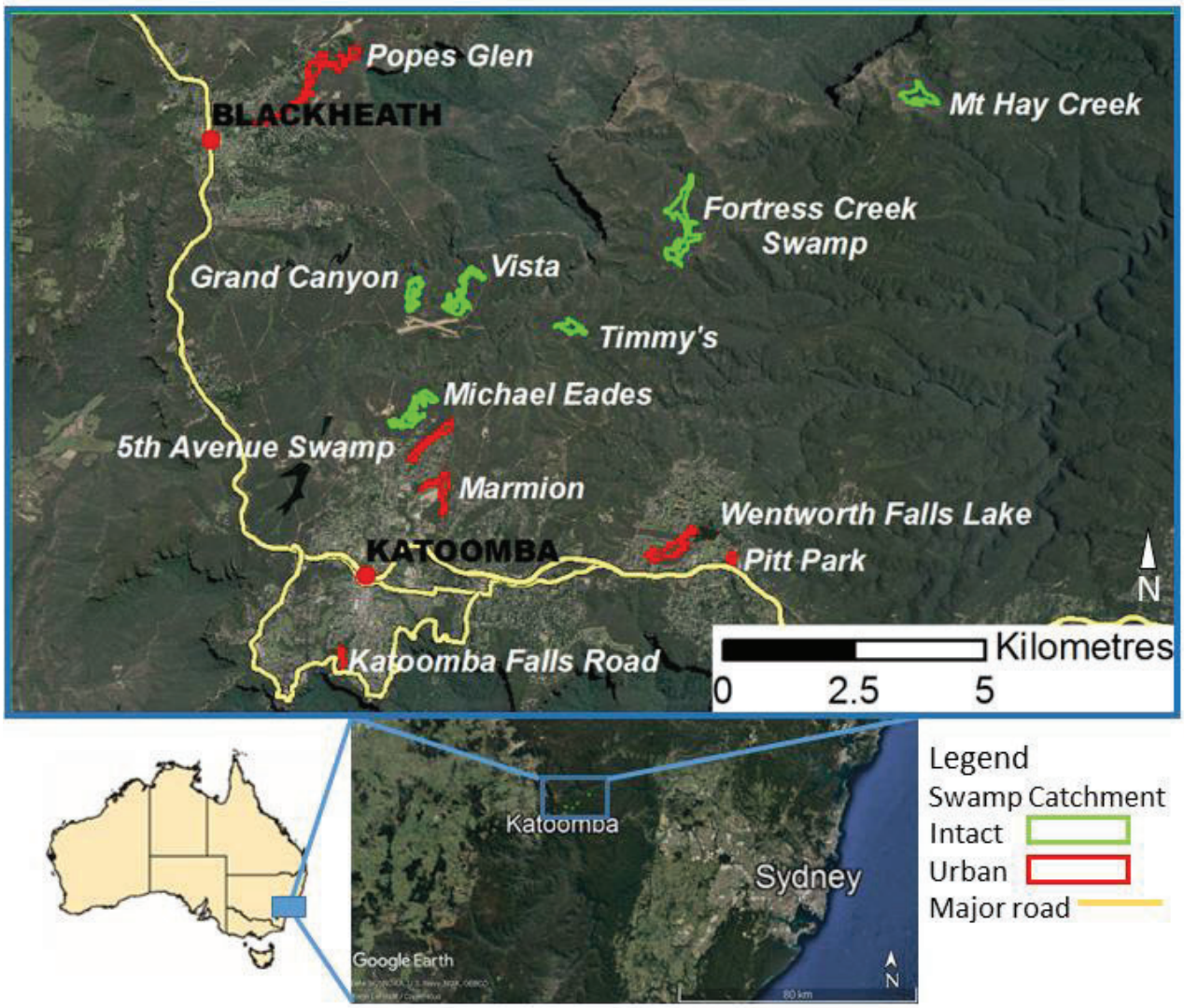

Figure 5.1 Study site locations. Red outlines show swamps in urbanised catchments and green outlines show swamps in intact catchments modified from (Christiansen *et al.*, 2019). Source: Basemaps produced with ArcGIS® software by Esri. Sources: Esri, DigitalGlobe, GeoEye, Earthstar Geographics, CNES/Airbus DS, USDA, USGS, AeroGRID, IGN, and the GIS User Community. Swamp outlines taken from the Macquarie University, K Fryirs & G Hose 2016, THPSS mapping layer. 6 maps showing the spatial distribution of THPSS were produced for the following areas: Blue Mountains - VIS_ID 4480 Budderoo - VIS_ID 4481 Gosford - VIS_ID 4482 Newnes - VIS_ID 4483 Woronora - VIS_ID 4484 Penrose - VIS_ID 4485. Creative Commons license at: https://data.nsw.gov.au/data/dataset/temperate-highland-peat-swamps-on-sandstone-thpss-vegetation-maps-vis-ids-4480-to-4485

Table 5.1. Summary of environmental conditions of sampled swamps.

Catchment type	Swamp	Latitude, Longitude	Sample depth	Soil Moisture (%)	Organic Content (%)	Ammonium (mg/kg)	pH	EC (µS/cm)	Imperious Catchment (%)	Storm drains (#)	Elevation (m)	Channel
Intact	Fortress Creek (FC)	S33.6551, E150.365	0	229.7 (151.7)	26.1 (13.4)	2.6 (0.6)	4.6 (0.2)	80.3 (5.9)	0	0	860	N
			50	42.6 (24.9)	6.2 (6)	0.9 (0.3)	4.7 (0.2)	35.8 (18.5)				
	Grand Canyon (GC)	S33.664, E150.319	0	259.4 (189.7)	21.5 (14.3)	4.3 (1.6)	5 (0.3)	99.5 (41.9)	4.9	0	962	N
			50	46.9 (24.2)	5.8 (4.7)	0.9 (0.4)	4.8 (0.5)	24 (21.2)				
	Michael Eade's Reserve (ME)	S33.6843, E150.319	0	302.6 (12.2)	46 (3.6)	12 (1)	4.5 (0.2)	86.6 (47.5)	13.1	0	944	N
			50	27.7 (18.1)	3.5 (1.8)	1.8 (0.3)	4.6 (0.2)	35.3 (20.7)				
	Mt Hay Creek (MtH)	S33.6289, E150.405	0	521.5 (338.1)	42 (17.5)	3.2 (0.7)	4.7 (0.4)	68 (13)	0	0	782	N
			50	57.2 (42.3)	7.6 (5.4)	2 (1)	4.4 (0.1)	67.1 (22.4)				
	Timmy's (TS)	S33.6686, E150.347	0	311 (138.3)	34.8 (14.2)	1.9 (3.1)	4.6 (0.1)	87.9 (10.5)	0	0	924	N
			50	81.3 (48.4)	21.9 (16.1)	1.6 (0.5)	4.5 (0.2)	42.5 (15)				
	Vista (VS)	S33.665, E150.328	0	221.1 (55.8)	31.9 (4.5)	5.9 (1.6)	4.6 (0)	87.8 (40.3)	0	0	957	N
			50	44.6 (15.7)	9.2 (4.7)	0.9 (0.7)	4.7 (0)	41.5 (27)				
Urbanised	Fifth Avenue (5A)	S33.6865, E150.324	0	170.3 (112.6)	19.5 (10.6)	7.6 (2)	5.4 (0.8)	49.8 (25.1)	44.3	4	920	Y
			50	61.6 (53.9)	7.4 (10.2)	2.8 (2.2)	5.4 (0.2)	25.3 (23.3)				
	Katoomba Falls Rd (KF)	S33.7258, E150.307	0	307.3 (43.4)	35.1 (13.9)	15 (8.1)	5.6 (0.2)	102 (14.5)	51.9	2	948	Y
			50	62.9 (34.8)	10.8 (6.7)	3.6 (2.3)	5.4 (0.3)	21.2 (12.9)				
	Marmion Rd (Ma)	S33.6956, E150.325	0	135.9 (101.1)	19.8 (13.5)	6.8 (3.4)	4.9 (0.5)	110.3 (47.7)	51.6	0	943	Y
			50	28 (4.6)	4.8 (0.8)	3 (3.5)	5.1 (0.5)	26.1 (13.8)				
	Pitt Park (PP)	S33.7087, E150.374	0	201.8 (20.1)	19.1 (0.5)	10.7 (3.1)	5.9 (0.2)	94.4 (18.7)	31.9	8	872	Y
			50	120.5 (91.7)	10.4 (7.9)	2.8 (0.3)	6.2 (0.2)	45.5 (28.2)				
	Popes Glen (PG)	S33.6336, E150.293	0	177.1 (117.4)	23.7 (16.3)	5.3 (3)	6 (0.1)	85.2 (72.3)	38.5	6	1024	Y
			50	31.4 (10)	3.6 (2.1)	2.7 (1.6)	5.8 (0.1)	30.3 (24.7)				
	Wentworth Falls Lake (WFL)	S33.7077, E150.362	0	462.6 (19.4)	47 (8.8)	8.3 (2.7)	5.9 (0.2)	75.5 (41.4)	62.7	11	893	N
			50	61.1 (2.2)	6.3 (2.3)	4.6 (3)	5.3 (0.2)	23.8 (12.8)				

Table adapted from (Christiansen *et al.*, 2019) sediment condition measures are averages (n=3), and numbers in parenthesis are standard deviation. Impervious catchment and storm drain data are from (Fryirs *et al.*, 2016).

SAMPLE COLLECTION

Samples were collected in September 2015 from twelve swamps. Six were considered 'urbanised catchment swamps' with impervious catchment area in excess of 30% and between zero and eleven stormwater drains in the swamp catchment (Fryirs *et al.*, 2016) (Fig 5.1, Table 5.1). These swamps were located within the towns of Katoomba, Wentworth Falls, Leura or Blackheath. The other six swamps, considered reference swamps, were located nearby in catchments with intact natural vegetation. The 'intact catchment swamps' had less than 15% impervious catchment area and no stormwater drains.

Sediment was collected at three random representative locations along the central axis of each swamp using a Russian D-corer. From each core, sediment was collected from the top 1-2 cm and at 50 cm depth. These depths target contrasting sediment composition and conditions (Cowley *et al.*, 2016). The surface is comprised of *surficial organic fines* of living and decomposing organic matter and fine silt and sand and is likely to favour aerobic microbial metabolic processes. Sediments at the 50 cm depth are more mineralised *alternating organic sands* that often includes striations of peat and, being deeper in the sediment profile, are more likely to favour anaerobic microbial metabolic processes (Cowley *et al.*, 2018a). Samples for molecular and chemical analysis were placed directly into separate sterile tubes (2 ml Eppendorf for molecular and 50 ml Falcon centrifuge tubes for sediment analysis). All sediment samples were snap-frozen immediately in the field using dry ice and stored upon return to the lab at -80° C and -20° C, respectively.

SEDIMENT ANALYSIS

Data for sediment conditions are from Christiansen *et al.* (2019, Chapter 4). Sediment was analysed for electrical conductivity and pH with benchtop probes in a 1:1 deionised water sediment slurry solution (Hamman *et al.*, 2007). Ammonium was analysed using APHA Standard Methods for the Examination of Water and Wastewater by the National Association of Testing Authority (NATA), accredited Sydney Analytical Laboratories, Seven Hills, NSW. Soil moisture was measured gravimetrically after drying to a stable weight and total organic content was determined by loss on ignition (Heiri *et al.*, 2001). Soil moisture and organic content were calculated as a percentage of dry weight.

MOLECULAR ANALYSIS

EXTRACTION

Environmental DNA was extracted from sediment samples using the PowerSoil Total RNA Isolation Kit and DNA Elution Accessory Kit (MoBio) according to the manufacture's protocol. The RNA elution was not used as part of this study, to focus on the clearer results found in the DNA community fingerprint (Chapter 4). The purity and yield of the DNA extraction was quantified using a NanoDrop 2000 spectrophotometer (Thermo Scientific Inc.). Eluted DNA was diluted with sterile MilliQ water (1:150) to avoid PCR inhibitors based on qPCR efficiency curves of serial dilutions.

SEQUENCING AND PIPELINE PROCESSING

Amplicon sequencing was performed on the V1-V3 region of the 16S rRNA bacteria gene via the Illumina MiSeq platform using the primers 27f and 519r (Lane *et al.*, 1985, Lane, 1991). PCR amplification and sequencing were completed by Ramaciotti Centre for Genomics, University of New South Wales, Sydney (ISO/IEC 17025 accredited laboratories).

Sequencing reads were processed using the EBI genomics project processing pipeline Version 4.0 (Mitchell *et al.*, 2017). In summary, paired overlapping raw reads were merged to form longer reads using SeqPrep (v 1.1). For quality control, low quality ends and sequences (>10% undetermined nucleotides) were removed using Trimmomatic (v 0.35), and sequences less than 100 nucleotides were removed using Biopython (v 1.65). Infernal (v 1.1.2) was used to identify ncRNA and lower quality overlaps removed by Cmsearch deoverlap script (v 1.0). Taxonomy and OTU classifications were assigned using MAPseq (v 1.2) with SILVAs SSU/LSU (v 128) database. Data are publically available in the European Nucleotide Archive (ENA) http://www.ebi.ac.uk/ena/data/view/PRJEB23103.

STATISTICAL ANALYSIS

Diversity, measured with Shannon diversity index, richness, estimated by Chao1 index, the relative phylum proportions and the ratio of *Proteobacteria* to *Acidobacteria,* which has been suggested as a broad indicator for trophic status (Smit *et al.*, 2001, Hartman *et al.*, 2008), were compared using a 3-factor analysis of variance (ANOVA). In these analyses, catchment type (between-subject factor) and depth (within-subject factor) were considered fixed factors and site was considered a random factor and nested within catchment type. Data were assessed for normality using Q-Q plots and log transformed where necessary to meet this assumption. Sphericity was tested using Mauchley's Test and the Geisser-Greenhouse Adjustments used where the assumption was not met. Tukey's post hoc pair-wise comparisons were used to test for differences between levels where there was a significant interaction. Relationships between phyla relative proportions, diversity and richness were explored using Pearson's correlation.

The community assemblages were compared at the OTU level by catchment type and depth using a 3-factor PERMANOVA, replicating the ANOVA analysis described above. The 'catchment type' and 'depth' were considered fixed factors with 'site' nested within catchment type as a random factor. Profiles at the OTU level were visualised using non-metric multidimensional scaling (NMDS). The OTU reads were standardised by total number of reads and square root transformed (Hellinger transformation) and made into a resemblance matrix using the Bray-Curtis similarity index (Clarke, 2006). Where differences were found, we followed up with SIMPER analysis to determine the OTU(s) that contributed to the variation as potential species indicators of degradation.

Relationships between bacterial community assemblages and sediment condition variables were analysed using distance based linear models (DistLM) with stepwise selection. Distance-based redundancy analysis is a constrained ordination method similar to Redundancy Analysis, which allows the use of non-euclidean distance measures, here, Bray-Curtis. Sediment variables that were significant

in explaining the community variation were explored further using Pearson's correlation to phylum proportions.

Analysis of taxonomic data was based on relative proportion (number of reads per OTU over the total number of reads for that sample). The phyla level dataset was limited to those contributing at least 1% of the total, and the OTU level dataset was limited to those contributing at least 0.1% of the total dataset. The diversity and richness index calculations were based on the complete dataset. The significance level (α) for all analyses was 0.05. The 3-factor ANOVA and Pearson's correlation analyses were performed using Minitab (Minitab Inc, PA, USA). The PERMANOVA, SIMPER and DistLM analyses were performed with PRIMER 6 & PERMANOVA + add on version 1.0.8 (PRIMER-E Ltd., Plymouth, UK).

RESULTS

SAMPLE READS

The mean number of sequence reads per sample was 97,430, ranging from 25,877 to 175,294. A total of 2754 operational taxonomic units (OTU) were found. The mean number of OTUs per sample was 544 OTU and ranged from 128 to 1098. There were 179 OTUs that individually made up at least 0.1% of the dataset. The greatest number of those were found in the surface of the urbanised catchments at 154 OTU (86%) and 79 OTU (44%) were found in both depths and treatments (Fig 5.2).

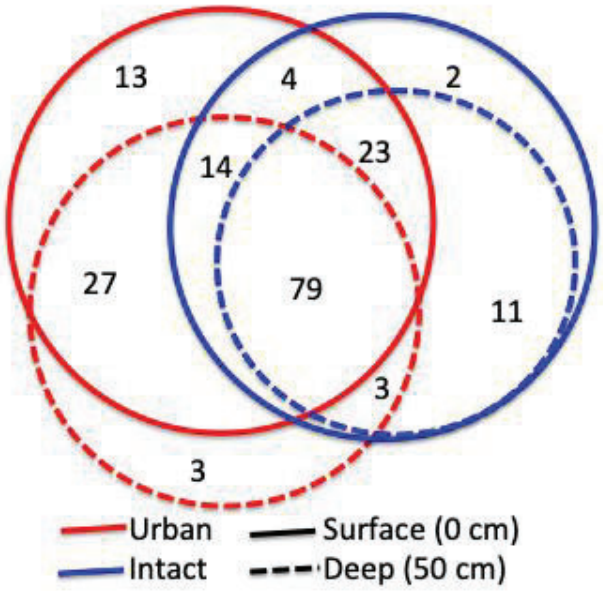

Figure 5.2 Venn diagram depicting unique and shared OTU at the species level in surface and deep sediments of upland swamps with urbanised or intact catchments.

DIVERSITY AND RICHNESS INDEX:

Shannon Diversity index differed significantly between catchment types ($p=0.022$), depths ($p<0.001$) and sites (nested within catchment type) ($p=0.041$). Overall, mean (± standard deviation) diversity was greater in the urban surface samples (4.53 ±0.33) than intact catchment surface samples (4.17 ±0.206), which were greater than deep urban (3.84 ±0.388) and intact catchment (3.57 ±0.355) samples (Fig 5.3). Richness differed significantly by catchment type ($p=0.032$) and depth ($p<0.001$), but not sites nested within catchments. Mean richness (± standard deviation) was greatest in the urban surface (1058 ±233) followed by intact catchment surface (782 ±112), deep urban (574 ±180) and deep intact samples (500 ±143) (Fig 5.3).

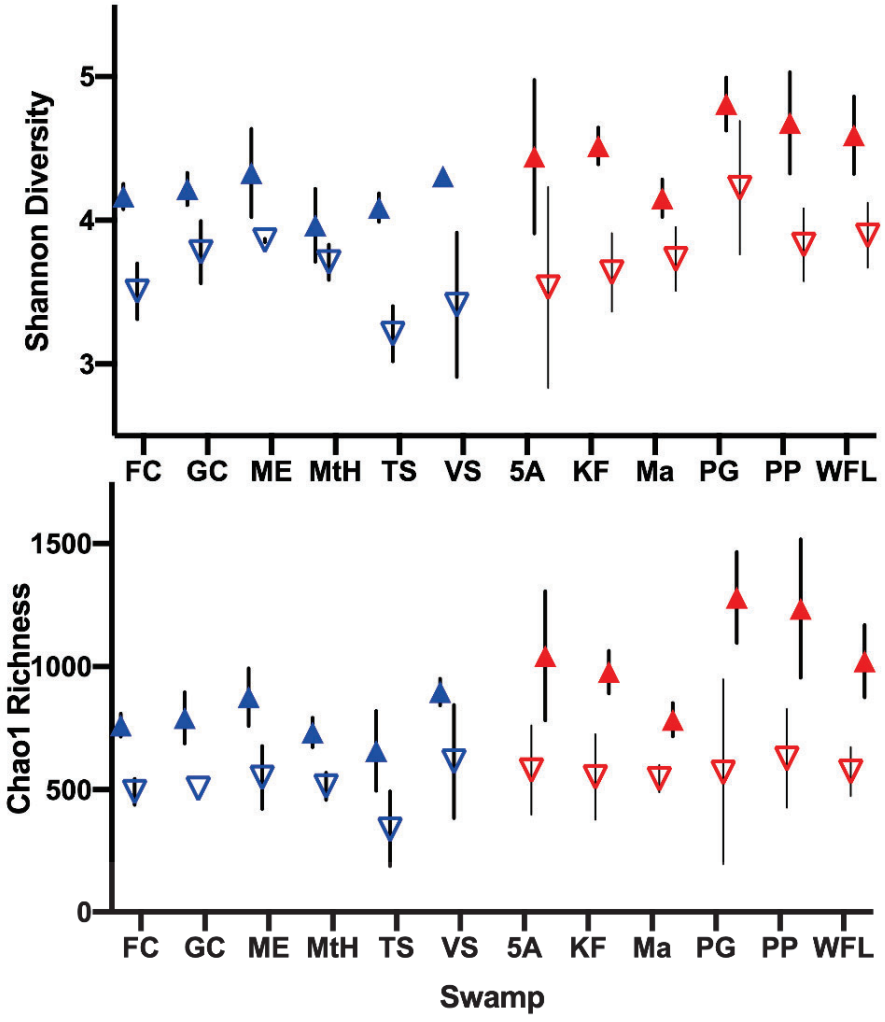

Figure 5.3 Bacterial community diversity and richness by site, depth and catchment type. Blue symbols are from intact catchment swamps, red symbols are from urbanised catchment swamps. Solid symbols are for surface and open are for deep. Error bars indicate 1 standard deviation. For site names and acronyms see Table 5.1.

PHYLA ANALYSIS

Proteobacteria was the most common phylum, comprising 32.2% of reads of the dataset, followed by *Acidobacteria* with 23.6% and *Chloroflexi* with 10.7%. Other phyla that comprised more than 1% of the total reads were: *Planctomycetes* (9.0%), *Nitrospirae* (3.3%), *Bacteroidetes* (3.0%), *Actinobacteria* (2.5%), *Verrucomicrobia* (2.3%), *Firmicutes* (1.4%) and Spirochaetes (1.1%). Reads for which there was no reliable match in the database were classified as 'Unassigned' and made up 7.1% of all and 'Other' and made up 3.9%.

Several phyla were relatively more common in either the deeper or surface samples. *Proteobacteria*, *Bacteroidetes*, *Actinobacteria* and *Verrucomicrobia* made up a greater proportion of reads in the surface sediments, while *Chloroflexi* and *Nitrospirae* were made up a greater proportion of reads in the deeper sediment (all $p<0.001$). *Acidobacteria* was relatively more common in the intact catchment swamps ($p=0.046$), while *Bacteroidetes* ($p=0.008$), *Nitrospirae* ($p=0.008$), and *Actinobacteria* ($p=0.007$) were more common in the urbanised catchment swamps. *Proteobacteria* as a whole did not differ significantly by catchment type, but there were differences of some *Proteobacteria* classes between catchment types. The proportion of *α-proteobacteria* reads was greater in the intact catchment swamps ($p=0.002$) than the urban swamps, while the proportion of *β-proteobacteria* ($p=0.004$) and *δ-proteobacteria* ($p=0.005$) was greater in the urbanised than the intact catchment swamps. The proportion of each *Proteobacteria* class varied with depth. There was a significant interaction between catchment type and depth for *Planctomycetes* ($p=0.025$), *Nitrospirae* ($p=0.012$), *Actinobacteria* ($p=0.003$) and *Firmicutes* ($p=0.002$). In the intact catchment swamps, *Planctomycetes* was relatively more common in the deep sediments and *Firmicutes* was relatively more common in surface sediment compared to the urbanised catchment swamps. In the urbanised catchment swamps *Nitrospirae* had higher proportion in surface sediments and *Actinobacteria* had higher proportion in deep sediments compared to the intact catchment swamps (Fig 5.4).

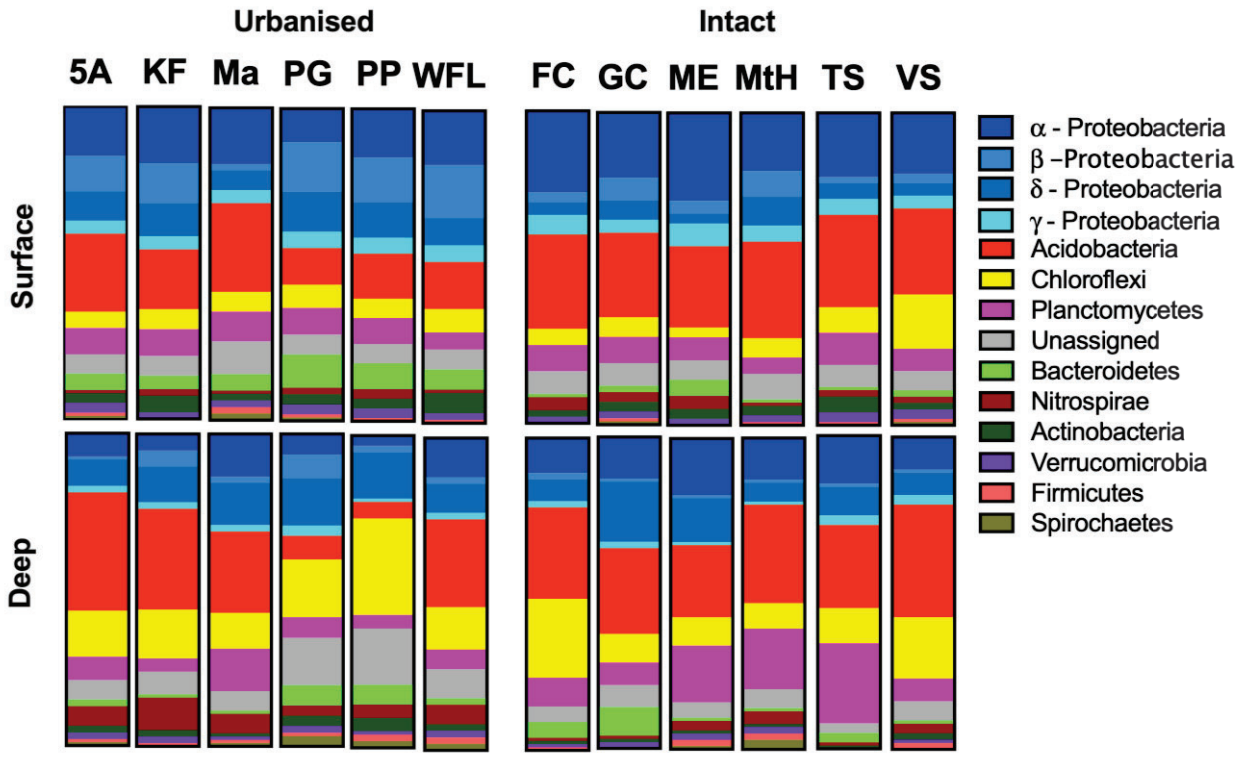

Figure 5.4. Proportional phyla composition. Mean phylum composition (including *Proteobacteria* classes) for each swamp by depth and arranged by catchment type (urbanised or intact). Site names and acronyms are defined in Table 5.1.

RATIO OF *PROTEOBACTERIA* TO *ACIDOBACTERIA*, INDICATOR OF TROPHIC STATUS

Three factor ANOVA revealed ratios of *Proteobacteria* to *Acidobacteria* were higher in urbanised catchments ($p=0.016$) than intact catchments. They were also higher in the surface sediments than the deep ($p=0.029$). There were significant differences between sites within catchments ($p<0.001$). Pairwise comparisons show Pitt Park (PP) and Popes Glen PG) had significantly higher mean *Proteobacteria* to *Acidobacteria* ratio than other sites except the Wentworth Falls Lake (WFL), driven by high value for the surface sediment at that site (Fig 5.5).

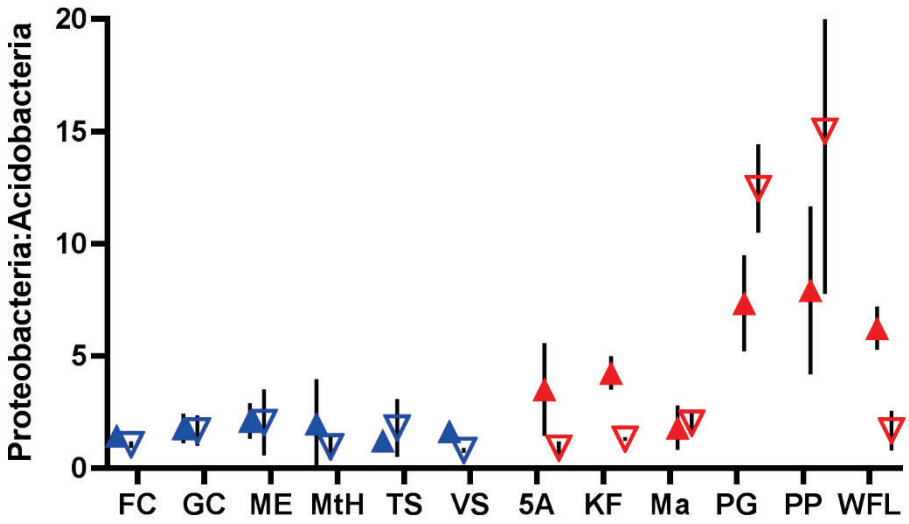

Figure 5.5 Ratio of* Proteobacteria *to* Acidobacteria *by swamp, an indicator of trophic status. Blue symbols are from intact catchment swamps, red symbols are from urbanised catchment swamps. Solid symbols are for surface and open are for deep. Error bars indicate 1 standard deviation. For site names and acronyms see Table 5.1.

BACTERIAL COMMUNITY ANALYSIS

The ordination of the bacterial community shows a division among samples along the X axis that reflects catchment types (Fig 5.6). There was further division along the Y axis, with surface samples clustering toward the top of the plot and samples from depth toward the bottom. In general, the surface samples were clustered together more tightly, suggesting greater within group similarity, than those from depth (Fig 5.6). This visualisation was supported by the PERMANOVA results. The bacterial community composition varied significantly by catchment type ($p=0.001$) and depth ($p=0.001$), site within catchment ($p=0.001$) and there was an interaction between catchment and depth ($p=0.037$) and site and depth ($p=0.004$). Pairwise tests of the sites revealed that Pitt Park (PP) and Popes Glen (PG) were significantly different from the other urbanised catchment swamps, but not each other. Vista Swamp (VS) was different from all but Fortress Creek (FC), and Grand Canyon (GC) and Timmy's Swamp (TS) were different.

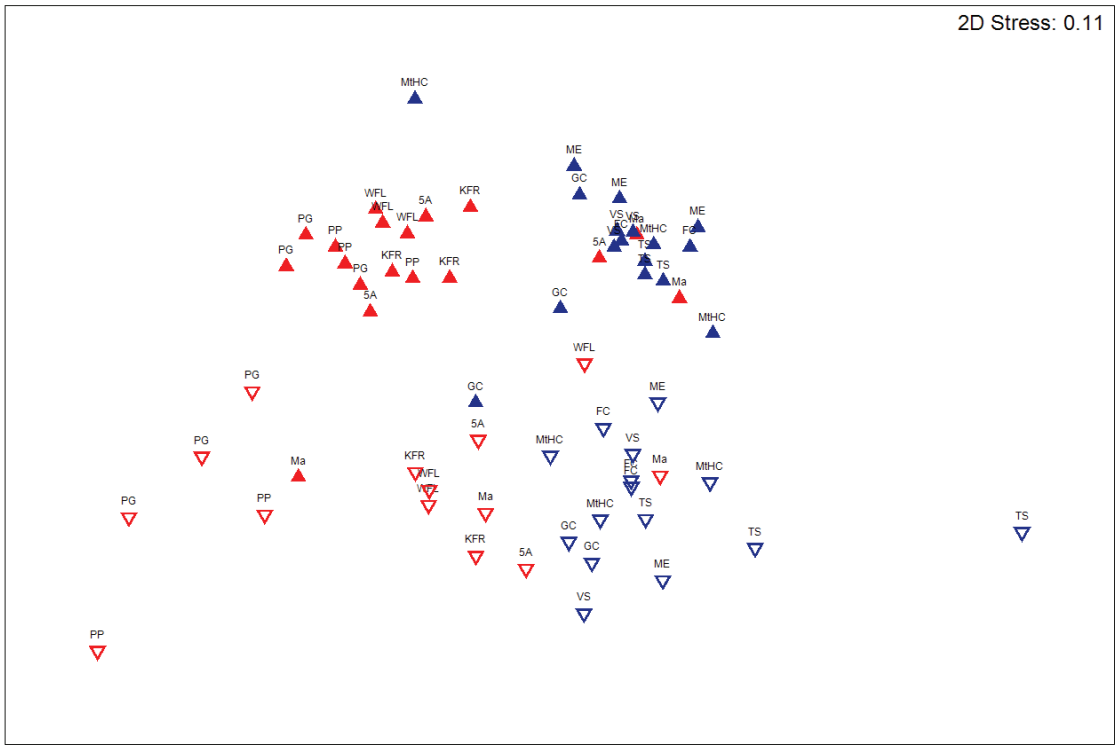

Figure 5.6. NMDS of bacterial community. Red symbols indicate samples from urbanised catchment swamps, blue indicate intact catchment swamps. Solid symbols are surface sediment bacterial community and open are deep sediment (50 cm). For site names and acronyms see Table 5.1.

Having found a significant difference between the depths and a significant interaction between catchment type and depth, we ran a second PERMANOVA and the SIMPER analysis on the surface and deep samples separately. In the surface, the catchment type (p=0.004) and sites (within catchment) (p=0.036) were significantly different. The taxa that contributed most to the differences between catchment types were *Candidatus_Koribacter* (genus) and order *Acidobacteriales* (order) from *Acidobacteria*, and *Roseiarcus* (genus) from *α-proteobacteria* which were all relatively more common in the intact catchment than the urbanised catchments. *Nitrosomonadaceae* (Family) from *β-proteobacteria* and *Geobacter* (genus) from *δ-proteobacteria* were both relatively more common in the in urbanised than the intact catchment swamps. The assemblage composition at depth differed significantly with catchment type (p=0.002) and site (within catchment type) (p=0.002). The taxa that contributed most to the differences between catchment type were *Ktedonobacterales* (order) from phylum *Chloroflexi*, *Candidatus_Koribacter* (genus) from *Acidobacteria*, *Isosphaera* (genus) and *Isosphaeraceae* (family) from *Planctomycetes*, and *Acidobacteriales* (order) from *Acidobacteria*, which all made up a greater portion of reads in the intact than the urbanised catchment swamps (Fig 5.7).

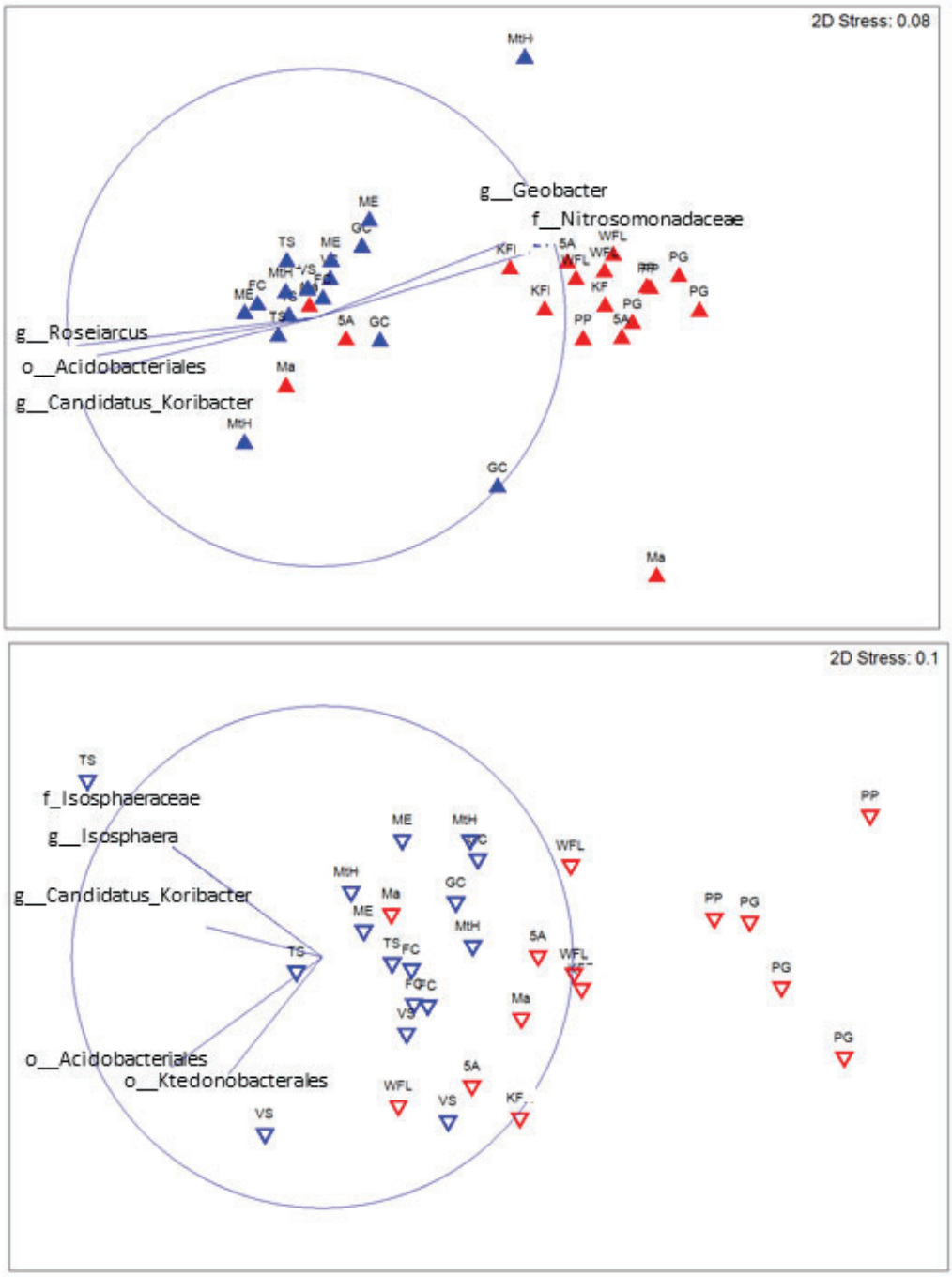

Figure 5.7 NMDS of surface (top) and deep (bottom) bacterial communities with the five OTU contributing the most variation between the catchment types. Blue symbols are from intact catchments and red symbols are from urbanised catchments. The vectors show the relationship of the OTUs that contributed the most variation between the catchment types. The OTU are reported to the lowest taxonomic level possible and the level of identification is indicated by the letter preceding the taxonomic name as follows: o = order, f = family, g = genus. For site names and acronyms see Table 5.1.

SEDIMENT ENVIRONMENT AND BACTERIAL COMMUNITY

Of the sediment environment variables measured, only pH explained a significant portion of the variation in the microbial community composition (p=0.001) in the surface sediment. Several phyla had significant relationships with pH in the surface sediment. *Acidobacteria* (r= -0.820, p<0.001) and *Firmicutes* negatively correlated with pH (r= -0.688, p<0.001). *Nitrospirae* (r= 0.795, p<0.001) and *Bacteroidetes* (r= 0.755, p<0.001) positively correlated with pH in the surface sediments. *Proteobacteria* (r = 0.385, p<0.025) and *Chloroflexi* (r= 0.384, p=0.025) also had a significant correlation with pH but the relationship was weak. In the deeper community, the pH (p=0.001) and electrical conductivity (p=0.027) were significant in explaining the variability of the microbial community. *Planctomycetes* (r= -0.527, p=0.003) and *Acidobacteria* (r= -0.469, p=0.009) and negatively correlated with pH. *Acidobacteria* (r =0.701, p<0.001) and *Bacteroidetes* (r = 0.632, p<0.001) and *Chloroflexi* (r = 0.418, p<0.021) positively correlated with pH in the deep sediments. *Planctomycetes* had a weak positive correlation with electrical conductivity (r= 0.402, p=0.028).

DISCUSSION

Applying high-throughput amplicon sequencing techniques to the high conservation value (THPSS) swamps of the Sydney basin, we have found catchment urbanisation coincided with shifts in bacterial taxon and community structure, suggesting that ecosystem degradation is occurring. Despite relatively small towns situated in the catchment of these swamps (e.g. Blackheath (population 5,000), Wentworth Falls (6,000) Katoomba (8,000) Leura (4,600)) (ABS, 2019), the patterns of change in the microbial assemblages were similar to those observed in highly urbanised or disturbed aquatic environments elsewhere (Hosen *et al.*, 2017, Jani *et al.*, 2018, Saxena *et al.*, 2018, Wang *et al.*, 2018). This highlights the usefulness of bacterial assemblages as indicators for trophic status (Fierer *et al.*, 2007, Hartman *et al.*, 2008, Ho *et al.*, 2017) and ecosystems health (Sims *et al.*, 2013, Urakawa and Bernhard, 2017). Critically, our results demonstrate that these swamps are being degraded by urbanisation.

Taxa such as, *Bacteroidetes* and *β-proteobacteria*, that have been associated with copitrophic life strategies, (Fierer *et al.*, 2007), were present in higher proportions in the urbanised catchment swamps compared to the intact swamps. While *Bacteroidetes* are common in freshwater environments, they respond positively to pollution, which is a likely consequence of their ability to degrade high molecular weight organic compounds (Thomas *et al.*, 2011, Martin *et al.*, 2012, Zhang *et al.*, 2012a). Others have reported greater proportions of *Bacteroidetes* in disturbed urban waters including sites along the River Ganges, India (Jani *et al.*, 2018), at industrial outfalls in the Nanxijiang River, China (Lu and Lu, 2014) and in urban streams in Chicago, USA (Drury *et al.*, 2013). On average, *Bacteroidetes* made up about three times the proportion of reads in the urbanised catchment swamps than the intact catchment swamps. *β-proteobacteria*, which are important for nitrogen cycling (Garrity, 2005), were twice as common in the surface sediments of urbanised catchment swamps than in the intact catchment swamps. Greater *β-proteobacteria* is often associated with nutrient enriched environments (Wobus *et al.*, 2003, Hartman *et al.*, 2008, Saxena *et al.*, 2018). The family *Nitrosomonadaceae*, within *β-*

proteobacteria, made up a greater portion of reads in the urbanised catchments, was one of the top contributors to the dissimilarity of the bacterial communities between the urbanised and intact catchment swamps in the surface sediment. *Nitrosomonadaceae* includes ammonia oxidisers (Garrity, 2005) and may be a useful indicator of ammonia and nutrient loading (Sims *et al.*, 2013, Urakawa and Bernhard, 2017). Ammonia oxidising bacteria (AOB) are abundant in eutrophic conditions such as wastewater processing (Sims *et al.*, 2012). Ammonia levels were highest in the surface sediments of urbanised catchment swamps (Christiansen *et al.*, 2019) and, not surprisingly, were positively correlated here with the proportion of *β-proteobacteria* (Pearson's correlation $r = 0.542$, $p<0.001$). Taxa within *β-proteobacteria* have also been linked to hydrocarbon pollution, which may reflect an influence from stormwater inputs and road runoff in the urbanised catchment swamps.

In contrast to the urbanised catchment swamps, the intact catchment swamps had a higher proportion of reads of an oligotrophic phylum, *Acidobacteria* (Fierer *et al.*, 2007). While *Acidobacteria* are diverse and ubiquitous (Janssen, 2006, Jones *et al.*, 2009, Kielak *et al.*, 2016), they are generally competitively advantaged in low resource (Smit *et al.*, 2001, Eichorst *et al.*, 2007, Fierer *et al.*, 2007, Ward *et al.*, 2009, Ho *et al.*, 2017) and low pH environments (Sait *et al.*, 2006, Hartman *et al.*, 2008), although variation exists between taxonomic subdivisions (Kielak *et al.*, 2016). The study swamps are naturally low nutrient and acidic environments (Keith and Myerscough, 1993, Pemberton, 2005) and impacts of urbanisation are locally associated with augmented nutrients and pH (Belmer *et al.*, 2015, Lane, 2016, Christiansen *et al.*, 2019). Therefore, a higher proportion of *Acidobacteria* in the intact catchment swamps would be expected. Elsewhere, similar results have been found with *Acidobacteria* being more common in streams from forested than in urbanised catchments (Hosen *et al.*, 2017) and in natural wetlands compared with artificial wetlands (Peralta *et al.*, 2013). Within *Acidobacteria*, *Candidatus_Koribacter*, a genus adapted to low resource conditions and slow growth (Ward *et al.*, 2009), and order *Acidobacteriales* were identified as contributors to the dissimilarity between the catchment communities.

Several taxa identified as being correlated with catchment type do not have a clear relationship (e.g *α-proteobacteria, Planctomycetes*) or have not received significant attention for their trophic traits (*δ-proteobacteria* and *Nitrospirae*) (Fierer *et al.*, 2007, Ho *et al.*, 2017). Additional insights can be gained by considering the function and processes of these taxa and how they might relate to differences between the catchment types. *Actinobacteria*, *δ-proteobacteria* and *Nitrospirae* made up a greater proportion of reads in the urbanised catchment swamps. Of interested is the genus *Geobacter* (class *δ-proteobacteria*), which was one of the top taxa explaining the dissimilarity between the catchments, being relatively more common in the urbanised catchment swamps. *Geobacter* are capable of oxidising organic compounds and metals, and are recognised as an important for biogeochemical processes and bioremediation (Lovley *et al.*, 2011, Cologgi *et al.*, 2014). They have been associated with contaminated environments (Lovley *et al.*, 2011) including polluted river systems (Liao *et al.*, 2019). In this study, increased *Geobacter* abundance may indicate contamination in the urbanised catchment swamps. Given *Nitrospirae* undergo chemolithoautotrophic growth through nitrite oxidation (Daims, 2014) and have been documented to have greater relative abundances associated with wastewater treatment plant outfalls (Drury *et al.*, 2013). It follows that they would also be favoured in higher nitrogen

conditions, however there was no correlative relationship between ammonium and *Nitrospirae* in this study. It has been suggested that *Nitrospirae* is favoured under less oligotrophic conditions compared to *Nitrosomonas* (Sims *et al.*, 2013), and so may not be an accurate indicator of nutrient loading. However, the genus *Leptospirillum* (Order *Nitrospirae*) is capable of iron (II) oxidation (Daims, 2014) and this may be reflective of conditions suitable for iron oxidation. In nearby swamps, evidence of bacterial iron oxidation have been linked to local, historical excavation disturbance (Hardwick, 2019). Supporting this, at Katoomba Falls Road (KF) swamp had the highest proportion of *Nitrospirae* and iron flocs were observed during sampling indicating active iron cycling (Fig 5.8).

Figure 5.8 Katoomba Falls Road swamp at the time of sampling showing red flocs indicative of iron cycling.

In the intact catchment swamps, *α-proteobacteria* made up a greater proportion of reads. The trophic status of *α-proteobacteria* is unclear (Fierer *et al.*, 2007) and has been considered both copiotrophic and oligotrophic depending on the environmental conditions or more specific taxa (Ho *et al.*, 2017). A top contributor to differences between the catchments was the genus *Roseiarcus* (Class *α-proteobacteria*) which is considered slow growing and moderately acidophilic (Kulichevskaya *et al.*, 2014). The phylum *Planctomycetes* was relatively commonly found in the deeper sediments of intact catchment swamps than urbanised swamps. Taxa in the family *Isosphaeraceae* (phylum *Planctomycetes*) were top contributors to the dissimilarity of the deeper sediments by catchment type. *Isosphaeraceae* are commonly found in boreal peatlands and are slow decomposers and some may be capable of anaerobic ammonium oxidation (Youssef and Elshahed, 2014, Fuerst, 2017, Ivanova *et al.*, 2017). Bacteria anaerobic ammonium oxidation has been associated with lower nutrient levels

(Burgin and Hamilton, 2007, Urakawa and Bernhard, 2017), which agrees with ammonium concentration levels being lower in the intact catchment swamps.

Another difference in bacterial communities between the catchment types were the diversity and richness measures. While high diversity is often associated with healthy ecosystems (Urakawa and Bernhard, 2017), we found intact catchment swamps had lower diversity and richness. Contamination and disturbance can negatively influence diversity (Baker and Banfield, 2003, Lenart-Boroń and Boroń, 2014), however, in naturally acidic and nutrient poor systems the inverse is often true (Griebler and Lueders, 2009, Horton *et al.*, 2019). We found the diversity and richness indexes correlated positively with pH. Acidic sediment conditions restrict the species of bacteria to those that are adapted to metabolising in acidic conditions (Fierer and Jackson, 2006) and the pH is a primary driver of bacteria biogeography, with highest diversity occurring around neutral pH (Fierer and Jackson, 2006, Hartman *et al.*, 2008). Any shift from the reference condition, whether positive or negative, indicates a departure from the normal condition. Importantly, bacterial diversity is related to functionality and ecosystem services (Bell *et al.*, 2005, Delgado-Baquerizo *et al.*, 2016) and is therefore important to consider for ecosystem health (Urakawa and Bernhard, 2017). In the case of peatlands, peat formation and storage is underpinned by slow microbial decomposition, therefore microbial changes may result in carbon exportation. Indices (phyla ratios) and/or indicator species (SIMPER results) explored in this chapter may be useful for tracking this departure from the norm.

The highly urbanised swamps, Popes Glen and Pitt Park stood out for a number of measures, deviating the furthest from the intact catchment swamps in terms of bacterial community composition (Figs 5.4 and 5.6). These sites have a history of disturbance and have ongoing stormwater influences as evidenced by the high pH levels (Table 5.1). Popes Glen has been severely impacted by stormwater runoff and invasive vegetation, and has since undergone major restoration works (Lane, 2016). Pitt Park is located immediately downstream of rail infrastructure, is in poor geomorphic condition and suffers altered carbon cycling and exporting relatively high levels of methane compared to other swamps nearby (Fryirs *et al.*, 2016, Cowley *et al.*, 2018a). Both swamps have altered vegetation communities with non-native species present (Christiansen, personal observation). The bacterial communities in Pitt Park and Popes Glen had high diversity index values compared to the other swamps, higher proportion of copiotrohic taxa (*β-proteobacteria* and *Bacteroidetes*), the lowest proportions of oligotrophic taxa *Acidobacteria* and very different values for the ratio of *Proteobacteria* to *Acidobacteria*. These microbial results suggest that the function of these swamps has been severely changed, which is likely responsible for the altered carbon exports occurring at Pitt Park (Cowley *et al.*, 2018a).

In conclusion, the results suggest that the impacts of urbanisation have altered the microbial community of ecologically significant swamps in ways that are consistent with ecosystem degradation. The results also indicate that microbial indicators of ecosystem health can also be applied to these swamp systems (Smit *et al.*, 2001, Hartman *et al.*, 2008, Sims *et al.*, 2013, Urakawa and Bernhard, 2017). Taken together, using microbial indicators are a potential biomonitoring tool for ecosystem health monitoring and assessment of these swamps.

CHAPTER 6
GENERAL DISCUSSION

Prior to the research of this thesis, the microbial ecology of the ecologically significant and endangered Temperate Highland Peat Swamps on Sandstone (THPSS) of the Sydney Basin had not been explored. Microbes, as mediators of biogeochemical cycling and decomposition, are fundamental for important peatland attributes such as carbon storage, nutrient cycling and greenhouse gas emissions (Fenchel *et al.*, 1999). Anthropogenic pressures are affecting ecosystems on an ever-increasing scale (Sanderson *et al.*, 2002). Therefore, understanding ecosystem community responses to these pressures is critical for management and protection of functioning ecosystems (Cooke *et al.*, 2018). The aim of this thesis was to increase understanding of microbial assemblages and functions in THPSS to improve their management. The program of research filled knowledge gaps in the sediment microbial component of the THPSS ecosystem and its response to significant anthropogenic disturbances through a series of studies undertaken to address the following research objectives:

1. Determine the structure of microbial communities and identify where ecologically important processes are occurring with emphasis on the processes of methane and nitrogen cycling.
2. Identify sediment properties that influence microbial communities.
3. Determine microbial community response to key disturbances that affect THPSS: urbanisation and fire.
4. Determine which taxa are associated with healthy and degraded systems and explore the potential of microbial indicators for assessing THPSS ecosystem health.

In this discussion, the results of the thesis are summarised (Table 6.1) and synthesised. Implications for the development of broader management strategies and tools to maintain, restore and monitor THPSS are discussed. Included is a discussion that compares and assesses the primary molecular techniques, T-RFLP, high-throughput 16S rRNA gene sequencing, and qPCR used in this thesis that could be used for ongoing THPSS health assessment. Although the focus of this thesis was on THPSS, the research has broader application for global peatlands and wetlands, where there have been limited studies into the effects of urbanisation on microbial communities and the impact of fires in wetlands (Andersen *et al.*, 2013, Bixby *et al.*, 2015).

STUDY OUTCOMES AND APPLICATION TO THPSS MANAGEMENT

The findings of this thesis highlight both the sensitivity and resilience of THPSS systems to disturbance. While a hazard reduction burn of THPSS did not appear to cause long-term changes to the microbial community or sediment conditions (Chapter 3), the ongoing disturbance of catchment urbanisation has (Chapters 4 and 5). The sediment pH appears to be a significant driver in the microbial differences found in the THPSS in urbanised catchments (Chapter 4).

Table 6.1 Summary of research for this thesis.

Chapter	Aim	Objectives*	How aims and objectives were addressed	Key findings and significance
2	To characterise microbial diversity and structure of a THPSS	1	• Assessed microbial community along depth profiles along downstream gradient • Determined by qPCR location of greatest gene abundances of methane cycling and ammonia oxidation • Showed T-RFLP microbial community shifts with depth	• First investigation of microbial ecology of THPSS • Depth stratified bacterial community • Genes (*pmoA*, Archaea *amoA* & Bacteria 16S rRNA) decreased abundance with depth • Deeper community more variable than surface • Area of disturbance and stormwater influence was different
3	To determine impact of a Hazard reduction burn on THPSS microbial communities	3	• Assessed changes with before and after impact study of a hazard reduction burn • Compared gene abundances (qPCR) in affected THPSS to before and controls • Compared community structure (T-RFLP) to before and controls • Investigated changes to microbial environment - sediment pH, organic and moisture content – associated with the burn	• After 1 year microbial communities were not different due to burn • Functional genes related to methane cycling (*mcrA* and *pmoA*) were not different 1 year post burn. • Sediment properties (pH, organic and moisture content) were not different after 1 year post burn. • System appears to have recovered within one year of hazard reduction burn
4	To determine the impact of an urbanised catchment on microbial community and function	2, 3	• Assessed differences in the microbial community between THPSS in urbanised catchments and those with intact vegetation • Compared gene abundances and activity (qPCR) • Compared community structure and active community (T-RFLP) • Assessed differences in the microbial environment – sediment pH, ammonium, electricity conductivity, organic and moisture content • Related environmental differences to community structure and gene abundances/activity.	• Sediment microbial community was different in swamps from urbanised catchments • Greater bacteria and archaea activity in urbanised swamps • pH and ammonium concentrations were higher in urbanised catchment swamps • Significant relationship with pH and microbial community variation and gene abundance/activity • Identified pH as important for maintaining microbial function in THPSS
5	To identify taxa responsible for differences in urbanised THPSS	4	• Sequenced DNA dataset from the urbanisation study • Compared phyla make up between catchment types • Identified taxa that contributed to dissimilarity between urbanised and intact catchment types • Compare results using previously suggested indicator (ratio of *Proteobacteria* to *Acidobacteria* for ecosystem assessment	• First bacterial community sequencing of THPSS • urbanised catchment swamps had more copiotrophic bacteria species • Intact catchment swamps had more oligotrophic species • Taxa shift indicates a change in trophic condition • Potential for microbial indicators to assess ecosystem health

*Objectives as listed in preceding text.

REPERCUSSIONS OF A HAZARD REDUCTION BURN

Chapter 3 provides evidence that hazard reduction burns do not have ongoing repercussions on the microbial communities of the affected swamps. Additionally, the measured sediment conditions were not different due to the fire, and the vegetation had largely recovered 12 months after the fire. The aim of hazard reduction burning is to prevent more intense, hotter bushfires that are more likely to impact deeper into the sediment profile (Agee and Skinner, 2005, Penman and Towerton, 2008). Northern peatlands affected by severe wildfires, for example, continued to have altered conditions and microbial communities in excess of 2-7 years after the disturbance (Belova *et al.*, 2014, Taş *et al.*, 2014, Danilova *et al.*, 2015). Therefore, hazard reduction burns may be important for protecting microbial function of THPSS and peatlands generally from a catastrophic fire. Of course, this depends on the assumption that hazard reduction burns are effective in preventing bushfires, which remains a contentious topic (Fernandes and Botelho, 2003).

A potentially important factor influencing the resilience of THPSS to fire disturbance, including hazard reduction burns, is the condition of the swamps prior to the disturbance. The study swamps documented in Chapter 3, both control and burnt, were located in a national park, within naturally vegetated undisturbed catchments in good geomorphic condition (Fryirs *et al.*, 2016) (e.g. reference condition). Ecologically healthy condition may have contributed to the lack of ongoing consequences associated with the fire, which has been found in subtropical wetlands (Medvedeff *et al.*, 2013). Degraded THPSS are often channelised and do not maintain a stable high water table (Cowley *et al.*, 2018b), which can result in the peat sediments becoming drier (Gorissen *et al.*, 2017). Dried or drained peat is more vulnerable to subsurface smouldering fires, which can cause drastic changes to peatland ecosystems (Page *et al.*, 2002, Kettridge *et al.*, 2015). Therefore, investigation into how a burn would affect degraded THPSS is needed. Another area where research is required is how the frequency of fire disturbance affects the microbial ecology. Already, fire occurs regularly in THPSS (Gorissen *et al.*, 2015) and is expected to continue to be an increasing threat with future climate conditions (Keith *et al.*, 2006, Keith *et al.*, 2010).

Management considerations: Under the assumption that hazard reduction burns do prevent high intensity bushfires, which can severely impact peatland ecosystems, hazard reduction burns may protect microbial function of ecologically healthy THPSS.

Further research considerations: Greater understanding of the impacts of severe fires, tracking THPSS recovery at different time scales and the response in degraded habitats is still needed. The extreme 2019/2020 fire season (see Fryirs *et al.* in review) offers opportunities to improve understanding of how these systems respond to fire.

URBANISATION INCREASES pH AND ALTERS MICROBIAL COMMUNITIES AND FUNCTIONS

Microbial communities are different in THPSS in catchments with and without urbanisation. Significantly, the sediment pH was correlated with measures of urbanisation (stormwater drains and catchment imperviousness) and was also the driver of microbial community differences (Chapter 4 and 5). Naturally, the sediment of THPSS is acidic (pH c.a. 4-5), however, runoff over concrete surfaces, including stormwater infrastructure, causes the dissolution of calcium, bicarbonate and potassium ions thereby increasing the alkalinity of waters entering the swamps from urbanised catchments (Davies *et al.*, 2010, Tippler *et al.*, 2014, Carroll *et al.*, 2020). Consequently, the pH measured in the urbanised catchment swamps was about

1 pH unit higher (pH c.a. 5-6) than that in intact catchment swamps. Visualising the sequenced bacterial community NMDS ordination (Chapter 5) by pH provides clear evidence of its influence (Fig 6.1). Samples from urbanised catchment swamps that had lower pH values were more closely clustered with the intact catchment swamp samples and samples from intact catchment swamps with higher pH were closer to the urbanised clusters (Fig 6.1). The phyla make up from the urbanised catchment swamp Marmion Rd, which had the lowest average pH (mean pH 5 compared with mean 5.7 for the other urbanised swamps and 4.6 for the intact catchment swamps), was most similar to intact catchment swamps (Chapter 5). Bacterial and archaeal activity was higher in the surface sediments of urbanised catchment swamps (transcription of 16S rRNA genes) and this was also correlated with pH (Chapter 4). Greater microbial activity has implications for decomposition rates and thus implications for carbon storage. Indeed, decomposition of leaf litter was faster in urbanised swamp streams than in undisturbed swamp streams (Hardwick, 2019). Methane emissions and carbon exports are also greater in swamps affected by urbanisation (Cowley *et al.*, 2018a) and methane cycling genes (*mcrA* and *pmoA*) were more actively transcribing (RNA abundances) in urbanised catchment swamps (Chapter 4) than intact catchments, and the methanogen gene (*mcrA*) was most abundant adjacent to stormwater drains (Chapter 2). Together these results indicate higher pH as a consequence of an urbanised catchment, alters THPSS microbial functions with unwanted side effects of potentially greater carbon export, decomposition and methane production.

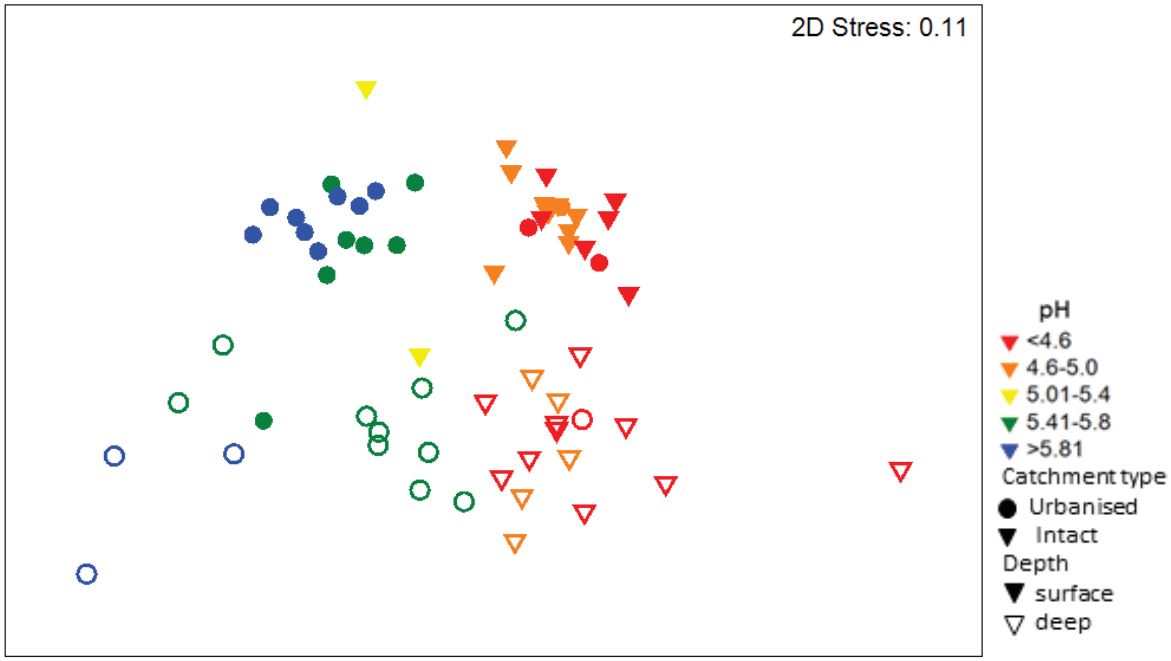

Figure 6.1 NMDS demonstrating the influence of pH on the bacterial community. NMDS of the bacterial community from the Illumina amplicon sequencing to the lowest taxonomic level coloured to emphasise the relationship of pH and the bacterial community make up. Colour indicates the pH, triangle symbols represent intact catchment swamps, circle symbols represent urbanised catchment swamps, solid symbols are surface sediments and open symbols are 50 cm deep sediments.

Management considerations: To protect the microbial function of THPSS, including important carbon cycling processes, effort should be directed to maintaining natural pH levels in urbanised catchment swamps. Alternative materials that will not alter the water chemistry should be used for stormwater drainage

infrastructure. PVC pipe, for example, does not increase bicarbonate, calcium and potassium ions like concrete (Davies *et al.*, 2010). Other alternatives include locally sourced sandstone guttering that matches the geology (Carroll, 2018), or coating concrete with resin to decrease its solubility (Grella *et al.*, 2016).

Further research considerations: Research teasing out the effects of other anthropogenic influences on THPSS microbial community such as altered land use (e.g. mining, agriculture), fluctuating water tables and more detailed study of water chemistry influences on microbial community using a broader suite of indicators is still needed.

TAXONOMIC CHARACTERISATION FOR THE DEVELOPMENT OF MICROBIAL INDICATORS

THPSS are endangered ecosystems of high ecological value. With the restoration of degraded swamps a management priority of local governments and landcare groups, monitoring the health of these ecosystems and establishing benchmarks of condition are critical. Monitoring allows for adaptive management and can lead to improved outcomes as well as demonstrating the success and value of rehabilitation efforts (Kohlhagen *et al.*, 2013). Key to successful environmental health assessment is monitoring relevant indicators for valued ecosystem properties. The use of microbes as biological indicators of ecosystem health has been gaining attention (Sims *et al.*, 2013, Urakawa and Bernhard, 2017). Microbes are relevant since they are fundamental for ecosystem function, and are also highly sensitive and responsive to environmental changes and pollution (Urakawa and Bernhard, 2017, Sutcliffe *et al.*, 2019). Biological indicators, generally, have recognised advantages, biota experience and respond to both physical, chemical and biological stressors including subtle or intermittent disturbances (Cullen, 1990). With recent advances in molecular techniques, such as next generation sequencing, characterisation of whole microbial communities, including taxa that are difficult to culture, has become increasingly accessible. Using the sequencing results from Chapter 5, I will consider applicability of microbial indicators that have been suggested by other researchers for ecological assessment of THPSS.

The ratio of *Proteobacteria* to *Acidobactera* reads has been suggested as a microbial indicator for ecosystem assessment (Smit *et al.*, 2001, Hartman *et al.*, 2008). In wetlands, this ratio has been shown to respond to land use and restoration status (Hartman *et al.*, 2008). The principle of using *Proteobacteria* and *Acidobactera* as a ratio is that they broadly represented copiotrophic and oligotrophic life strategies (respectively) and so a change in the ratio indicates a trophic shift (Smit *et al.*, 2001). Supportive of this, the ratio of *Proteobacteria* to *Acidobactera* was greater in urbanised catchment swamps (Chapter 5). This was primarily driven by *Acidobactera* making up a greater proportion of the community in the intact catchment swamps, since the relative proportion of *Proteobacteria* was not different by catchment type. *Acidobacteria* has been considered oligotrophic based on predictable characteristic responses to C substrate availability (Fierer *et al.*, 2007). *Proteobacteria* is a diverse phylum with classes that are not consistently copiotrophic (Fierer *et al.*, 2007). It may be more reliable if the indicator ratio included a more reliably copiotrophic taxonomic group, such as *Bacteriodetes* or *β-proteobacteria* (Fierer *et al.*, 2007) to replace *Proteobacteria*. In light of the results from this thesis, both *Bacteriodetes* and *β-proteobacteria* made up a significantly higher proportion of the bacterial community in urbanised catchment swamps than in undisturbed swamps. *Bacteriodetes* also had significant positive correlations (Pearson's) with measures of urbanisation (impervious catchment area $r=0.427$, $p<0.001$, and stormwater drains $r=0.491$, $p<0.001$) as

did *β-proteobacteria* (r=0.410, p<0.001 and r=0.479, p<0.001 respectively). Of note, *Acidobacteria* had significant negative correlations with measures of urbanisation (impervious catchment area r=-0.311, p=0.012, stormwater r=-0.457 p<0.001).

In their review of microbial indicators for wetland assessment, Sims *et al.* (2013) recommend focusing on microbial communities responsible for key biogeochemical processes for wetland assessment and monitoring. Specifically, they suggest monitoring the ratio of ammonia oxidising archaea (AOA) to ammonia oxidising bacteria (AOB) (Sims *et al.*, 2013). The AOA are generally more persistent and competitive than AOB in lower nutrient conditions such as natural wetlands, whereas AOB dominates in nutrient rich situations such as constructed wetlands receiving wastewater (Sims *et al.*, 2013). In essence, the ratio of AOA to AOB also represents a ratio of oligotrophic to copiotrophic life strategists. In Chapter 4, the abundance of AOA *amoA* gene was quantified using qPCR, however it was not consistently detected across all samples. Most detections (12 out of 15, out of a total 71 samples) were from urbanised catchment swamps. Despite attempts using tested universal primers for AOB *amoA* gene (Rotthauwe *et al.*, 1997), no successful isolation or reliable detections of the gene was made. Naturally THPSS are nutrient poor systems (Keith and Myerscough, 1993), so the genes associated with ammonia oxidation may not be abundant enough to isolate and therefore I was unable to clone and develop the qPCR assay. However the detailed sequencing results (Chapter 5), highlighted *Nitrosomonadaceae* (Family), which includes AOB (Garrity, 2005), as a defining taxon found urbanised catchment swamp surface sediments. Perhaps, along these lines, the *Nitrosomonadaceae* taxonomic group could serve as an indicator of urban degradation. Alternatively, greater refinement of assays to detect the AOA and AOB genes in THPSS could also lead to a promising measure.

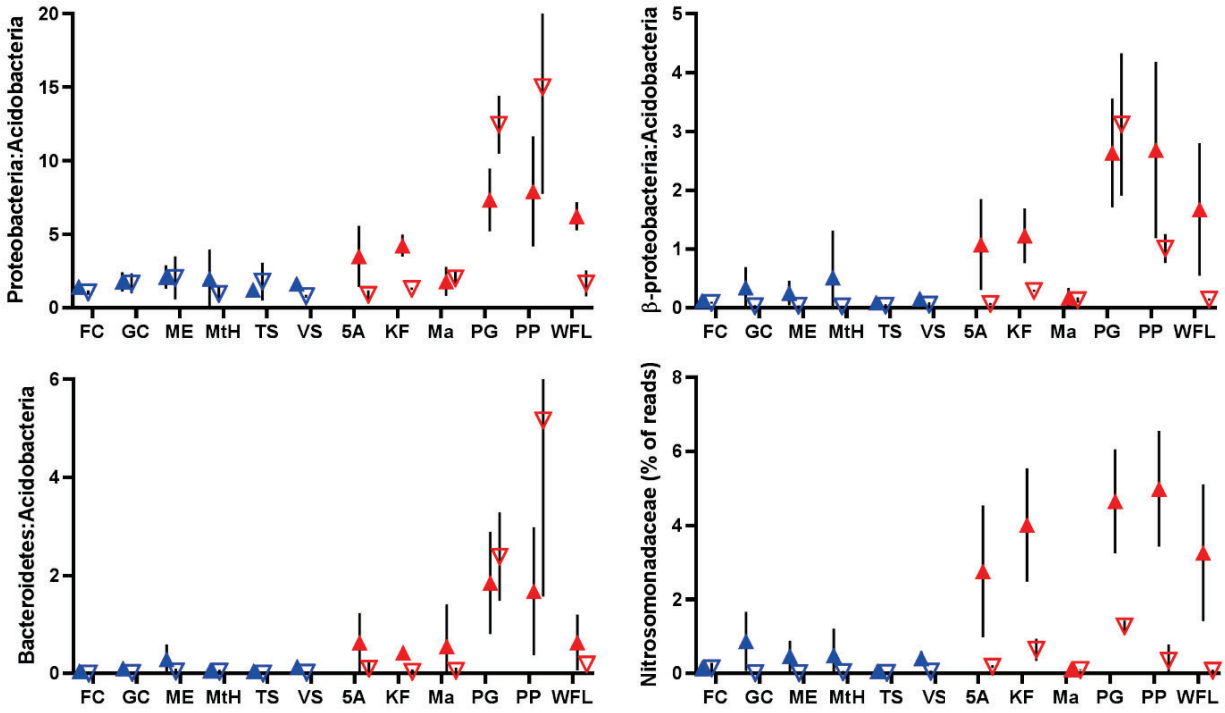

Figure 6.2 Potential microbial indicators to assess THPSS. Comparing THPSS affected by urbanised catchment disturbance (red) to those with natural intact vegetation catchments (blue). Surface sediment are represented by solid symbols, open symbols represent 50 cm deep

There were clear differences in the ratios of copiotrophic and oligotrophic taxa, and the proportion of *Nitrosomonadaceae* (Fig 6.2) between the catchments and these metrics show promise as an indicator of disturbance. Consistently, each potential indicator highlighted the sites Pitt Park and Popes Glen as the most disturbed within the urbanised catchment swamps. The indicators that use ratios are, to a degree, inherently related as each has *Acidobacteria* as a component. All of the ratios differed significantly with catchment types (urbanised vs non-urbanised, Chapter 5), but the strongest differences were found with the proportion of *Nitrosomonadaceae* ($p=0.002$), ratios of *Bacteriodetes* to *Acidobacteria* ($p=0.004$) and *β-proteobacteria* to *Acidobacteria*, whereas *Proteobacteria* to *Acidobactera* had the highest p value ($p=0.016$).

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Management considerations: Microbial indicators could be a useful biological indicator, particularly when combined with assessment of the hydro-geomorphic structure and water chemistry to assess ecosystem health of THPSS.

Further research considerations: Greater research is needed to develop reliable, fit for purpose microbial indicators. Detailed assessment of potential indicators is still needed for THPSS and other systems more broadly.

MOLECULAR TECHNIQUES FOR THPSS MICROBIAL RESEARCH AND ASSESSMENT

Policy makers and land managers need cost effective tools for the ongoing assessment of THPSS ecosystem health. This program of research used qPCR, T-RFLP and next generation sequencing to describe attributes of the microbial communities. Each of these have inherent advantages and disadvantages. T-RFLP (Chapters 1-4) and next generation sequencing (Chapter 5) were used for comparing microbial community composition (van Dorst *et al.*, 2014), while qPCR (Chapters 1-4) and next generation sequencing (Chapter 5) microbial function was used to infer microbial functional attributes and potential. Next generation sequencing provides insight into microbial function through analysis of taxonomic groups (Kwon *et al.*, 2011) and their known function and qPCR can be used to quantify functional gene abundances (Smith and Osborn, 2009). The following section will compare these techniques in light of the results from this thesis and makes recommendations for the applicability of molecular assessment tools for THPSS.

For microbial community analysis, the advantages of sequencing is that it provides detailed taxonomic resolution, which allows for inferences of microbial processes and functions to be made within the same analysis (Shokralla *et al.*, 2012, Hugerth and Andersson, 2017). Sequencing also provides better estimates of diversity and richness than T-RFLP as rarer OTU are identified with sequencing and multiple OTU are not binned together as can occur with analysis of T-RFLP data (Orcutt *et al.*, 2009), although sequencing errors may result in overestimated diversity (Kunin *et al.*, 2010). The primary advantage of T-RFLP, on the other hand, is the lower cost of analysis. Lower costs permits greater replication within a set budget. Sufficient replication is especially important for ecological studies where there will be inherent variability (Chapter 2), and replication may be sacrificed to meet budget constraints (van Dorst *et al.*, 2014).

Researchers have assessed the relevance of T-RFLP community fingerprinting as amplicon next generation sequencing becomes more accessible and have found that T-RFLP offers an adequate low cost alternative to sequencing for comparing community differences (Orcutt *et al.*, 2009, van Dorst *et al.*, 2014, De Vrieze *et al.*, 2018, Lindstrom *et al.*, 2018). Although, significant differences between microbial communities found by sequencing can be missed with T-RFLP (Kasai *et al.*, 2016).

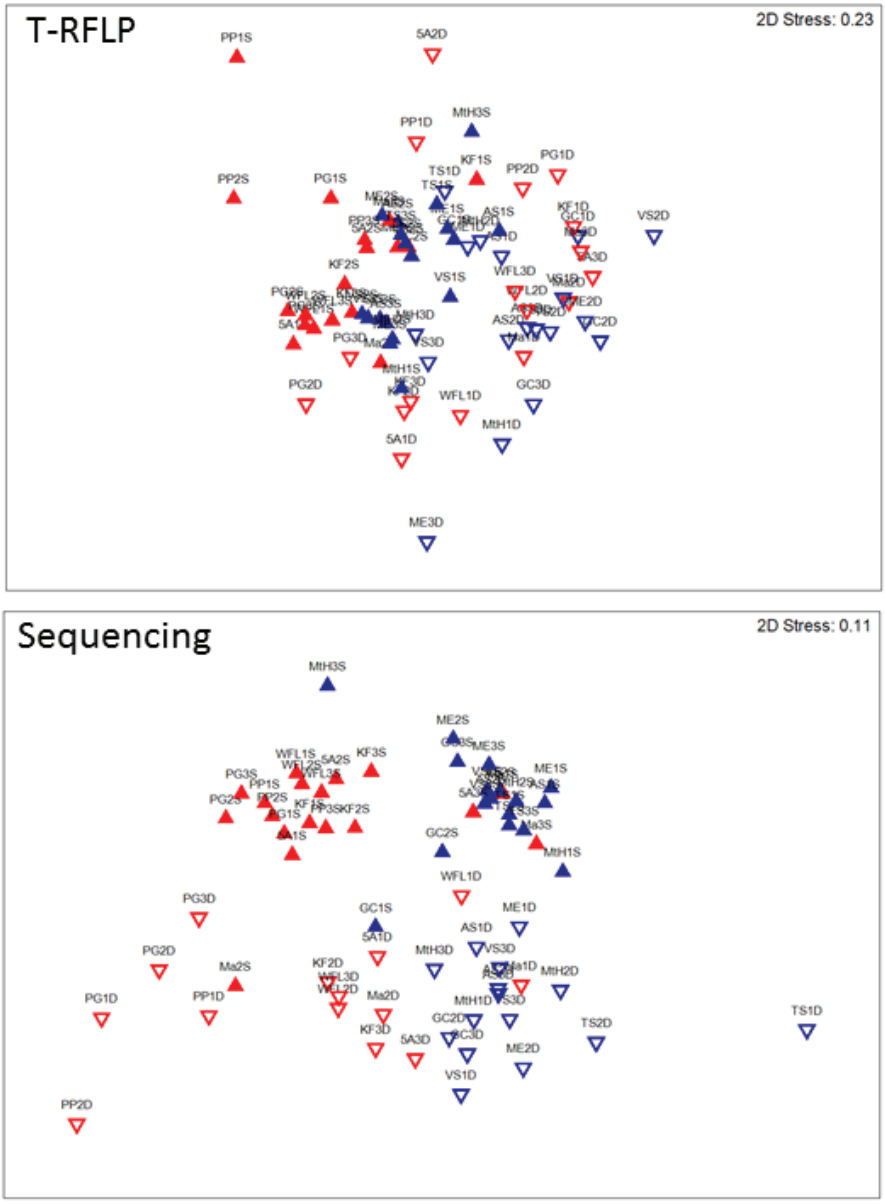
Since recent research indicated that T-RFLP is an adequate method to characterise differences in microbial communities in soils (van Dorst *et al.*, 2014), I used T-RFLP to broadly analyse microbial community patterns. Then, I targeted the most noteworthy results with high throughput sequencing. With the urbanisation study (Chapter 4), multiple depths, bacteria and archaea, as well as the transcribing community (RNA) with replicates within twelve swamps were evaluated with T-RFLP. From this initial analysis, I was able to gain an understanding of what results were significant and would be of the most value to sequence. The bacterial DNA community, which was found to be different between the urbanised and intact catchment swamps, was selected for further analysis by sequencing to determine what taxonomic groups were responsible for these differences.

In this study, the T-RFLP analysis had generally lower richness and diversity than the sequencing analysis. This matches other studies (Orcutt *et al.*, 2009, van Dorst *et al.*, 2014, De Vrieze *et al.*, 2018, Lindstrom *et al.*, 2018). On average, the number of T-RFs (mean 46.4, stdev ±17.3) from the T-RFLP analysis was about 9.6% (stdev± 5.5%) of the number of OTU (mean 542, stdev±195) identified per sample by sequencing. Diversity was also greater with the sequencing analysis at Shannon's diversity H=4.0 (±0.48) compared with H=2.7 (±0.43). These figures are very similar to those reported in van Dorst *et al.* (2014). In this study, it was expected that sequencing would provide greater resolution with richness and diversity, surprisingly, however, there was no correlation between two methods with these measures. De Vrieze *et al.* (2018) found Shannon's diversity index was correlated between T-RFLP and Illumina sequencing in their study of anaerobic digesters. However, comparison of the methods (T-RFLP and 454 pyrosequencing) on polar soils samples also did not have correlations between diversity (H') or richness (S) measures (van Dorst *et al.*, 2014).

Overall, similar conclusions were reached from the T-RFLP and sequencing analyses. Both showed microbial community differences by catchment type and depth (all with $p<0.01$). Sequencing provided greater resolution and detected differences between sites within the catchments that T-RFLP did not. Distance based Linear Modelling (DistLM) of both analyses indicated that depth and pH were the significant factors explaining the variation (both $p<0.001$) and ammonium, electrical conductivity, organic and moisture content were not significant. Comparing the NMDS ordination (Fig. 6.3), the differentiation between catchment types and depth was much clearer with sequencing showing four general quadrants in the ordination, left to right separating the catchment types and upper and lower separating the depths. Comparing richness and diversity, there were some divergences between the methods. Shannon diversity based on sequencing data differed with depth ($p<0.001$) and catchment type ($p=0.045$), while with T-RFLP, differences were only found by depth ($p=0.02$). Further, there were significant differences in sequence-based OTU richness between the depths ($p<0.001$) but not for T-RF richness ($p>0.05$).

For assessment of microbial function, qPCR has the advantage of being rapid, relatively simple, and low cost (Ginzinger, 2002, Smith and Osborn, 2009). However, it is limited to a single targeted microbial function (e.g. chosen gene) at a time (Smith and Osborn, 2009), although developing technology, such as

microfluidic qPCR, allows high throughput quantification of multiple genes per assay (Crane *et al.*, 2018). Next generation sequencing, as discussed above, can provide broader non-targeted assessment based on taxonomic make up (Baird and Hajibabaei, 2012, Shokralla *et al.*, 2012). However, inferences of microbial ecological function must be approached with caution since only small number of genome sequences and cultivation studies have been done relative to the enormous diversity of microbes (Land *et al.* 2015). A potential useful application would be to use sequencing to identify significant taxa and develop targeted and relevant qPCR assays that are cost effective for ongoing monitoring. For example, the next generation sequencing analysis in Chapter 5, highlighted the family *Nitrosomonadaceae* as a potential indicator of urban disturbance, developing primers that target this taxa (Thornton and Basu, 2011), and monitoring their abundance through qPCR. In addition to the functional genes and primers utilised in this thesis, numerous primers have been developed targeting various metabolic functions and taxa (Smith and Osborn, 2009).



6.3 Side by side comparison microbial communities based on T-RFLP and sequencing. NMDS of the urbanisation study microbial communities based on T-RFLP analysis (left) and amplicon sequencing (right). Sequencing ordination shows clearer groupings. Colours represent catchment type - red are urbanised catchment swamps, blue are intact catchment swamps, solid symbols are surface sediment and open symbols are deep sediment (50 cm).

***Management considerations*:** While T-RFLP is a suitable, cost effective technique for comparing microbial communities, sequencing provides greater resolution and more reliable assessment of diversity. For a cost-effective, targeted THPSS (or other wetland and/or peatland) assessment, sequencing can be used to inform and develop indicators that can be assessed ongoing through qPCR technology.

Further research considerations: Developing and accessing microbial indicators that can be applied easily and cost effectively such as qPCR.

RESEARCH IN CONTEXT

THPSS in the Sydney Basin have been deemed ecologically significant and endangered ecosystems, however, many management and restoration efforts have been carried out in absence of detailed knowledge of the systems (Kohlhagen *et al.*, 2013). This research, as outlined above, and other recent work on THPSS, have sought to fill knowledge gaps and ultimately improve management outcomes (Mooney and Martin, 2016). Here, I place the findings of this thesis in the context of the region, other recent findings and more broadly to other peatlands.

A common finding of recent THPSS research has been negative impacts of urbanisation. Despite areas of northern peatlands occurring in and near urbanisation (Chapman *et al.*, 2003), research into the consequences of urban pressures on peatland microbial communities (Andersen *et al.*, 2013) or peatlands structure and function generally (Cowley, 2017) has been overlooked. With global increases in urbanisation, knowledge of peatland response will be important to manage and protect these valuable systems. The recent research of urbanisation on THPSS is therefore important to global understanding of peatlands. It has been found that impervious catchment area and concentrated stormwater flows have incised the natural valley fill structure of many THPSS located in urbanised catchments (Fryirs *et al.*, 2016). Consequences of channelisation are numerous. It reduces the ability of THPSS to store water and sediment, which reduces the nutrient and pollution filtering capacity of these systems (Cowley *et al.*, 2018b). The loss of these functions potentially affects both the base flow and quality of Sydney's domestic water supplies (Cowley, 2017). THPSS channelisation was also shown to increase carbon exports, reducing the capacity of THPSS to store carbon (Cowley *et al.*, 2018b), an important attribute of peatland ecosystems that is fundamental for long-term climate stability (Parish *et al.*, 2008). Impervious catchment area has also affected the water chemistry of THPSS (Belmer *et al.*, 2015). Swamps from urbanised catchments have distinct urban geochemical signature of higher pH and alkalinity with greater concentrations of calcium, bicarbonate, potassium and strontium ions (Carroll *et al.*, 2020). The biota that is affected includes the endangered Blue Mountains water skink (*Eulamprus leuraensis)* (Gorissen *et al.*, 2015, Gorissen *et al.*, 2017), aquatic macroinvertebrates (Belmer *et al.*, 2018) and stygofauna (Hardwick, 2019). As a consequence, increased decomposition rates have been observed in THPSS streams with urbanised catchments (Hardwick, 2019). The results and conclusions from Chapters 4 and 5 add to a body of growing scientific evidence that urbanisation is altering THPSS function and affecting microbial structure and function. As noted above, microbes are the foundation of decomposition and nutrient cycling processes and together with the recent research on urbanisation impacts to THPSS highlights the interrelatedness of the abiotic and biotic aspects of ecosystems (Fig 6.4).

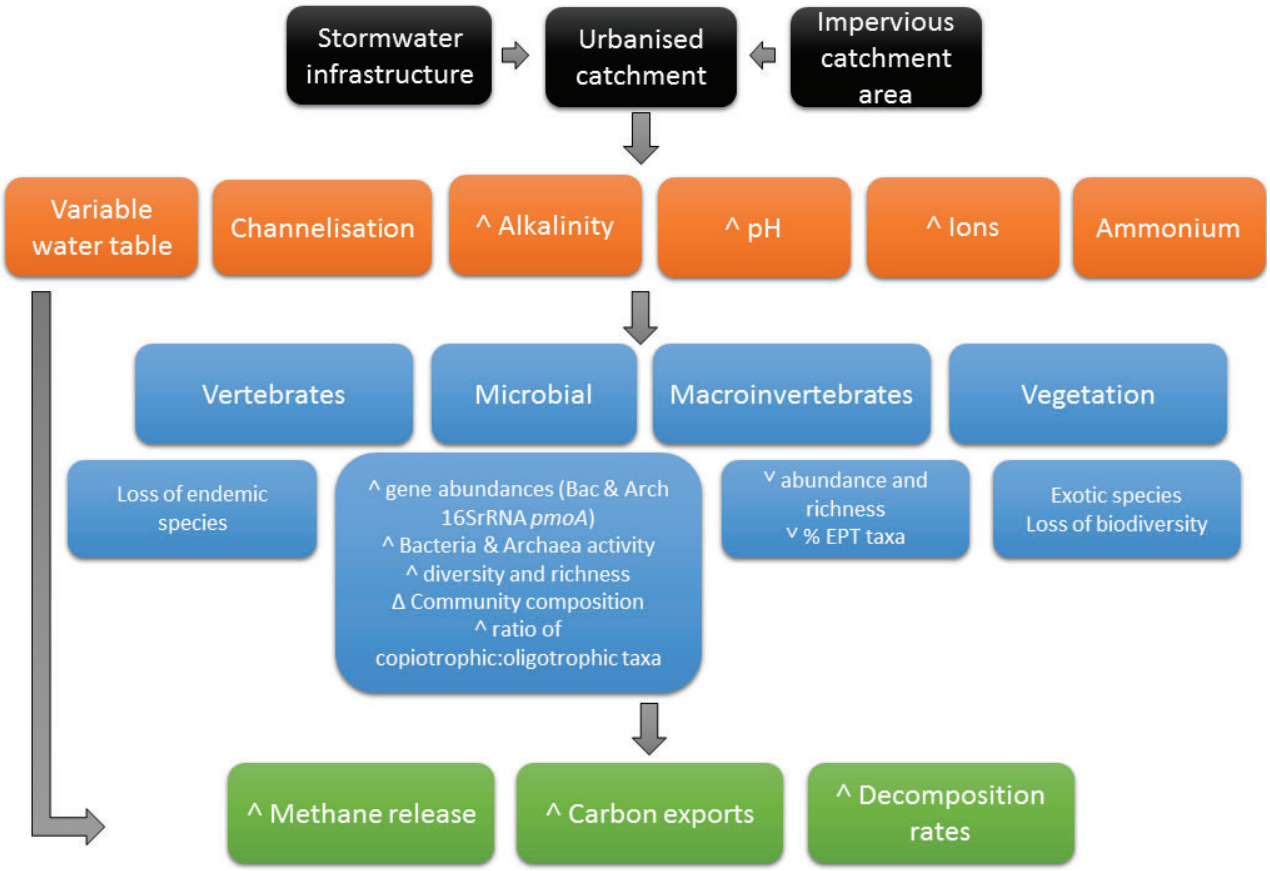


Figure 6.4 Conceptual diagram of recent research of THPSS response to urbanisation. Recent research considered together highlights the interrelated effects of urbanisation on abiotic (orange) and biotic factors (blue) and the resulting changes to key processes (green). ^ indicates increase, ᵛ indicates decrease, Δ indicates changed.

Fire has also been a focus of recent research in THPSS. In the Blue Mountains fires occur regularly affecting THPSS (Gorissen *et al.*, 2015). The endangered Blue Mountains skink seems to be resilient to fires occurring in swamps (Gorissen *et al.*, 2015, Gorissen *et al.*, 2018). Gorissen *et al.* (2018) also reported rapid recovery of vegetation with sedge sprouting within the first weeks after a fire. The understory and sunlight penetration to substrate were largely recovered within 15 months of the fire and total vegetation was no different to unburnt controls (Gorissen *et al.*, 2018). These findings are similar to observations noted in Chapter 3 after a hazard reduction burn. Taken together, these observations and results indicate THPSS flora and fauna are adapted to fire disturbance that regularly occurs around the Sydney Basin (Hammill and Tasker, 2010). From the results of Chapter 3, the same appears to be true of microbes, paralleling findings elsewhere if subsurface burning does not occur (Levine *et al.*, 1990, Zhao *et al.*, 2012b, Medvedeff *et al.*, 2013). However, increasing frequency and intensity of fires, which is expected to be a consequence of climate change (Keith *et al.*, 2006), may result in more lasting and severe consequences, particularly if subsurface burns occur which are more likely within dried or drained peat (Hammill and Tasker, 2010, Kettridge *et al.*, 2015).

The studies for this thesis were undertaken in Blue Mountains region where the highest number of THPSS occur (Fryirs *et al.*, 2018). Although there are other areas where THPSS and coastal upland swamps are concentrated (Fig 1.2) (Fryirs *et al.*, 2018), these ecosystems are similar across the regions (Keith and Myerscough, 1993, Fryirs *et al.*, 2014a, Cowley *et al.*, 2016, Hose *et al.*, 2017) and share many similarities with other peatlands around the world (Rydin and Jeglum, 2006). For example, the water chemistry of the Blue Mountains THPSS (Belmer *et al.*, 2015, Chapter 4), was similar to Budderoo (Hose *et al.*, 2017) as is the vegetation and fauna (Keith and Myerscough, 1993, Hose *et al.*, 2014, Hose *et al.*, 2017). Of particular importance for microbial communities (Fierer and Jackson, 2006, Hartman *et al.*, 2008, Chapter 4), the pH reported for undisturbed swamp in Budderoo (4.6 – 5.0) was comparable to the average found in the intact catchment swamps (4.6) (Chapter 4). Together, with the profound changes that are associated with urbanisation discussed above (hydrological, faunal and water chemistry), catchment land use would likely be than greater driver of differences to microbial communities and functions than regional differences in otherwise highly similar habitats (Hartman *et al.*, 2008).

FUTURE OF THPSS

With peatlands being a vital store of atmospheric carbon globally, the protection of peat ecosystem function should be an important part of climate change policy (Keith *et al.*, 2010, Della Bosca and Gillespie, 2019. However, the future of such systems, including THPSS, remains in the balance due to climate and policy uncertainty (Parish *et al.*, 2008). While peatland habitats are rare in Australia and are restricted to patches of suitable topography and climate conditions (Pemberton, 2005), around the Sydney Basin, THPSS are a relatively common feature (Fryirs *et al.*, 2018). This area is projected to become drier and hotter and therefore less suitable for THPSS in the future (Keith *et al.*, 2010, Keith *et al.*, 2014). Already, THPSS are marginally peat forming, in part due to the variable climate conditions that prevail in south eastern Australia (Fryirs *et al.*, 2014a). They may be the canaries of global peatland ecosystems. As areas of northern peatlands are likely to be facing drier or more variable conditions (Gagnon and Gough, 2005), responses of THPSS may provide insights into the future of other peatland systems in more temperate or higher latitude regions (Fryirs *et al.*, 2014a). Since peatland microbial communities will continue to play a role in determining whether these systems serve as sinks or sources of carbon, results of this thesis and recent research highlight that maintaining good geomorphic condition and naturally acidic conditions will be critical.

CONCLUSIONS

Using a suite of molecular techniques (T-RFLP, qPCR, next generation sequencing), the work of this thesis has determined that urbanisation, but not hazard reduction burns, are a threat to the naturally functioning THPSS microbial communities. Sediment pH, which was higher (more neutral) in THPSS affected by urbanisation is an important determinant of the microbial community make up, gene abundances and expression in THPSS. Associated with urbanisation was a taxonomic shift favouring copiotrophic over oligotrophic life strategists. Potential biological indicators to track urbanisation impact and rehabilitation of THPSS include the family of ammonia oxidising bacteria, *Nitrosomonadaceae*, which were associated with catchment urbanisation, and/or ratios of copiotrophic to oligotrophic taxanomic groups such as *Bacteriodetes* to *Acidobacteria* or *β-proteobacteria* to *Acidobacteria*.

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Table S1. Relationship between environmental variables and microbial assemblages as determined by T-RFLP of 16S genes in upland swamps of the Blue Mountains, NSW, Australia. Marginal tests indicate the relationship between environmental variables and assemblages individually. The sequential test indicates the relationship between environmental variables and assemblages determined by stepwise multiple regression. Partial R2 values indicate the relationship of a variable once those listed above it have already been fitted to the stepwise model. Bold indicates $p < 0.05$.

		Marginal test		Sequential test		
	Variable	Individual R^2	P	Partial R^2	Cumulative R^2	P
Bacterial 16S DNA	Electrical conductivity (µS/cm)	**0.174**	**0.001**	**0.174**	**0.174**	**0.001**
	pH	**0.170**	**0.001**	**0.171**	**0.345**	**0.001**
	Soil Moisture (%)	**0.118**	**0.004**	0.035	0.381	0.320
	Ammonium (mg/Kg)	**0.125**	**0.002**	0.026	0.407	0.676
Bacterial 16S RNA	Soil Moisture (%)	**0.085**	**0.010**	**0.085**	**0.085**	**0.012**
	Electrical conductivity (µS/cm)	**0.077**	**0.025**	0.063	0.148	0.080
	pH	0.044	0.373	0.039	0.187	0.510
	Ammonium (mg/Kg)	0.067	0.063	0.042	0.228	0.407
Archaea 16S DNA	Ammonium (mg/Kg)	0.110	0.078	0.110	0.110	0.073
	pH	0.105	0.100	0.058	0.168	0.319
	Electrical conductivity (µS/cm)	0.027	0.819	0.035	0.202	0.646
	Soil Moisture (%)	0.033	0.724	0.015	0.217	0.917

S2 Table. Summary of T-RFLP amplification. Number of samples out of a total 3 replicates that had sufficient amplification for analysis of DNA and RNA transcribing community analysis (T-RFLP).

Catchment type	Swamp	Sample depth	T-RFLP Amplification (n/3)			
			RNA		DNA	
			Bacteria	Archaea	Bacteria	Archaea
Intact	Fortress Creek	0	3	0	3	0
		50	3	0	3	2
	Grand Canyon	0	3	1	3	1
		50	2	2	3	2
	Michael Eade's Reserve	0	3	0	3	0
		50	3	0	3	2
	Mt Hay Creek	0	3	0	3	1
		50	3	0	3	3
	Timmy's	0	3	0	3	0
		50	3	0	3	1
	Vista	0	3	0	3	0
		50	3	0	3	0
Urbanised	Fifth Avenue	0	3	1	3	1
		50	3	1	3	1
	Katoomba Falls Rd	0	3	0	3	1
		50	3	0	3	2
	Marmion Rd	0	3	1	3	1
		50	3	0	3	1
	Pitt Park	0	3	2	3	2
		50	2*	1*	3	2*
	Popes Glen	0	3	2	3	3
		50	2*	1*	3	3
	Wentworth Falls Lake	0	3	0	3	1
		50	3	0	3	2

* Indicates a sample replicate was lost, so these are out of a total of 2 possible replicates rather than 3.