Characterising swamp biodiversity and community structure with environmental DNA

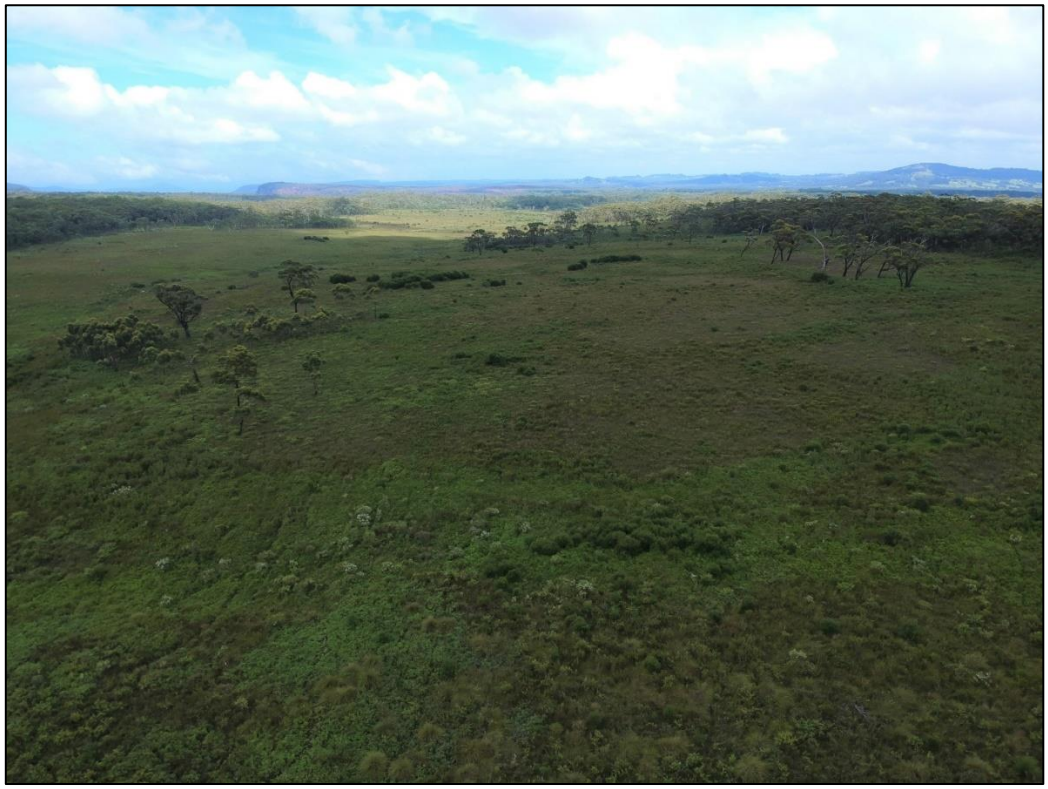

Photo: Upland swamp in the Upper Nepean State Conservation Area, taken by Natalie Caulfield

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This thesis is written in the form of manuscript for submission to the journal of Environmental DNA, with the following exceptions: 1.5 text spacing, line numbers and running title is not included, subheadings are included in the Introduction and Discussion sections, an Aims section has been added, and the Introduction, Methods and Discussion sections are extended.

Declaration

I wish to acknowledge the following assistance in the research detailed in this report:

Anthony Chariton, Rachael Dudaniec, Johan Pansu and Grant Hose for assistance with experimental design. Anthony Chariton, Rachael Dudaniec, Johan Pansu, Jessica O'Hare, Emma Petrolo and Adam Hejl for comments on a draft of this manuscript. Johan Pansu, Anthony Chariton, Grant Hose, Paul Greenfield and Frederic Boyer for assistance with analysis. The New South Wales Government's Saving our Species program through its Office of Environment and Heritage, the Environmental Trust New South Wales and Macquarie University for funding this project.

All other research detailed in this report is my own original work.

Signature:

Natalie Caulfield

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Abstract

In the coastal highlands of the Sydney Basin, Australia, upland swamps are a characteristic environment that supports a unique diversity of biota. These ecosystems carry out essential services including hydrological functions and carbon sequestration. They are currently listed as endangered ecosystems under the Threatened Species Conservation Act 1995, with some of the main threats including mining practices and urbanisation. Previous research on these environments have focused on their geomorphological and hydrological features, with little research on the interactions between local environmental factors and species diversity across spatial scales. This thesis used environmental DNA and metabarcoding techniques to characterise prokaryotic and eukaryotic components of biodiversity across three distinct swamp regions; the Blue Mountains, Upper Nepean State Conservation Area and Budderoo National Park. The relationship between these communities and environmental variables was investigated, as well as their diversity and composition across local and regional scales. Our results indicated that prokaryotic communities were shaped by local environmental variables to a greater extent compared to eukaryotic communities, which were more influenced by geographic distance. This research provides an insight into the uniqueness of swamp biodiversity across multiple spatial scales whilst also demonstrating a novel approach for the future monitoring of swamp ecosystems.

Key Words: biomonitoring, eukaryotes, metabarcoding, prokaryotes, spatial scales, swamp

Table of Abbreviations

Abbreviation	Definition
THPSS	Temperate Highland Peat Swamps on Sandstone
BM	Blue Mountains
BNP	Budderoo National Park
SCA	Upper Nepean State Conservation Area
BMGC	Blue Mountains Grand Canyon
BMPR	Blue Mountains Pulpit Rock
BMTR	Blue Mountains Trail Road
BNP3	Budderoo National Park site no.3
BNP4	Budderoo National Park site no.4
BNP6	Budderoo National Park site no.6
SCA2	Upper Nepean State Conservation Area site no.2
SCA3	Upper Nepean State Conservation Area site no.3
SCA8	Upper Nepean State Conservation Area site no.8

1. Introduction

Swamps are ecologically important environments found throughout the world (Lehner & Doll, 2004). They carry out essential ecosystem services including: hydrological functions (Cowley et al., 2018a); carbon sequestration (Treague & Abbott, 2003); nutrient cycling (Akamatsu et al., 2009); and microclimate regulation (McLaughlin & Cohen, 2013). These services support life on Earth and are sustained by a healthy diversity of plant, animal and microbial life (Cardinale et al., 2011). However, many of these environments are under threat due to anthropogenic impacts and environmental degradation, such as: mining practices (Krogh, 2007); urbanisation (O'Driscoll et al., 2010); eutrophication (Smith, 2003); pollution; and the invasion of exotic plants and animals (Wright et al., 2017; Wright et al., 2011).

All swamps consist of three typical features: 1) soils that have poor drainage; 2) a unique flora that has adapted to characteristic wetland conditions; and 3) periodically waterlogged soils (Burton, 2009). The Ramsar Convention on Wetlands classifies swamps based on their specific geomorphological and hydrological features as well as the biota they support (Ramsar, 1971). These distinguishing features classify unique swamp environments, for example: peatlands (Fryirs et al., 2014; Pemberton, 2005; Posa et al., 2011); swamp forests (Deb et al., 2016; Villa & Mitsch, 2015; Zhao et al., 2018); and mangroves (Akamatsu et al., 2009; Cordeiro & Costa, 2010). The biotic communities that flourish in these environments are often unique and differ from the surrounding areas (Hose et al., 2014; Webb et al., 2012). Both the physical aspects of swamps and the biotic communities that they support drive the ecological functions that these systems carry out, most notably hydrological processes (Bullock & Acreman, 2003; Horwitz & Finlayson, 2011) and the ability to store carbon (Adame & Fry, 2016; Kayranli et al., 2010).

The subsequent introduction aims to provide background information on the upland swamps in the Sydney Basin Region. Here, I will discuss the ecological value of these ecosystems, their unique biota, and the impact of anthropogenic threats on these environmentally sensitive areas. I also provide an overview of the use of molecular techniques for monitoring ecosystems; specifically using environmental DNA (eDNA) metabarcoding to investigate the diversity and community composition of taxa within an area. In addition, I explore how measuring patterns of biodiversity across landscapes can assist in our understanding of species connectivity between suitable habitats. The introduction concludes by detailing the overall objectives of the thesis and postulates several hypotheses specifically regarding swamp community composition driven by environmental factors and geographical distance.

1.1. Upland Swamps in the Sydney Basin

In the coastal highlands of the Sydney Basin, New South Wales, Australia, upland swamps are a characteristic environment found in low-lying sandstone plateaus (Young, 2017). Research conducted by Fryirs et al. (2019) on the spatial distribution of these environments identified 3,208 individual swamps throughout the Sydney Basin. As well as being a predominant feature in the landscape, these areas also carry out important ecological functions which influence both the immediate and wider environment. Many of these upland swamps are classified as peat-forming (Fryirs et al., 2014), with three main types defined by their location in relation to the catchment area as well as the primary source of water: headwater, valley infill and hanging swamps (Commonwealth of Australia, 2014) (Figure 1). It is important to understand the different types of swamps within the region as this directly affects the ecological niches and services they provide. It also informs researchers of their vulnerability to disturbance and uniqueness in order to implement correct protection and management strategies. Peat-forming swamps perform valuable ecosystem services as they are able to accumulate and store large amounts of carbon (Clymo et al., 1998). They also play an important hydrological role, storing water that enters the system via rainfall, runoff or groundwater aquifers (Hose et al., 2014), creating a wet refuge in an otherwise typically dry Australian environment (Keith et al., 2010). These swamps also filter water that flows into the Greater Sydney and Wollongong catchments, supporting an increasingly large urban population (Cowley et al., 2018a; Keith et al., 2006).

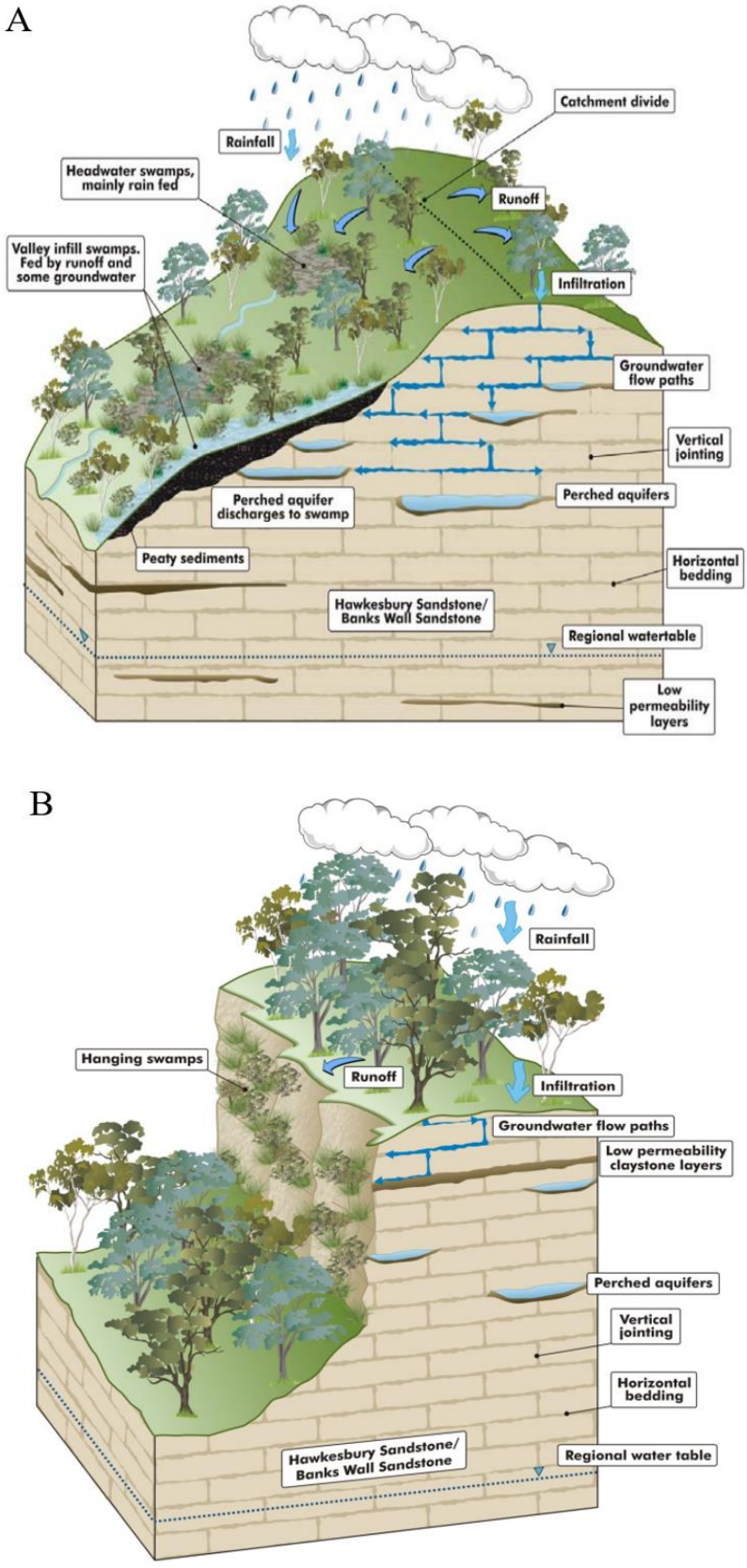

Figure 1: Illustration of the hydrological cycle within A) headwater and valley infill swamps, and B) hanging swamps. Water enters the systems via rainfall, runoff or grounwater from perched aquifers (Commonwealth of Australia, 2014).

The ecological communities that dominate these swamps are classified as Temperate Highland Peat Swamps on Sandstone (THPSS) (Young, 2017), and they are defined by the unique biota found within the swamps. The vegetation within these environments is diverse, with wet areas typically consisting of sphagnum bogs and fens, while drier parts support dense shrubs and heathland, with very few trees within their boundaries (Benson, 1997; Carey, 2007; Whinam et al., 2003). The vegetation creates a distinct border, effectively separating the swamp from the wider landscape which is typically woodlands or sclerophyll forests (Keith & Myerscough, 1993) (Figure 2).

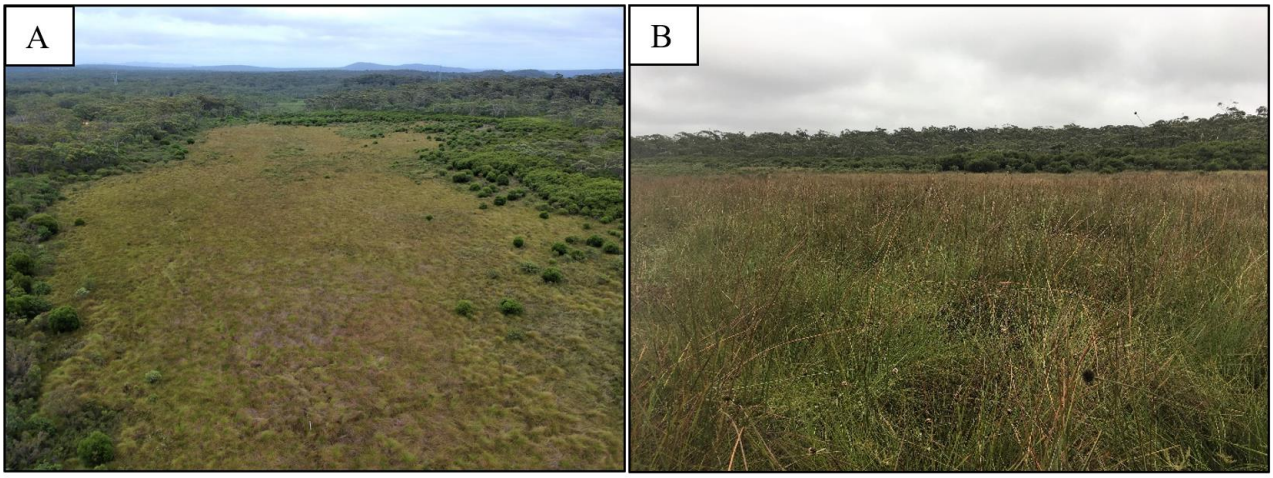

Figure 2: Photos of upland swamps highlighting the distinct vegetation differences and clear boundaries. A) An aerial photo of a swamp in the Upper Nepean State Conservation Area showing heathland vegetation within the swamp surrounded by bushland. B) A photo standing within the swamp heathland vegetation with sclerophyll bushland boundary, Upper Nepean State Conservation Area.

Due to the insular nature of upland swamp habitats, the communities that they support are vulnerable to disturbance (Nakamura et al., 2015). The THPSS are listed as Endangered Ecological Communities, defined as 'facing a very high risk of extinction in Australia in the near future' (Biodiversity Conservation Act, 2016). As a result of this risk and their importance to hydrological and ecological services, upland swamps are protected by both State (Biodiversity Conservation Act, 2016; Threatened Species Conservation Act, 1995) and Commonwealth legislation (Environment Protection and Biodiversity Conservation Act, 1999).

Research on the plants and animals found within upland swamps have identified many specialised species that are endemic to these habitats. This includes plants such as *Lepidosperma evansianum*,

Boronia deanei and *Pultenaea glabra*, which are listed as Vulnerable under the NSW Biodiversity Conservation Act (2016) (Carey, 2007; DEC, 2006; Keith & Benson, 1988). Animals such as the Endangered Blue Mountains Water Skink, *Eulamprus leuraensis*, have been described as a swamp specialist that has limited dispersal between swamps, even within close spatial ranges. A genetic study carried out by Dubey and Shine (2010) recommended individual populations be managed as discrete conservational units due to the limited gene flow and high divergence between swamps. A more recent study by Gorissen et al. (2017) found a reduction in soil moisture as a result of groundwater loss was a key threat to this species, due to habitat destruction. This is also a major threat to the Endangered Giant Dragonfly, *Petalura gigantea* (Baird & Burgin, 2016), which relies on moist sediment to burrow and lay its eggs, a crucial part of their reproductive phase. Invertebrates specialised for groundwater environments, known as stygofauna, rely on aquifers for habitat and have been found to have unique taxonomic structures across temporal and spatial scales (Hose, 2009; Hose et al., 2017). The dependence on groundwater to provide essential habitat within swamp environments increases a species vulnerability to disturbance, as swamps are often fragmented and isolated, limiting gene flow and dispersal (Benson & Baird, 2012).

Although there are multiple regulations in place to protect these ecologically important ecosystems, upland swamps continue to be threatened at both a local and regional scale (Pemberton, 2005). Mining practices are one of the biggest threats, and can have wide ranging and devastating impacts across the region (Jankowski, 2010; Krogh, 2007). Underground coal mining, specifically longwall mining, is classified as a Key Threatening Process under the Threatened Species Conservation Act (1995). Longwall mining can destroy upland swamp habitats by cracking the sandstone bedrock, draining a swamp of water within weeks of the initial damage. This permanently alters the water table and the ecosystem that upland swamps support (Young, 2017). A report from the NSW Department of Planning & Environment (2015) outlined that a number of swamps within the Illawarra Coal mining area underwent changes to their size, ecosystem function and composition, as well as impacting the structural integrity of the bedrock as a result of longwall mining practices in the area.

Land clearing for agricultural use and urban development near swamp regions are also a threat to these ecosystems. Large scale urbanisation can increase the risk of eutrophication (Smith, 2003), sedimentation, channelization and pollution from stormwater runoff (Mackintosh et al., 2015). Such threats have the capacity to alter the hydrological function and geomorphic structure of swamps and impact ecosystem services. Research by Christiansen et al. (2019) on several Blue Mountain upland swamps found that storm water drains increased pH and ammonia concentrations

and altered the community composition of microbial organisms; this in turn, shifted the swamps' functions towards carbon emissions and methane production. Research by Cowley et al. (2018b) added credence to this trend, with disturbed swamps exhibiting a four-fold increase in methane production and carbon emissions compared to undisturbed upland swamps in the Blue Mountains. A swamps' hydrological function is also impacted with the increase of impermeable surfaces built as part of urban developments; as this can lead to higher volumes of runoff and increased water flow into swamp environments. This damages the integrity of swamp morphology from erosion and sedimentation, impacting the water storage and filtering ability of the ecosystem (Banaszuk & Kamocki, 2008; O'Driscoll et al., 2010).

The added stress of anthropogenic climate change further increases the vulnerability of freshwater ecosystems (Bush et al., 2012; Finlayson et al., 2013; Hughes, 2003). Research into the historical wetland-woodland boundaries of a mire wetland within Australia by Keith et al. (2010) found that there was a strong relationship with the availability of moisture and increasing size of mire wetlands. The study concluded that these systems were sensitive to fluctuations in moisture levels and that a reduction in size could be expected under current climate change projections. An assessment by Wrona et al. (2006) found the impacts of climate change were wide ranging with knock-on effects throughout the ecosystem, such as a decrease in suitable habitat, as well as disruption to food-web interactions and ecological function. Collectively, these threats damage the environment, decreasing the health and resilience of an ecosystem, and if left unchecked, can push them to the point of complete ecological collapse (Yule, 2010).

Upland swamps have been described as an ecological indicator due to their sensitivity to disturbances (Young, 2017). If a swamps ecological functions are impacted, it will have knock-on effects for the wider environment, reducing the amount of available water in the area as well as their filtering capacity (Freidman & Fryirs, 2015). By understanding the connection between upland swamps and the wider community, swamps could be used as an indicator ecosystem to measure and monitor the health of the wider environment.

1.2. Monitoring biodiversity

To effectively manage and protect upland swamps from the threats described above, it is essential to monitor them across temporal and spatial scales (Bunn et al., 2010; Bush et al., 2012). Assessing the biodiversity of an ecosystem is one approach for characterising the overall health of the environment (Hooper et al., 2012). Traditionally, monitoring and evaluating the biodiversity of a

given area often requires researchers with field-specific training and taxonomic expertise to identify various species of plant and animal life (Hopkins & Freckleton, 2002). These methods can be time consuming and limit a researcher's ability to detect sensitive subtle changes that could indicate larger ecological tipping points (Chariton et al., 2016).

Molecular tools provide a powerful solution for characterising ecological community structures. Environmental DNA (eDNA) has been shown to have great potential for biomonitoring ecosystems (Stat et al., 2017; Thomsen & Willerslev, 2015). Here, I define eDNA as the DNA of organisms obtained directly from environmental samples, such as water (Ficetola et al., 2008) or sediment (Chariton et al., 2010). DNA metabarcoding is one such technique used within the field of eDNA, which takes advantage of polymerase chain reaction (PCR) to amplify a short taxonomically informative strand of DNA that has conservative regions at the start and end of the sequence (Pompanon et al., 2011). The amplicons are then sequenced with high-throughput sequencing (HTS) to identify the genetic material from organisms that are present within a sample (Shokralla et al., 2012). As a technique, it is non-invasive, can be implemented with standardised sampling procedures, and be carried out over large landscapes within a relatively short time frame (Bohmann et al., 2014). This gives researchers the ability to obtain large amounts of data on the biodiversity of a given area (Dafforn et al., 2014; Yoccoz, 2012).

Implementing eDNA metabarcoding methods allows for the simultaneous characterisation of organisms across numerous taxonomic groups, as well as potentially inferring their relative abundance within a specific location (Andersen et al., 2011). Organisms that hold key roles in facilitating ecological functions, such as microorganisms, should be an essential part of monitoring programs to obtain a full biodiversity assessment of an area (Rosenberg, 2001). Microbial communities are critical for numerous key ecological functions within ecosystems. They are a fundamental food source at the base of all food-webs; drive the cycling of nutrients within an ecosystem; and hold key roles as decomposers and autotrophs (Sigee, 2005). The 16S ribosomal marker gene is commonly used in eDNA metabarcoding studies to target prokaryotic organisms, as it is highly conserved within Bacteria and Archaea (Caporaso et al., 2012; Weisburg et al., 1991). Numerous studies focusing on prokaryotic communities in wetland ecosystems have linked their diversity and community composition to local environmental variables finding they are highly sensitive to factors such as changes in moisture levels, pH and nutrients (Christiansen et al., 2019; Farías et al., 2014; He et al., 2014; Li et al., 2018). Another common taxonomic group to include in biodiversity monitoring programs are eukaryotic organisms, i.e.: plants, fungi and animals, as this group encapsulates a broad range of organisms within the environment. The 18S ribosomal marker

gene has been successfully used to target eukaryotic organisms from environmental samples (Hardy et al., 2010). Many studies within wetland ecosystems have focused on understanding how eukaryotic community composition changes across spatial scales (Baldwin et al., 2013; Banerji et al., 2018). This spatial information can be used to detection disturbances within an ecosystem as well as create appropriate management plans (Chariton et al., 2016).

The high resolution data gained from eDNA metabarcoding allows researchers to investigate the differences in community composition, structure and diversity across temporal and spatial scales (Civade et al., 2016). Incorporating eDNA metabarcoding into biodiversity assessments will allow for more comprehensive and adaptive management plans, that are able to protect and conserve vulnerable ecosystems (Thomsen et al., 2012).

1.3. Research aims and hypothesis

Here, I investigate the biodiversity and community structure of upland swamps in the Sydney Basin, comparing differences at local versus landscape scales. Using environmental DNA metabarcoding methods for sediment samples collected from upland swamps in the Sydney Basin, I:

1) characterise prokaryotic and eukaryotic components of biodiversity across three distinct swamp regions;
2) investigate the relationships between swamp biodiversity and community structure and local environmental variables; and
3) examine the spatial structuring of species diversity and composition within swamps, between swamps and across regions.

I hypothesise that prokaryotic community assemblages will have a greater response to local environmental conditions at smaller spatial scales compared to eukaryotic communities. I also expect that community differentiation by distance will be greater in eukaryotic communities compared to prokaryotic.

This research aims to provide new insight into the uniqueness of swamp biodiversity across multiple spatial scales, both within and between swamps and across distinct regions of the Sydney Basin. Collectively, this information can be used to assist in managing these unique and endangered ecosystems.

2. Methods

2.1. Study area

The upland swamps selected for this study have been classified as Endangered Ecological Communities within NSW and Australia (Biodiversity Conservation Act, 2016; Environment Protection and Biodiversity Conservation Act, 1999; Threatened Species Conservation Act, 1995). Three regions within the Sydney Basin (New South Wales, Australia) were selected for this study: The Blue Mountains (BM), Upper Nepean State Conservation Area (SCA) and Budderoo National Park (BNP) (Figure 3). All swamps included in this research were located within the Greater Sydney drinking water catchment and protected within their respected areas.

Rainfall data in the three months prior to sampling showed the BNP region received the greatest amount of rainfall with a total of 716mm, compared to SCA which received 573.8mm and BM with 467.6mm (Bureau of Meteorology, 2019). Climate data revealed all regions experienced similar ranges in temperature. SCA and BNP had an average maximum temperature of 22.2^{o}C and average minimum of 11.2^{o}C, while BM had an average maximum of 20.5^{o}C and average minimum of 10.7^{o}C (Bureau of Meteorology, 2019). Climate measurements for SCA and BNP regions were taken from the same station in Moss Vale due to their close proximity.

2.2. Swamp sites

Sampling was performed over a four week period between January-February 2019, during which nine swamps from three regions were sampled (Figure 3, Table 1 and Figure S1-S3 in Supplementary material). In each region, three swamps, hereafter referred to as sites, were selected to maximise their distance from urban development using environmental and spatial data produced by Fryirs and Hose (2016). To enable spatial comparisons within and between regions, the distance between sites was also taken into consideration, with 2 sites selected closer together than the third. In general, one pair of sites was between 1-2kms apart, with the third site approximately 3-6kms away from its nearest site.

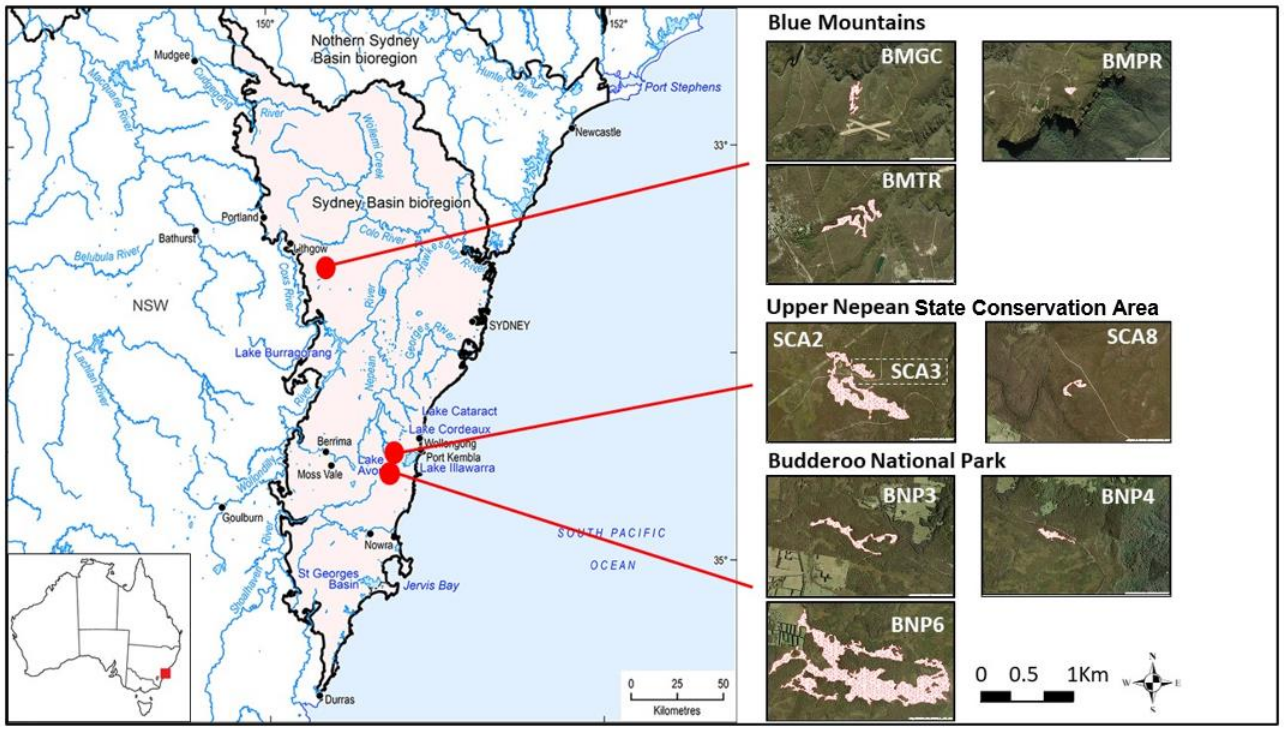

Figure 3: Study site locations within the Sydney Basin, New South Wales, Australia. Sites are outlined in red. Maps were created with ArcGIS software by Esri. Data on each swamps' spatial distribution was sourced from Fryirs and Hose (2016).

Table 1: GPS coordinates of study sites selected within each of the three regions.

Blue Mountains (BM)		Budderoo National Park (BNP)		State Conservation Area (SCA)	
BMGC	33°39'45.36"S, 150°19'10.20"E	**BNP3**	34°37'36.11"S, 150°40'45.54"E	**SCA2**	34°32'5.68"S, 150°38'48.12"E
BMPR	33°36'55.4"S, 150°19'36.1"E	**BNP4**	34°38'5.51"S, 150°41'44.79"E	**SCA3**	34°31'48.45"S, 150°38'54.77"E
BMTR	33°40'44.25"S, 150°17'38.07"E	**BNP6**	34°39'16.30"S, 150°41'44.11"E	**SCA8**	34°30'42.93"S, 150°34'26.71"E

2.3. Sample collection

Within each site, 3 x 9 meter plots were set up approximately 30 meters apart (Figure 4A). Plots were based on an L shape design, consisting of a 9 metre transect emanating in both a North-South direction and the other East-West (Figure 4B). At each plot, seven individual sediment samples of 100-200g were collected at a distance of 3 metres apart, from a surficial depth of approximately 5cm. Field controls were taken at each site by opening and exposing an empty sterile sample bag to the environment for the equivalent time required to take a sediment sample.

To prevent cross contamination during sample collection, new collection bags, gloves and sterilised spades were used for each individual sample. Spades were soaked in a 10% bleach bath for 2 hours each day, then rinsed multiple times with tap water and dried prior to reuse. In addition, care was also taken to avoid walking over sample collection areas.

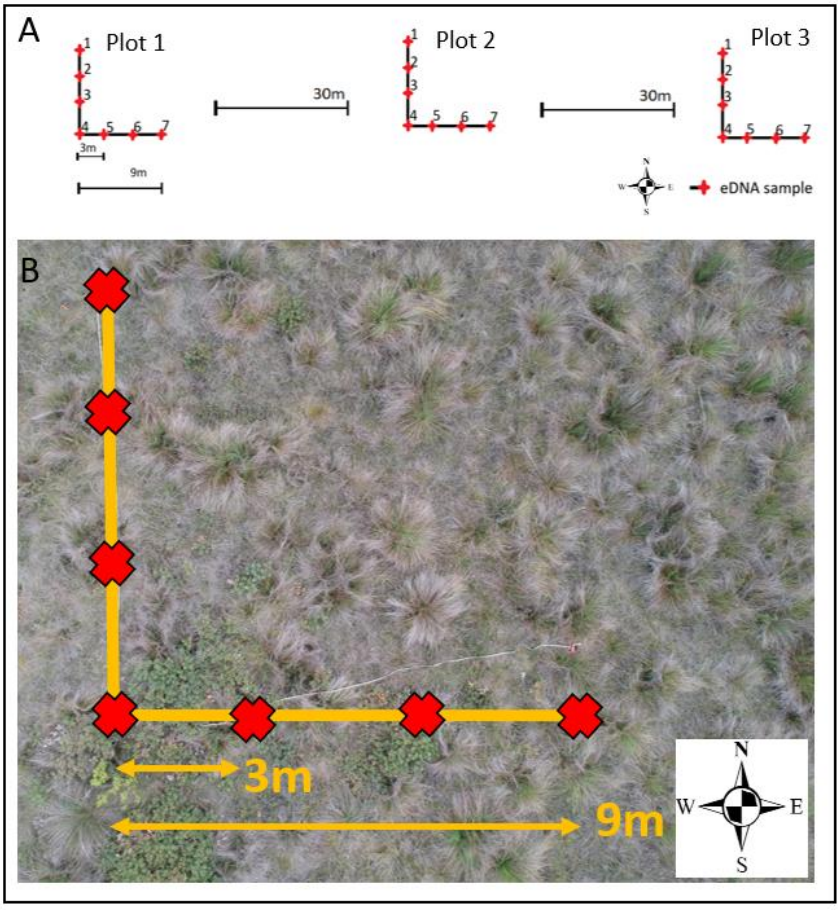

Figure 4: Sediment sample collection design. A) Three plots (1, 2, 3) were set up per site, with a 30m space between them. B) Seven samples were collected per plot, approximate locations illustrated by the red crosses, 1-3 taken in a North-South direction, 5-7 in an East-West direction and sample 4 the corner point.

Samples were transported to the field laboratory at room temperature where extractions were carried out the same day. A portion of each sample was stored separately at room temperature in airtight containers for transport to the laboratory, once there samples were then stored at -25^{o}C before sediment analysis was carried out.

2.4. DNA extraction

DNA extractions were carried out in the field within 8 hours of sample collection using a phosphate buffer extraction protocol derived from Taberlet et al. (2012). Briefly, 15g of sediment was added to 15mL of phosphate buffer solution (1.97g of NaH_2PO_4 + 14.7 g of Na_2HPO_4 for 1L of DNA free water) and mixed by hand for 15 minutes in a 50mL sterilised falcon tube. When sediment samples were overly dry, additional buffer was added to the mixture to ensure fluid consistencies. After shaking, 2mL of the supernatant was subsampled by pouring into a 2mL collection tube, then centrifuged for 10 minutes at 12,000g. The supernatant was then used as starting material for DNA extraction using the NucleoSpin Soil Kit (Macherey-Nagel, Düren Germany), following manufacturer's instructions with the exception of excluding the cell lysis stage. In practice, this means pipetting 800µL of the centrifuged supernatant into a 2mL collection tube with 400µL of SB binding buffer and then vortexing the mixture for 5 seconds. The DNA was then bound to a NucleoSpin Soil Column by filtering 600µL of the mixture through the column and centrifuging for 2 minutes at 10,000g. This step was repeated twice to filter all the sample through the spin column collection tube. The spin column was then washed with 500µL of SB binding buffer, 550µL of SW1 wash buffer and 650µL of SW2 wash buffer; at each stage the column was centrifuged for 2 minutes at 10,000g and flow through was discarded. Field extractions were paused at this stage, with the samples on the NucleoSpin Soil Columns secured in airtight containers containing silica gel for DNA preservation and stable transport at room temperature. Once in the laboratory, samples underwent a final wash with 650µL of SW2 buffer. The DNA was then eluted from the spin columns using 100µL SE buffer, centrifuged at 11,000g for 30 seconds. The final product was stored at -25^{o}C.

Field and laboratory control samples followed the same DNA extraction protocol as stated above. Field control sample collection bags were washed with the phosphate buffer solution and processed as described above, extraction controls consisted of 400µL of SB binding buffer and 800µL of phosphate buffer solution.

2.5. DNA amplification

Two DNA metabarcodes located on 16S rDNA V4 and 18S rDNA V7 gene regions were amplified to characterise prokaryotic and eukaryotic communities, respectively (details provided in sections 2.5.1. and 2.5.2.). To assess PCR inhibition and maximise DNA amplification (Murray et al., 2015), samples were initially subjected to quantitative PCR (qPCR) on a Lightcycler 480 (Roche Molecular Systems Inc, America), using non-tagged primers.

Positive controls for 16S and 18S rDNA were created to represent a mock community of seven to eight species that would not appear naturally within the study area. Using extracted DNA of known species and concentrations, three mixtures were created by varying the molarity of each DNA sample (Table S1 in Supplementary material). Given the ubiquity of many bacteria, a novel approach was used for the 16S rDNA positive controls. In this case, a synthetic oligonucleotide was created using a fungal sequence flanked by the conserved 3' and 5' prime regions of the 16S V4 (515F-806R). *In silico* testing of the sequence ensured that no represented hits were found on either GenBank or SILVA. The synthetic 16S rDNA sequence was developed and tested by Dr David Midgley, CSIRO, Australia (unpublished data). Based on the qPCR results, all samples were diluted 1:10 with DNA free water.

2.5.1. Prokaryote 16S rDNA amplification

The 16S rDNA V4 region for prokaryotic organisms (approximately 350bp) was amplified using the non-tagged primers 515F (5'-GTGYCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACNVGGGTWTCTAAT-3') (Caporaso et al., 2012). The qPCR mixture per sample for 16S rDNA included: 7.5µL AmpliTaq Gold 360 Master Mix (Life Technologies, Australia), 0.5µL Sybr Green Nucleic Acid Gel Stain (Qiagen, Germany), 0.3µL of non-tagged 16S forward primer at 0.2µM concentration, 0.3µL of non-tagged 16S reverse primer at 0.2µM concentration, 4.4µL of DNA free water and 2µL of DNA sample. 16S rDNA qPCR conditions were as following: initial denaturation at 95°C for 10 minutes, followed by 40 cycles of denaturing (94°C for 45 seconds), annealing (50°C for 60 seconds) and extension (72°C for 90 seconds), with a final extension period of 72°C for 10min.

Based on the 16S qPCR results, all samples were diluted 1:10 with DNA free water. Three 96 well plates were set up with diluted DNA samples, which included: 2 positive controls, 2 negative controls, 5-8 extraction controls and 84-41 samples per plate. Samples were arranged in a randomised pattern across plates to prevent systematic bias (Burns & Ellison, 2011). The plates were sent to the Ramaciotti Centre (University of New South Wales, Australia), for 16S V4 PCR

amplification and library prep for HTS. Amplicons were then purified and sequenced on an Illumina MiSeq (2 x 250 bp paired-end).

2.5.2. Eukaryote 18S rDNA amplification

Amplification of the 18S rDNA V7 region for eukaryotic organisms (approximately 160bp) was performed using the universal primers All18SF (5'-TGGTGCATGGCCGTTCTTAGT-3') and All18SR (5'-CATCTAAGGGCATCACAGACC-3') (Hardy et al., 2010). The qPCR mixture per sample for 18S rDNA non-tagged primer included: 7.5µL AmpliTaq Gold 360 Master Mix (Life Technologies, Australia), 0.5µL Sybr Green Nucleic Acid Gel Stain (Qiagen, Germany), 0.6µL of forward non-tagged 18S primer at 0.4µM concentration, 0.6µL of reverse non-tagged 18S primer at 0.4µM concentration, 3.8µL of DNA free water and 2µL of DNA sample. 18S rDNA qPCR conditions were as following: initial denaturation at 95°C for 10 minutes, followed by 35 cycles of denaturing (94°C for 60 seconds), annealing (50°C for 60 seconds) and extension (72°C for 90 seconds), with a final extension period of 72°C for 10min. Based on the 18S qPCR results, all samples were diluted 1:10 with DNA free water.

A 96 well plate was set up for tagged 18S rDNA PCRs with 73 samples and 23 controls, including: 12 blanks, 4 extraction controls, 4 PCR controls, and 3 positive controls. Samples were arranged in a randomised pattern across plates to prevent systematic bias (Burns & Ellison, 2011). PCRs for HTS were carried out in duplicates using a Mastercycler X50s (Eppendorf, Germany). For each reaction carried out, the mix included: 2µL of DNA sample, 1.6µL of DNA free water, 10µL of AmpliTaq Gold 360 Master Mix (Life Technologies, Australia), 3.2µL of a tagged forward fusion-primer and 3.2µL of a tagged reverse fusion-primer at a concentration of 0.4µM. 18S rDNA PCR conditions were as following: initial denaturation at 95°C for 10 minutes, followed by 35 cycles of denaturing (94°C for 60 seconds), annealing (50°C for 60 seconds) and extension (72°C for 90 seconds), with a final extension period of 72°C for 10min. Post-PCR products were tested on an agarose gel electrophoresis to ensure amplification was successful.

2.6. Eukaryote 18S rDNA purification and sequencing

A PicoGreen double stranded DNA (dsDNA) protocol (Life Technologies, Australia) and a plate reader (BMG Labtech, Germany) was used to measure the post-PCR concentrations of each sample with PHERAstar software (BMG Labtech, Germany). The concentration of each sample was measured twice. In brief, the PicoGreen protocol included the use of flat bottom black plates with a

mixture of 180µL TE buffer, 20µL of 1:10 diluted amplicon sample and 100µL of 200x dilution PicoGreen dsDNA reagent. The plate reader was calibrated prior to running each sample plate. Results were used to quantify the concentration of 18S amplicons and averaged across the duplicates. Using the results, samples were pooled in equimolarity into a sterilised 50mL falcon tube to create an 18S amplicon library of 200ng/µL.

Purification of the 18S amplicon library was carried out using 1080µL AMPure XP beads (Beckman-Coulter Life Sciences) mixed with 600µL of pooled amplicon sample. This mixture was incubated at room temperature for 10 minutes then placed on a magnetic separator for 10 minutes until the beads cleared. The supernatant was aspirated, then washed three times with 800µL of 70% ethanol, followed by 10 minutes of air drying. Beads were then mixed with 50µL DNA free water and left for 10 minutes on the magnetic separator. The supernatant was aspirated off and the sample concentration was verified on a Qubit® 3.0 Fluorometer (Thermo Fisher Scientific). The final amplicon library was diluted with DNA free water to obtain a concentration of 50ng/µL then sent to the Ramaciotti Centre (University of New South Wales, Australia) for Illumina MiSeq sequencing (2 x 250 bp paired-end).

2.7. Sediment analysis

Each sample was analysed for the following physical and chemical properties: moisture content (%), organic matter (%), total organic carbon (%), hydrogen (%) and nitrogen (%) nutrient content, pH and electrical conductivity (µS/cm). To determine the moisture content, samples were dried in an oven at 80^{o}C for 48hrs. Total organic matter was determined by loss on ignition (Heiri et al., 2001), a subsample of dried sediment was placed in a muffle furnace, at 550^{o}C for 5 hours. Samples were weighed before and after drying for both procedures to obtain the relevant moisture and organic matter content as a percentage of the dry weight.

For each sample, pH and electric conductivity were determined using a mixture of dried sediment and deionized water in a 1:5 weight to volume ratio, which was then placed on a rotative shaker for 1 hour to homogenise the mixture. pH was measured using a pH 3310 meter (WTW, Xylem Analytics, Australia) as per manufacturing instructions and electric conductivity measurements were taken using an Aqua-Conductivity meter (TPS Pty Ltd, Australia). Total carbon, hydrogen and nitrogen content were analysed using approximately 1.5-4mg of dried sediment material and processed in a LECO 900 analyser as per manufacture instructions (Isomass Scientific Inc., Calgary Canada) with regular calibration and checking of standards taking place throughout the analysis.

2.8. Bioinformatics and taxonomic assignment

Metabarcoding data received from the Ramaciotti Centre (University of New South Wales, Australia) was run through the Greenfield Hybrid Analysis Pipeline (GHAP) v2.1, created by CSIRO Australia (available at https://doi.org/10.4225/08/59f98560eba25). The GHAP pipeline utilises USearch tools (Edgar, 2013) (available at http://drive5.com/Usearch) along with additional taxonomic classification tools, specific to each amplicon, to cluster and classify sequences. This pipeline generates a table of Operational Taxonomic Units (OTUs) with taxonomic classifications and species assignments along with specified read counts for each sample.

The script runs through the following steps: 1) demultiplexing MiSeq data by assigning reads to their original sample ID based on known tagged primer pairs; 2) if necessary reads are trimmed to remove poor quality tail regions (i.e., with an Illumina base call quality score <25); 3) merge overlapping areas of paired reads using the *fastq_mergepairs* command from Usearch; 4) dereplicate reads with the command *fastx_uniques*, a step designed to reduce processing identical reads; 5) removing sequences that are outside the expected range length (i.e., 245-255bp for 16S and, 130-160bp for 18S); 6) cluster sequences into OTUs with a 97% similarity threshold using *cluster_otus*, this command also carries out chimera checking; 7) filter non-target OTUs as well as PCR artefacts and 8) classification of OTUs.

Classification of 16S rDNA OTUs used two independent tools: the RDP Naïve Bayesian Classifier - Ribosomal Database Project (Cole et al., 2013) (available at http://rdp.cme.msu.edu/); and the *usearch_global* command to improve the confidence of taxonomic assignment. The RDP 16S Training Set is supplemented with data from the RefSeq 16S to potentially allow for species level identification. The minimum confidence level for RDP to assign taxonomic classification is 0.5, while the *usearch_global* command searches for matches between OTUs and the reference set. 18S rDNA OTUs were classified by BLASTing sequences using the *ublast* command from Usearch, against a curated set of sequences derived from the SILVA v128 SSU reference set (Quast et al., 2013). Taxonomical assignment was set using command *taxoBLASTCutoffs*, with the following BLAST similarity cut-offs: phylum 0.77, class 0.8, order 0.85, family 0.9, genus 0.95, species 0.97. For each taxonomic level, if the assignment score was below the cut-off threshold, the OTU was unassigned at that specific level.

Filtering 16S rDNA and 18S rDNA OTU tables after the GHAP pipeline was conducted in R version 3.5.1 (R_Core_Team, 2018) using tools from the R package *vegan* (Oksanen et al., 2019). The in-house script carried out the following steps: 1) normalise data against positive controls; first

by identifying the positive controls, and then removing any OTUs found equal or below the positive control in each sample, i.e. to remove potential tag-jumping sequences; 2) removal of OTUs with a total count < 10 reads in the entire dataset; 3) for 16S OTUs only, removal of OTUs from Eukaryotic organelles (chloroplast and mitochondria); 4) removal of OTUs that were unassigned at the kingdom level, 5) rarefaction of count data (the minimum read count value used to rarefy data for 16S was 23,839 and 18S was 2,069); and 6) convert rarefaction data to relative abundance.

2.9. Statistical analysis

Data exploration and analysis was conducted in R version 3.5.1 (R_Core_Team, 2018).

2.9.1. Physio-chemical attributes of sites

Physical and chemical variables were recorded for each sample. A one way analysis of variance (ANOVA) test followed by a Tukey HSD post hoc was conducted to explore if there were any significant differences within and between regions and sites, and, if so, where the differences lie, respectively.

A log transformation was performed on site area and elevation prior to a Principal Component Analysis (PCA) to reduce the data skewness due to the large variation of values. A PCA of physical and chemical factors was carried out using function *dudi* and *fviz_pca_biplot* from the R package *ade4* (Dray & Dufour, 2007). A Pearson's correlation coefficient test was performed between all factors to identify collinear variables; variables which were significantly correlated ($P < 0.05$) and had a high correlation coefficient value ($r^2 > 0.80$) were removed from subsequent analysis.

2.9.2. eDNA data

The effectiveness of the experimental design and sample collection to capture site diversity was explored with a series of species accumulation curves, using the function *specaccum* from the R package *vegan* (Oksanen et al., 2019). This was also used to estimate the potential number of species that could be found in a site (Chao 1).

2.9.2.1. Diversity analysis

Rarefied 16S and 18S rDNA data was used to calculate ecological univariate indices using tools from the R package *vegan* (Oksanen et al., 2019), specifically calculating OTU Richness, Shannon Wiener Diversity Index (Equation 1) (Shannon, 1948) and Pielou's Evenness Index (Equation 2) (Pielou, 1966) at the region, site and plot level.

$$H = \sum_{i=1}^{k} p_i log\ (p_i) \quad (1)$$

$$J' = \frac{H}{H_{max}} \quad (2)$$

Where k is the total number of OTUs in the dataset, i indicates a specific OTU within the dataset, p_i is the proportion of reads belonging to the i^{th} OTU compared to the total number within the dataset. H is the Shannon Wiener Diversity and H_{max} is the maximum value of H.

A Kruskal-Wallis test followed by a Dunn multiple comparison test were conducted to explore if there was any significant difference in OTU Richness, Shannon Wiener Diversity and Pielou's Evenness Index within and between regions and sites, and, if so, where the differences lie, respectively.

2.9.2.2. Community composition

Prior to carrying out community analysis, both 16S and 18S rDNA datasets were transformed with a Hellinger transformation. The community composition of each site was explored at several taxonomic levels, with bar plots to visualise the differences. A non-metric multi-dimensional scaling (nMDS) analysis was performed to visualise dissimilarity patterns between the sites, using the Bray-Curtis dissimilarity index with the function *vegdist* from the R package *vegan* (Oksanen et al., 2019). Statistical differences in OTU community composition between and within regions, were explored using permutational multivariate analysis of variance (perMANOVA). For this, initially the dissimilarity between pairs of sample using Bray-Curtis distance was computed, and then used the *adonis* function from the R package *vegan* (Oksanen et al., 2019) to conduct the perMANOVA (based on 999 permutations), with 'sites' nested within 'regions', and 'plots' nested within 'sites'.

2.9.2.3. Relationship between communities and environmental variables

The relationship between environmental variables and OTU data was analysed with a distance-based Redundancy Analysis (dbRDA) with the function *dbrda* from R package *vegan* package (Oksanen et al., 2019) using the Bray-Curtis dissimilarity index.

2.9.2.4. Multi-scale spatial analysis of community assemblages

The proportion of variation within and between region, site and plot level were calculated using a variance component analysis with a two factor nested design (Quinn & Keough, 2002). A distance-decay analysis was carried out to examine if there was any correlation between community dissimilarity, using Bray-Curtis dissimilarity index and geographical distance. This analysis was performed across regions, within regions and within sites. An ANOVA was used to determine the R^2 value as an indication of the strength of the relationship between geographical distance and community dissimilarity.

3. Results

3.1. Physio-chemical attributes of sites

The physical and chemical properties of each site is shown in Table 2. Recorded pH levels of all sediment samples were classified as acidic, with a limited range between 3.20-4.75. The pH values of the sediment samples differed between regions (ANOVA: F = 14.09, $P < 0.001$). The pH of the sediment was significantly lower ($P < 0.001$) in the BNP region (pH = 3.87, SD = 0.27, min = 3.56, max = 4.75), than both SCA (pH = 4.06, SD = 0.23, min = 3.66, max = 4.59), and BM (pH = 4.04, SD = 0.24, min = 3.20, max = 4.57) regions (Table 2). At a site level there was no significant difference between BNP sites (ANOVA: F = 0.46, $P = 0.64$). SCA sites were significantly different to each other (ANOVA: F = 74.09, $P < 0.001$), with SCA8 having a higher pH value than both SCA2 and SCA3 ($P < 0.001$). A test of BM sites (ANOVA: F=3.12, $P = 0.05$), found BMTR had a lower pH value compared to BMPR ($P = 0.04$).

Soil moisture content differed between the regions (ANOVA: F = 65.39, $P < 0.001$). The BM region was drier ($P < 0.001$), than the other two regions (mean = 21.52%, SD = 12.67) (Table 2). Within the SCA region, SCA3 was wetter (mean = 72.83%, SD = 8.80) than SCA2 and SCA8 ($P < 0.001$). Soil moisture content varied within all sites in the BNP region ($P < 0.001$), with BNP4 having the greatest moisture content (mean = 71.62%, SD = 10.78) and BNP3 having the smallest (mean = 29.98%, SD = 3.00).

There were differences in the elevation of each region, (ANOVA: F = 6094, $P < 0.001$). On average, the BM sites had the highest elevations (mean = 989.92m, SD = 9.71m, min = 974.00m, max = 1011.00m), with the SCA (mean = 598.43m, SD = 22.55m, min = 578.00m, max = 630.00m) and BNP sites (mean = 617.05m, SD = 19.56m, min = 584.00m, max = 659.00m) being at similar elevations (Table 2).

The area of sites varied between (ANOVA: F = 19.72, $P < 0.001$) and within regions (ANOVA: F = $2.2*10^{29}$, $P < 0.001$). Site BNP6 was the largest site overall with an area of 1.79km^2 and BMPR was the smallest site with an area of 0.02km^2. The BNP region had the largest sites on average (mean = 0.68km^2, SD = 0.97km^2), followed by the SCA region (mean = 0.27km^2, SD = 0.26km^2) and BM (mean = 0.10km^2, SD = 0.09km^2) (Table 2).

Table 2: Summary of physical and chemical properties of each site, values were averaged per site (n=21). Values in parentheses are standard deviation. Variables; Elev: Elevation (m); SM: Soil moisture as a percentage of dry weight (%); OC: Organic content as a percentage of dry weight (%); EC: Electrical conductivity (µS/cm); C: Total Carbon content (%); H: Total Hydrogen content (%); N: Total Nitrogen content (%); C:N: Carbon to Nitrogen ratio.

Site	Area (km^2)	Elev (m)	SM (%)	OC (%)	pH	EC (µS/cm)	C (%)	H (%)	N (%)	C:N
BMGC	0.07	977	12.52 (3.02)	11.29 (3.11)	4.04 (0.14)	75.51 (28.74)	3.43 (1.79)	0.33 (0.21)	0.19 (0.07)	18.05
BMPR	0.02	994	13.39 (2.79)	8.88 (2.22)	4.14 (0.19)	74.11 (15.90)	2.27 (0.94)	0.23 (0.10)	0.14 (0.04)	16.21
BMTR	0.20	999	39.82 (18.36)	49.11 (36.37)	3.96 (0.32)	200.07 (108.93)	24.35 (19.45)	2.30 (2.15)	0.79 (0.59)	30.82
BNP3	0.18	597	29.98 (3.00)	15.24 (4.26)	3.89 (0.11)	108.97 (23.10)	8.56 (6.89)	0.78 (0.88)	0.32 (0.27)	26.75
BNP4	0.05	642	71.62 (10.78)	68.01 (21.11)	3.89 (0.35)	214.72 (71.48)	31.14 (12.66)	3.46 (1.66)	1.15 (0.50)	27.08
BNP6	1.79	612	49.53 (20.46)	30.26 (20.36)	3.83 (0.15)	201.10 (102.13)	14.46 (13.46)	1.27 (1.34)	0.59 (0.54)	24.51
SCA2	0.55	582	54.73 (18.15)	57.59 (20.07)	3.94 (0.13)	306.58 (98.91)	26.44 (8.97)	2.86 (1.81)	1.26 (0.35)	20.98
SCA3	0.21	584	72.83 (8.80)	85.08 (14.01)	3.92 (0.12)	456.66 (173.71)	38.36 (4.68)	4.07 (0.66)	1.34 (0.18)	28.63
SCA8	0.04	630	45.74 (9.49)	46.18 (9.34)	4.34 (0.12)	187.40 (63.98)	22.36 (4.03)	2.33 (0.45)	1.15 (0.25)	19.44

A PCA of physical and chemical factors was able to explain 81.8% of variation on the first two axes. Soil moisture, organic content, electrical conductivity, total Carbon, Hydrogen and Nitrogen content were the main drivers on the first axes, explaining a large proportion of variance (63.9%) (Figure 5). This axis groups sites SCA3, BNP4 and SCA2 as having high values of soil moisture, and organic content along with the other variables which contribute to this axis. The second axes of the PCA explained 17.9% of the variance and was driven by pH, site area, elevation and C:N ratio. Two BM sites, BMPR and BMGC, were grouped together with high elevation and low soil moisture. The influence of pH is shown by separating SCA8 with high pH from BNP6 and BNP3. Site area is also a contributing factor to this axis with the largest site BNP6 on opposite ends with SCA8, one of the smallest sites.

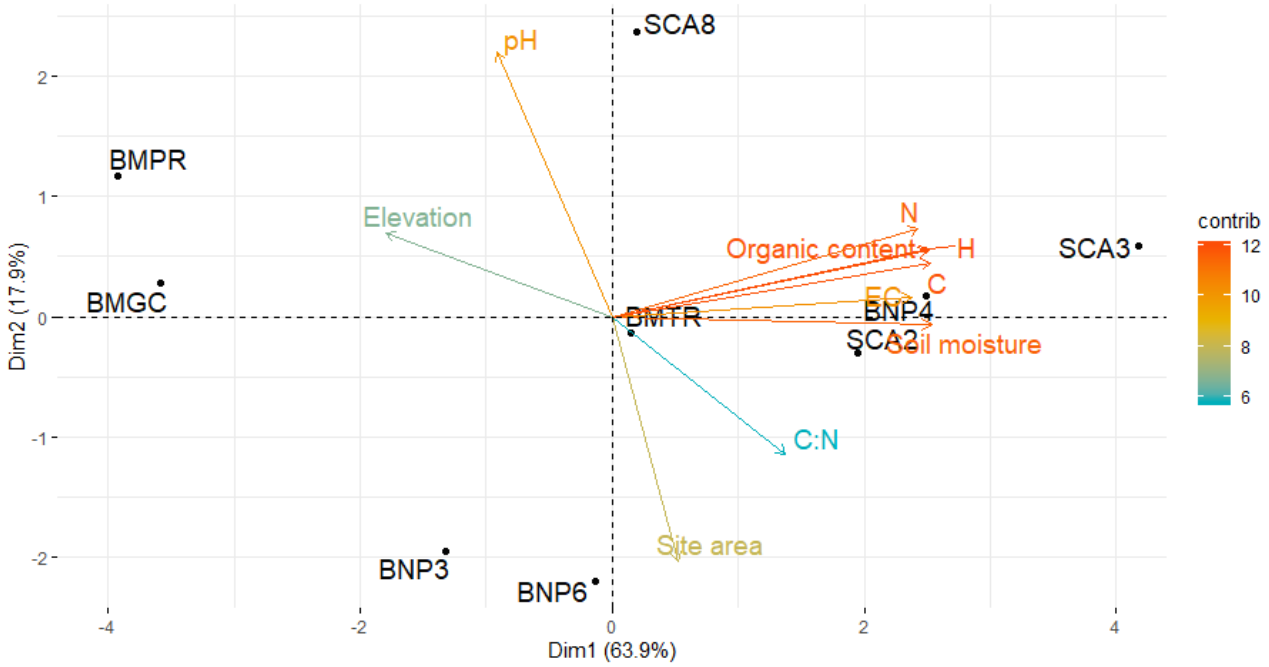

Figure 5: Principal component analysis (PCA) biplot of physical and chemical variables. The contribution of each variable to the two axes is displayed through the range of colours in the legend. Dim1 captures the most variation of the PCA at 63.9%, Dim2 captures 17.9% of the variation.

Pearsons' correlations identified several collinear variables; soil moisture, organic content, electric conductivity, carbon, hydrogen, and nitrogen. These variables were significantly correlated ($P < 0.05$) and had a high correlation percentage ($r^2 = 0.84\text{-}0.99$). The PCA indicated soil moisture was the strongest contributing factor, as such variables that were collinear to soil moisture were excluded from subsequent analysis.

3.2. Prokaryotic communities

The 16S rDNA sequencing run resulted in ~12 million reads before filtering. Subsequent to bioinformatics processing and taxonomic assignment the dataset contained ~10.8 million reads, with further filtering resulting in ~9.9 million reads capturing 11,770 OTUs prior to rarefaction. Two samples were removed due to their low number of reads (<10); specifically, BNP6_A1 and BNP3_B2, resulting in 187 samples included in the 16S rDNA analysis. The final rarefied dataset contained ~7.85 million reads encompassing 11,209 OTUs, with an average read count of ~42,000 per sample. The filtered 16S rDNA data, hereafter will be referred to as prokaryotic data.

The effectiveness of our experimental design and sample collection to capture the overall diversity of each site was tested with a series of accumulation curves (Figure 6). The curves showed a steady increase in the number of OTUs between 1-10 samples that gradually lessened across all sites with increasing sample size. Collecting 21 samples accounted for approximately 64% of estimated OTUs per site (Chao1) (Table S2 in Supplementary material).

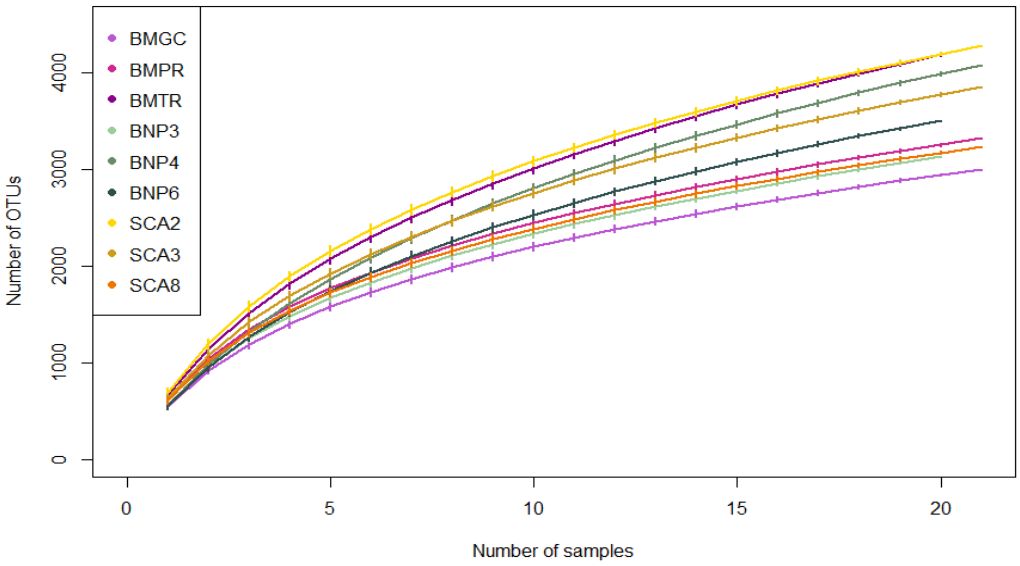

Figure 6: Accumulation curve for prokaryotic Operational Taxonomic Units (OTUs) recorded per site, each site consisted of 21 samples, with the exception of BNP6 and BNP3, which had 20 samples. BM – Blue Mountains, BNP – Budderoo National Park, SCA – Upper Nepean State Conservation Area.

3.2.1. Diversity of prokaryotic OTUs

At a regional level, SCA had slightly higher mean values of OTU richness, Shannon diversity and Pielou's evenness compared to BM and BNP (Figure 7 and Table S3 in Supplementary material). The BM region showed high variation for OTU richness counts compared to the other two regions (Figure 7A). Shannon diversity was relatively similar between the three regions ranging between 6.91-6.98 on average (Figure 7B) but showed higher variation in the SCA region. Pielou's evenness was similar for all regions, ranging between 0.845-0.847 (Table S3 in Supplementary material). Collectively, no difference in univariate indices were detected between the three regions (Kruskal-Wallis test: Chi square = 0.27, df = 2, P = 0.88 for OTU richness, Chi square = 0.36, df = 2, P = 0.84 for Shannon diversity, and Chi square = 0.36, df = 2, P = 0.84 for Pielou's evenness). A Dunns *post hoc* test of pairwise comparisons between regions showed there was no significance for any of the three measurements (P > 0.7).

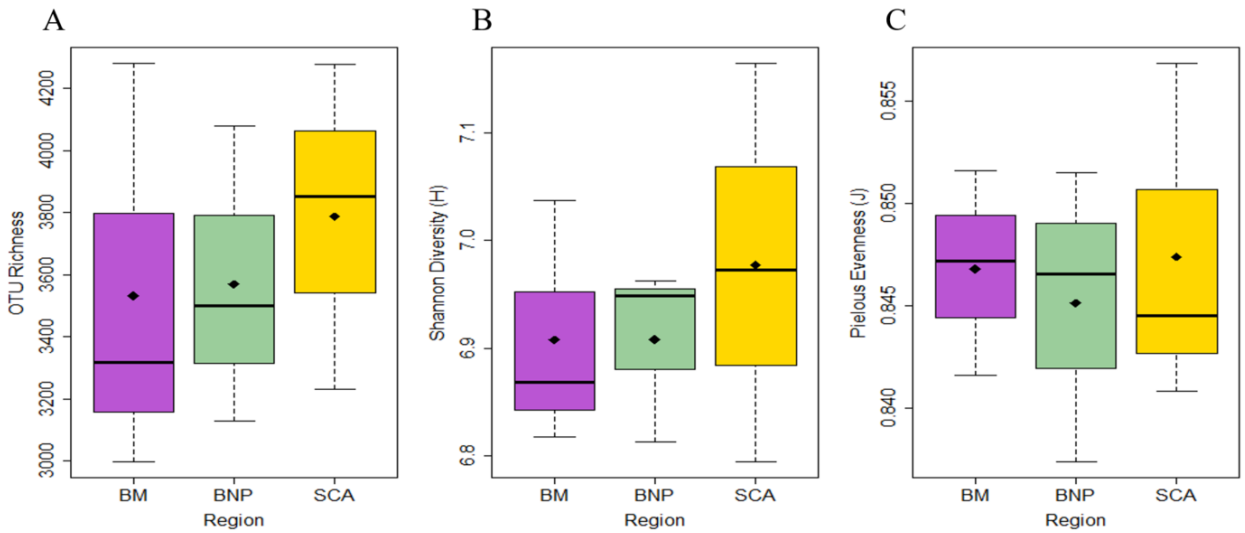

Figure 7: Boxplots of prokaryotic communities based on site averages per region; A) Operational Taxonomic Unit (OTU) richness, B) Shannon diversity (H), C) Pielou's evenness (J). Black lines within the coloured boxed indicate median values and black diamonds indicate the mean. Top lines of the boxes are the 75th percentile, bottom lines of the boxes are the 25th percentile, and whiskers show maximum and minimum values. BM – Blue Mountains, BNP – Budderoo National Park, SCA – Upper Nepean State Conservation Area.

At a site level no differences were identified, across the three ecological indices (Kruskal-Wallis test: Chi square = 10.12, df = 8, P = 0.26 for OTU richness, Chi square = 5.34, df = 8, P = 0.72 for

Shannon diversity, and Chi square = 14.91, df = 8, *P* = 0.06 for Pielou's evenness) (Figure 8 and Table S4 in Supplementary materials). A Dunns *post hoc* test of pairwise comparisons across the three ecological indices between sites was not significant (*P* > 0.6 for OTU richness, *P* = 0.9 for Shannon diversity, and *P* = 0.2 for Pielou's evenness).

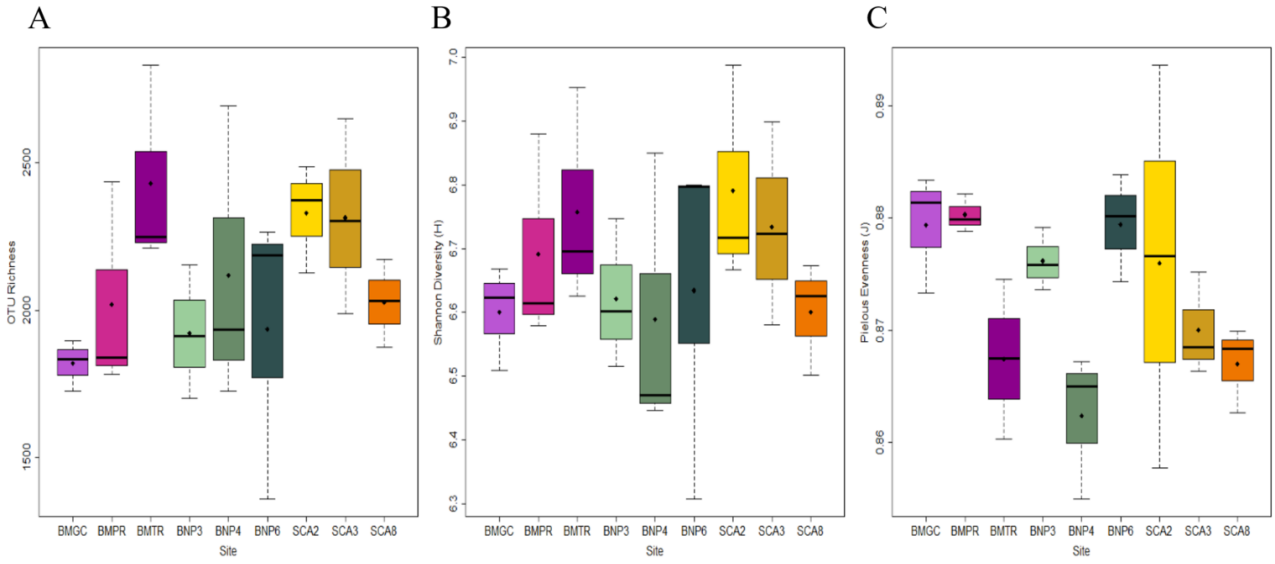

Figure 8: Boxplots of prokaryotic communities based on plot averages per site; A) Operational Taxonomic Unit (OTU) richness, B) Shannon diversity (H), C) Pielou's evenness (J). Black lines within the coloured boxed indicate median values and black diamonds indicate the mean. Top lines of the boxes are the 75th percentile, bottom lines of the boxes are the 25th percentile, and whiskers show maximum and minimum values. BM – Blue Mountains, BNP – Budderoo National Park, SCA – Upper Nepean State Conservation Area.

3.2.2. Prokaryotic community composition

Prokaryotic data consisted of an overwhelming majority of reads assigned to the kingdom Bacteria 99.88%, with Archaea making up the additional 0.12%. The distribution of reads within phyla were similar across regions and sites (Figure 9). The top 10 phyla accounted for approximately 90% of all reads. With dominant phyla including Proteobacteria, Acidobacteria, Verrucomicrobia Planctomycetes and Actinobacteria.

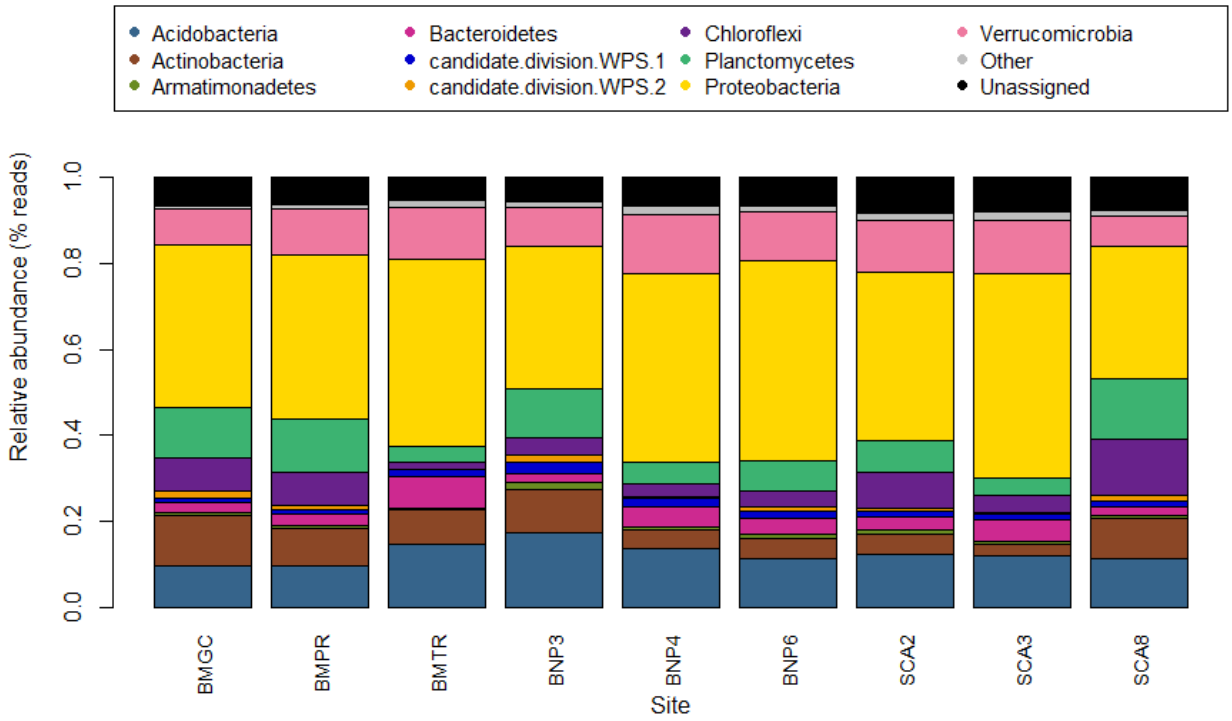

Figure 9: Taxonomic distribution of prokaryotic reads within top ten phyla per site. All phyla that were not within the top ten groups were included in the Other category, Operational Taxonomic Units that were not assigned at the phyla level were classified as Unassigned.

The composition of the prokaryotic communities differed between (perMANOVA: F = 10.75, $P < 0.001$) and within regions (perMANOVA: F = 5.24, $P < 0.001$). A *post-hoc* pairwise test found all regions and sites contained different prokaryotic community assemblages ($P < 0.001$). This is further illustrated by the nMDS ordination plot which showed the prokaryotic assemblages from each region and site had substantial overlaps in composition (Figure 10). Both the BM and SCA samples tended to group together within their region, and strong clustering is seen with sites BMPR and SCA3.

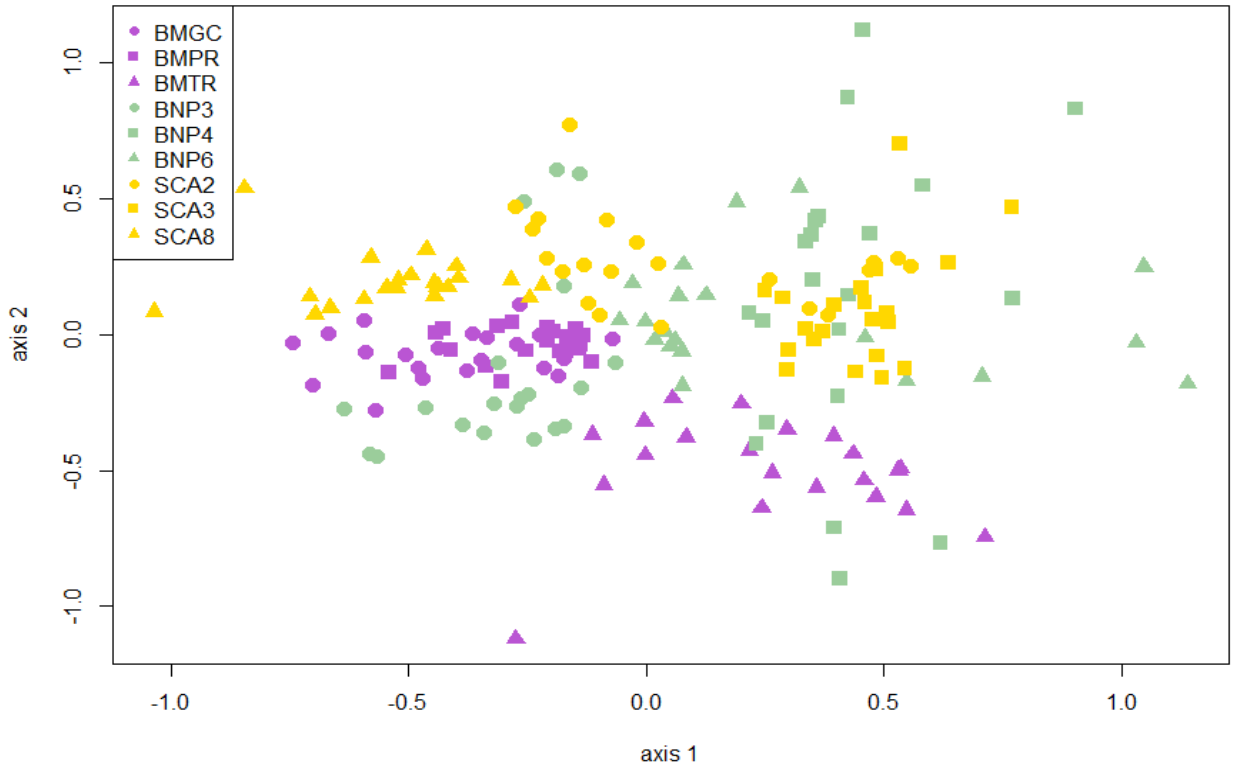

Figure 10: Non-metric multidimensional scaling (nMDS) ordination plot of prokaryotic communities illustrating composition dissimilarity across regions and sites (stress = 0.174). Each point represents a single sample, points that are closer together are similar in their community composition. BM – Blue Mountains, BNP – Budderoo National Park, SCA – Upper Nepean State Conservation Area.

3.2.3. Relationship between prokaryotic communities and environmental variables

A dbRDA analysis based on five environmental variables explained 18.37% of the total variation in the prokaryotic communities (Figure 11). The first two axis explained 15.9%, with the first axis explaining 11.3% of total variation influenced by site area and soil moisture. The second axis explained 4.6% of total variation with BM sites separated by elevation. Site area (9.78%, $P < 0.001$), elevation (3.60%, $P < 0.001$) and soil moisture content (3.44%, $P < 0.001$) were the largest contributing factors to the dbRDA, while pH (0.96%, $P = 0.003$) and C:N ratio (0.60%, $P = 0.98$) only explained a small amount of biotic variation.

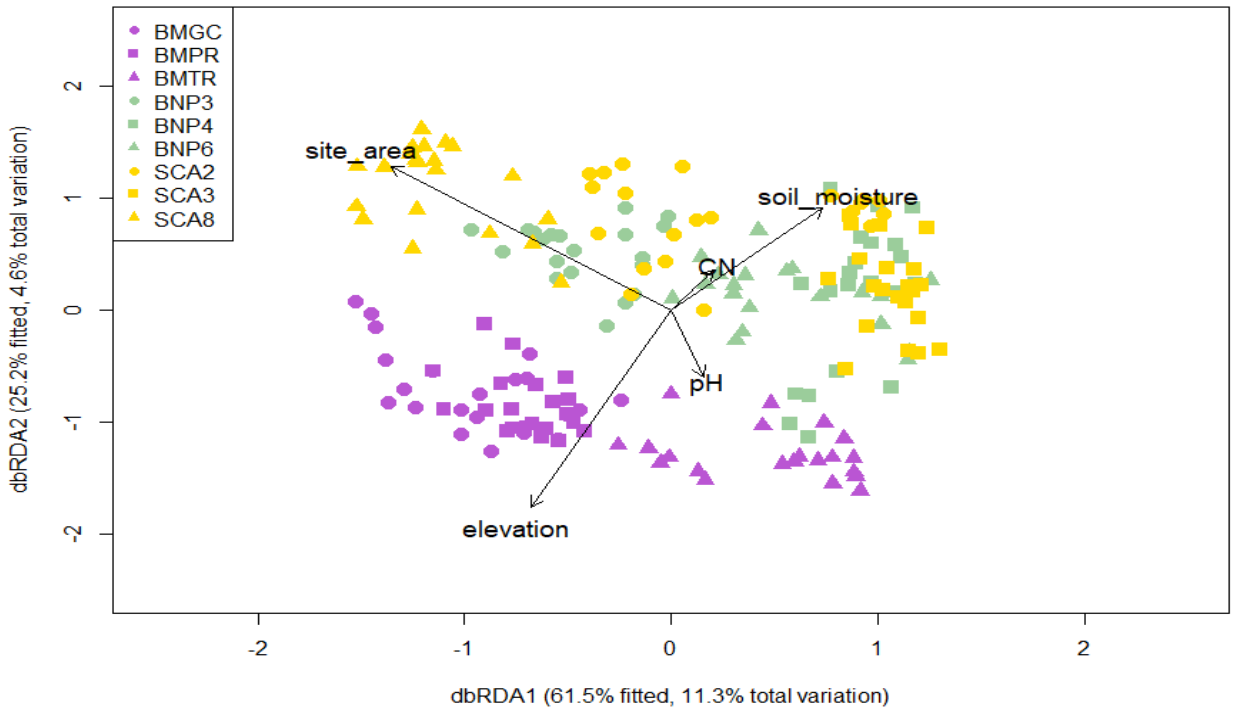

Figure 11: A distance-based redundancy analysis (dbRDA) ordination plot showing the relationship between prokaryotic communities and five environmental variables: site area, elevation, soil moisture content, pH and C:N ratio. BM – Blue Mountains, BNP – Budderoo National Park, SCA – Upper Nepean State Conservation Area.

3.2.4. Multi-scale spatial analysis of prokaryotic community assemblages

A variance component analysis indicated that the majority of variation occurred at the sample level, residuals 60% (Table S5 in Supplementary material). Sites explained a quarter of the variability of samples (25%), with plots explaining 10% and regions having a minimal 4% impact on variation.

Prokaryotic communities across regions became more dissimilar with increasing geographic distance ($F = 2667.4$, $R^2 = 0.133$, $P < 0.001$) (Figure 12). Within all regions, prokaryotic communities differed significantly from each other: BM ($F = 1086$, $R^2 = 0.357$, $P < 0.001$), BNP ($F = 342.5$, $R^2 = 0.157$, $P < 0.001$) and SCA ($F = 1224$, $R^2 = 0.385$, $P < 0.001$) (Figure 13). When comparing points sampled within sites, five out of nine sites showed significant differences ($P < 0.05$) in prokaryotic communities with increasing geographic distances (Figure S4 in Supplementary materials). There was no regional pattern in respect to where the community differences were found, but two sites had high correlation coefficient values; SCA2 ($R^2 = 0.52$, $P < 0.001$) and BNP4 ($R^2 = 0.23$, $P < 0.001$) (Figure S4 in Supplementary materials).

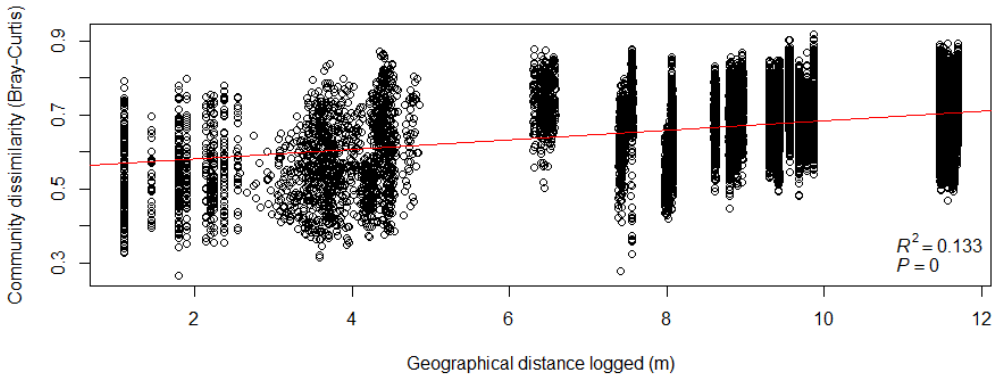

Figure 12: Prokaryotic community differentiation by distance plot across all regions, regression line in red showing adjusted R^2 value.

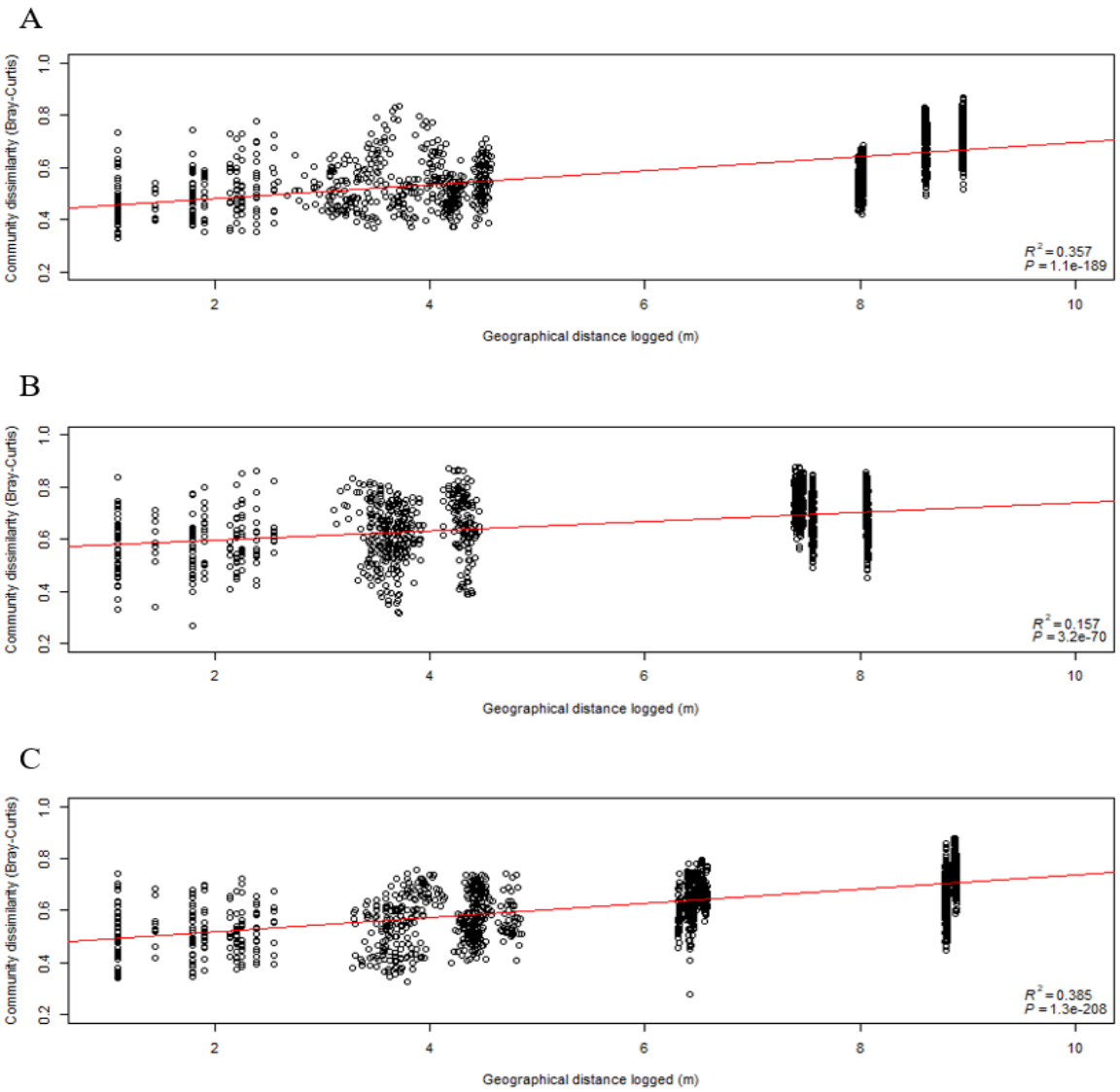

Figure 13: Prokaryotic community differentiation by distance plot for each individual region, regression line in red showing adjusted R^2 value. A) Blue Mountains region (BM), B) Budderoo National Park region (BNP), C) Upper Nepean State Conservation Area region (SCA).

3.3. Eukaryotic communities

The 18S rDNA sequencing run resulted in ~17 million reads before filtering, containing 189 samples, all sequenced successfully. Due to stringent parameters within the bioinformatics process and taxonomic assignment stages the dataset contained ~1.2 million reads, with further filtering resulting in ~920,000 reads capturing 1,395 OTUs prior to rarefaction. The final rarefied dataset contained 405,096 reads encompassing 1,334 OTUs with an average read count of ~2100 per sample. The filtered 18S rDNA data, hereafter will be referred to as eukaryotic data.

The effectiveness of our experimental design and sample collection to capture the overall diversity of each site was tested with a series of accumulation curves (Figure 14). The curves showed a steep increase between 1-5 samples then continues in a slower upward trend across all sites with increasing sample size. Collecting 21 samples per site accounted for approximately 67% of estimated OTUs (Chao1) (Table S6 in Supplementary material).

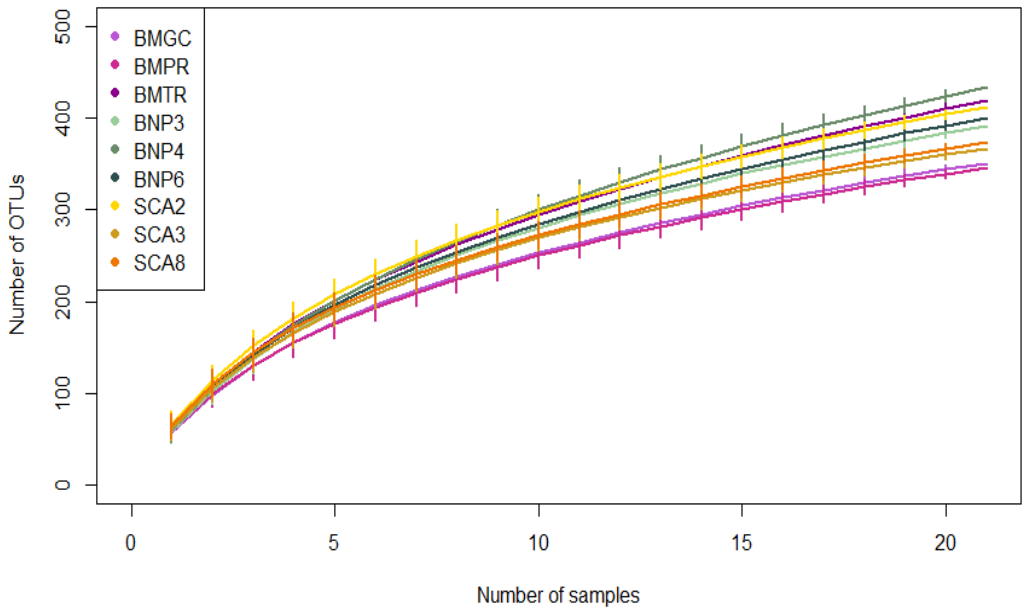

Figure 14: Accumulation curve for eukaryotic Operational Taxonomic Units (OTUs) recorded per site, each site consisted of 21 samples. BM – Blue Mountains, BNP – Budderoo National Park, SCA – Upper Nepean State Conservation Area.

3.3.1. Diversity of eukaryotic OTUs

At a regional level, BNP had slightly higher mean values of OTU richness and Shannon diversity values compared to BM and SCA (Figure 15 and Table S7 in Supplementary material). The BM region displayed great variation of OTU richness and Pielou's evenness (Figure 15). All three regions had similar values of Pielou's evenness, with average values of 0.862-0.863 (Figure 15C). However, there were no differences for any of the measured univariate indices between the three regions (Kruskal-Wallis test: Chi square = 1.87, df = 2, P = 0.39 for OTU richness, Chi square = 2.22, df = 2, P = 0.33 for Shannon diversity, and Chi square = 0.36, df = 2, P = 0.84 for Pielou's evenness). A Dunns *post hoc* pairwise comparisons test between regions was not significant (P > 0.5 for OTU richness, P > 0.4 for Shannon diversity, and P > 0.7 for Pielou's evenness).

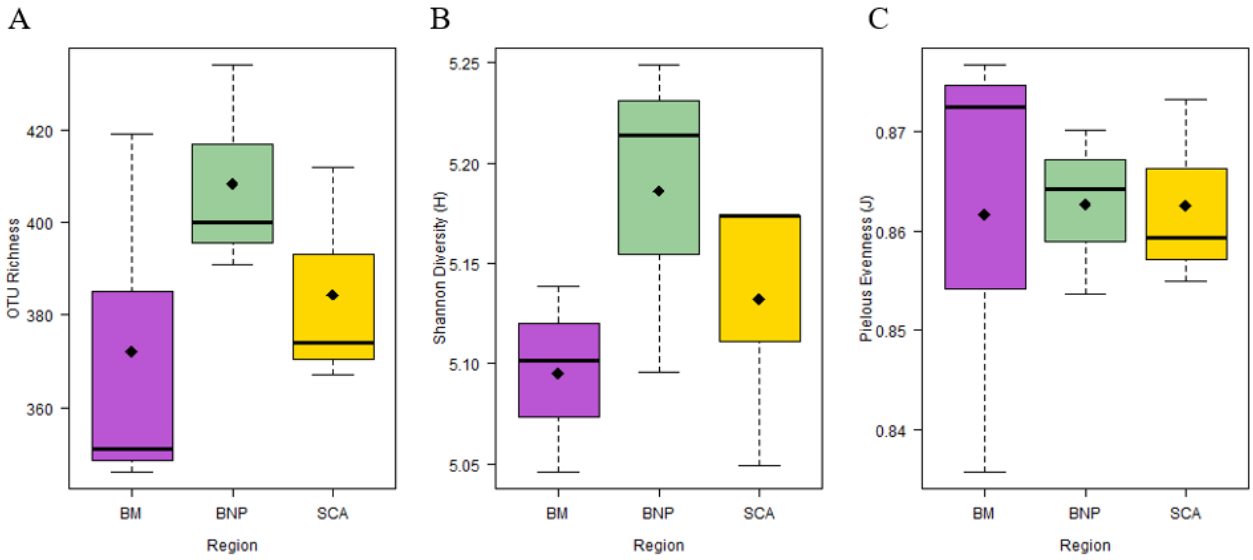

Figure 15: Boxplots of eukaryotic communities based on site averages per region; A) Operational Taxonomic Unit (OTU) richness, B) Shannon diversity (H), C) Pielou's evenness (J). Black lines within the coloured boxed indicate median values and black diamonds indicate the mean. Top lines of the boxes are the 75th percentile, bottom lines of the boxes are the 25th percentile, and whiskers show maximum and minimum values. BM – Blue Mountains, BNP – Budderoo National Park, SCA – Upper Nepean State Conservation Area.

At a site level, there is a consistent pattern for BNP and SCA sites across the three ecological indices (Figure 16 and Table S8 in Supplementary materials). Within the BM region, site BMTR exhibited altering values compared to BMGC and BMPR. However, none of the differences between sites were significant (Kruskal-Wallis test: Chi square = 9.55, df = 8, P = 0.30 for OTU

richness, Chi square = 6.21, df = 8, *P* = 0.62 for Shannon diversity, and Chi square = 12.22, df = 8, *P* = 0.14 for Pielou's evenness). A Dunns *post hoc* pairwise comparisons test between regions was not significant (*P* > 0.49 for OTU richness, *P* > 0.92 for Shannon diversity, and *P* > 0.25 for Pielou's evenness).

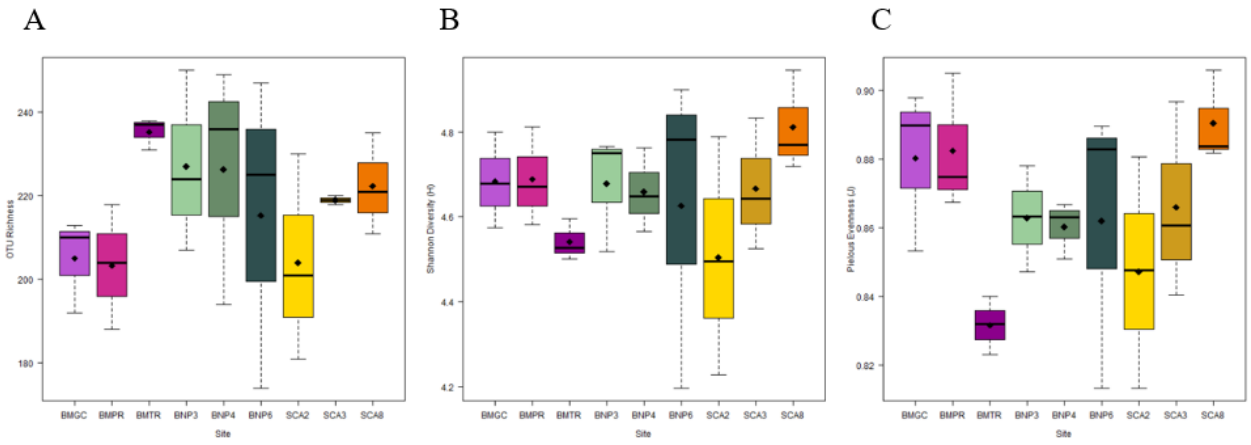

Figure 16: Boxplots of eukaryotic communities based on plot averages per site; A) Operational Taxonomic Unit (OTU) richness, B) Shannon diversity (H), C) Pielou's evenness (J). Black lines within the coloured boxed indicate median values and black diamonds indicate the mean. Top lines of the boxes are the 75th percentile, bottom lines of the boxes are the 25th percentile, and whiskers show maximum and minimum values. BM – Blue Mountains, BNP – Budderoo National Park, SCA – Upper Nepean State Conservation Area.

3.3.2. Eukaryotic community composition

Eukaryotic data contained ten kingdoms, with the top five accounting for 94.63% of reads; Fungi 36.98%, Metazoa 35.47%, Rhizaria 12.08%, Plantae 5.73% and Alveolata 4.37%. The top 10 phyla accounted for approximately 90-94% of reads in the BM region, 84-87% in the BNP region and 78-84% in the SCA region. The distribution of reads within phyla were similar within regions but varied between (Figure 17). Sites in the BM region were seen to have a larger proportion of reads assigned to the phyla Basidiomycota and Ascomycota compared to BNP and SCA sites.

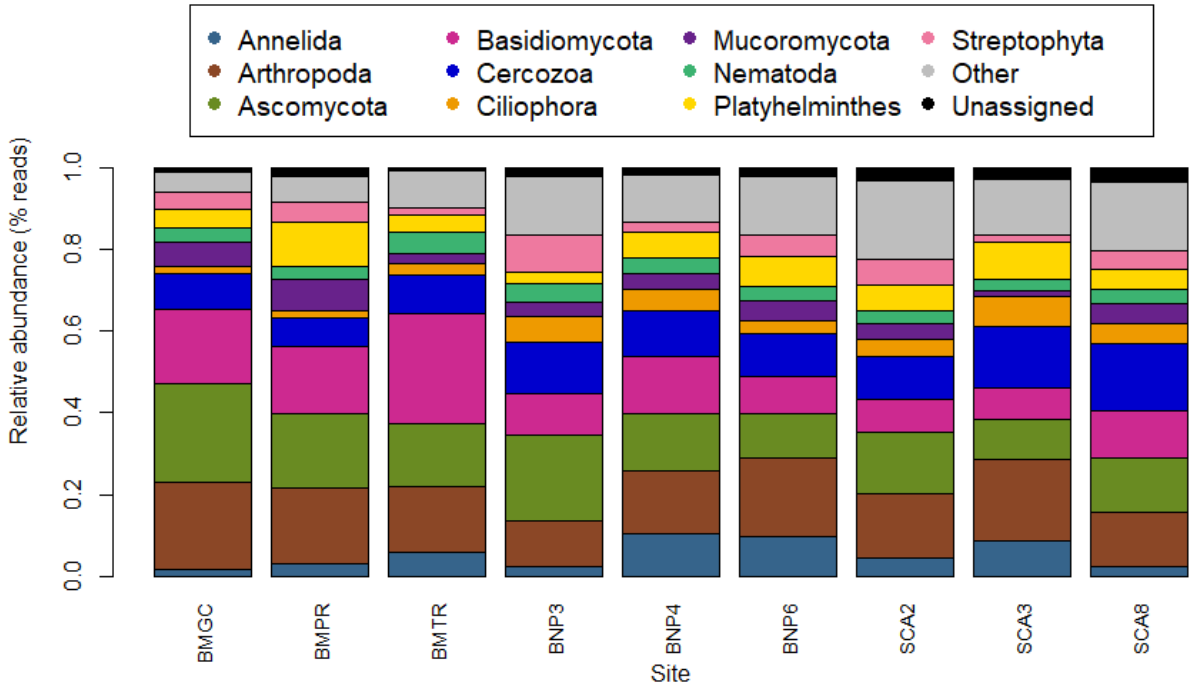

Figure 17: Taxonomic distribution of eukaryotic reads within top ten phyla per site. All phyla that were not within the top ten groups were included in the Other category, Operational Taxonomic Units that were not assigned at the phyla level were classified as Unassigned.

The composition of the eukaryotic communities differed between (perMANOVA: F = 9.25, $P < 0.001$) and within regions (perMANOVA: F = 5.19, $P < 0.001$). A *post-hoc* pairwise test identified all regions and sites contained different eukaryotic assemblages ($P < 0.001$). The nMDS ordination plot, which shows eukaryotic assemblages from each region and site shows BM sites separated from SCA and BNP (Figure 18). Sites from SCA and BNP display an overlapping of community composition. Overall sites within regions are mostly clustered together but do exhibit some spread.

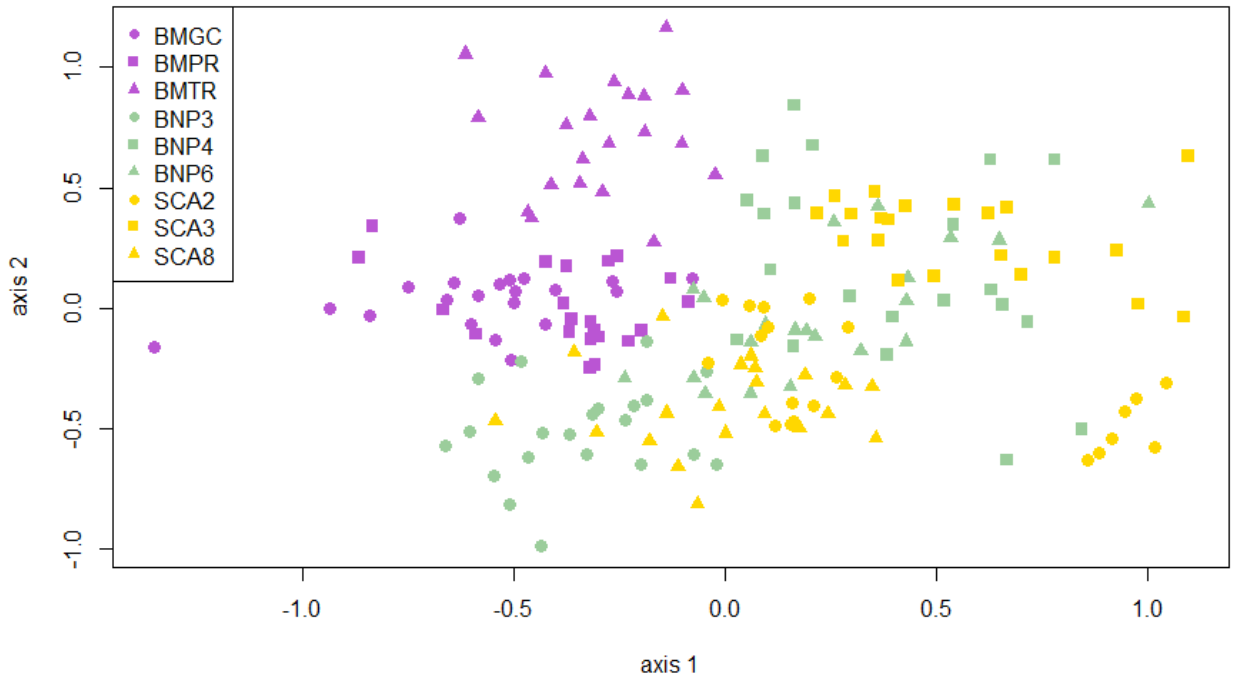

Figure 18: Non-metric multidimensional scaling (nMDS) ordination plot of eukaryotic communities illustrating composition dissimilarity across regions and sites (stress = 0.213). Each point represents a single sample, points that are closer together are similar in their community composition. BM – Blue Mountains, BNP – Budderoo National Park, SCA – Upper Nepean State Conservation Area.

3.3.3. Relationship between eukaryotic communities and environmental variables

A dbRDA analysis based on five environmental variables explained 15.7% of the total variation in eukaryotic communities (Figure 19). The first two axis explained 12.5%, with the first axis explaining 6.8% of total variation with high soil moisture and high elevation separating communities, specifically the BM sites. While the second axis explained 5.7% of total variation, with site area influencing changes within communities. Site area (5.76%, $P < 0.001$), elevation (4.56%, $P < 0.001$) and soil moisture content (3.90%, $P < 0.001$) were the largest contributing factors to the dbRDA, while pH (0.74%, $P = 0.013$) and C:N ratio (0.74%, $P = 0.014$) only explained a small amount of biotic variation.

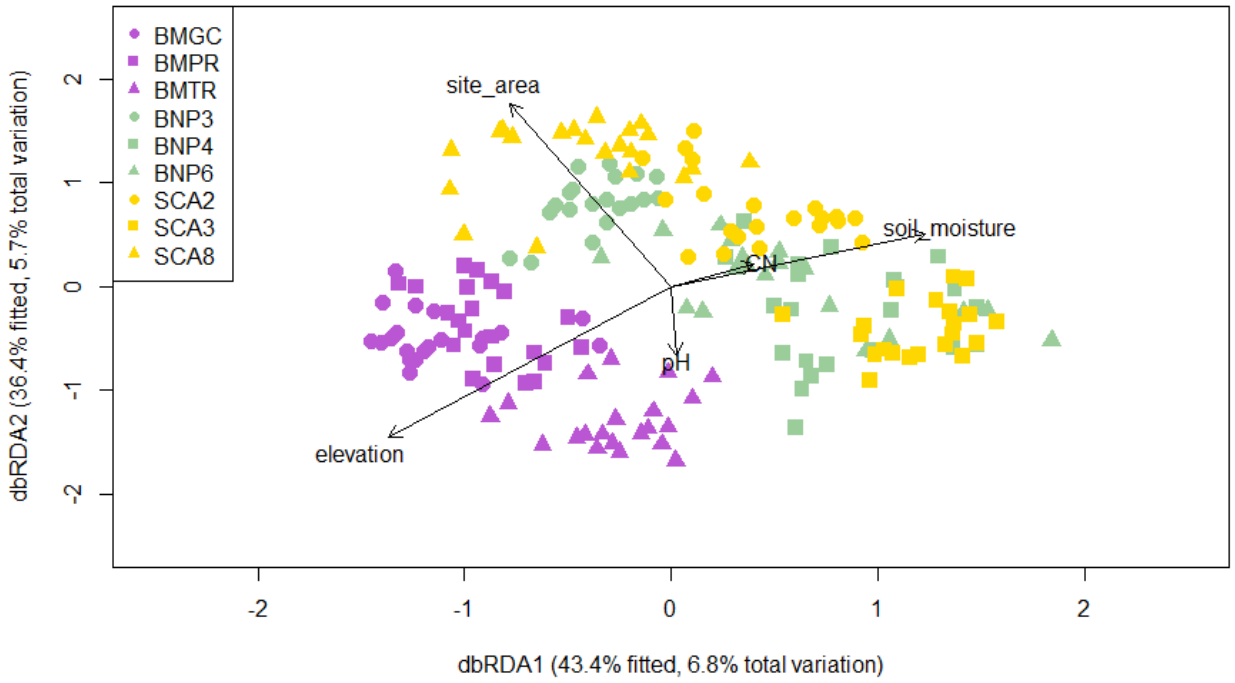

Figure 19: A distance-based redundancy analysis (dbRDA) ordination plot showing the relationship between eukaryotic communities and five environmental variables: site area, soil moisture content, C:N ratio, pH and elevation. BM – Blue Mountains, BNP – Budderoo National Park, SCA – Upper Nepean State Conservation Area.

3.3.4. Multi-scale spatial analysis of eukaryotic community assemblages

A variance component analysis indicated that the majority of variation occurred at the sample level, residuals 60% (Table S9 in Supplementary material). The effect of sites explained approximately 20% of variation, while plots explained 15% of the variation and regions only 5%.

Eukaryotic communities across regions were significantly dissimilar with increasing geographic distance ($F = 5834.3$, $R^2 = 0.247$, $P < 0.001$) (Figure 20). Within all regions, eukaryotic community dissimilarity increased with geographical distance between sites: BM ($F = 1244.3$, $R^2 = 0.389$, $P < 0.001$), BNP ($F = 631.4$, $R^2 = 0.244$, $P < 0.001$) and SCA ($F = 1191$, $R^2 = 0.379$, $P < 0.001$) (Figure 21). When comparing samples within sites, eight out of nine sites showed significant differences ($P < 0.05$), excluding within site BMGC, (Figure S5 in Supplementary materials). High correlation coefficient values were found in four sites, with the highest being SCA2 ($R^2 = 0.88$, $P < 0.001$), then SCA8 ($R^2 = 0.22$, $P < 0.001$), BNP4 ($R^2 = 0.21$, $P < 0.001$) and BNP6 ($R^2 = 0.20$, $P < 0.001$). Larger correlation coefficient values were found for eukaryotes compared to prokaryotes, suggesting eukaryotic organisms have a stronger community differentiation by distance pattern.

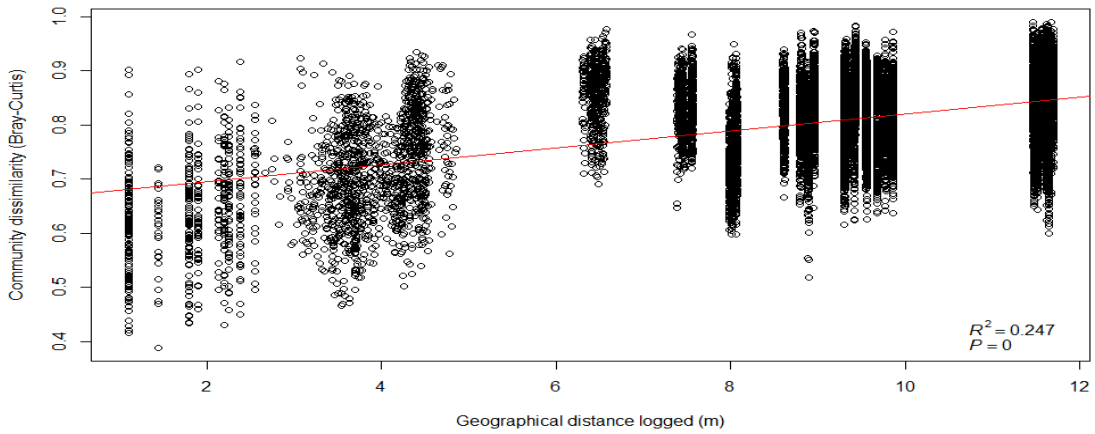

Figure 20: Eukaryotic community differentiation by distance plot across all regions, regression line in red showing adjusted R^2 value.

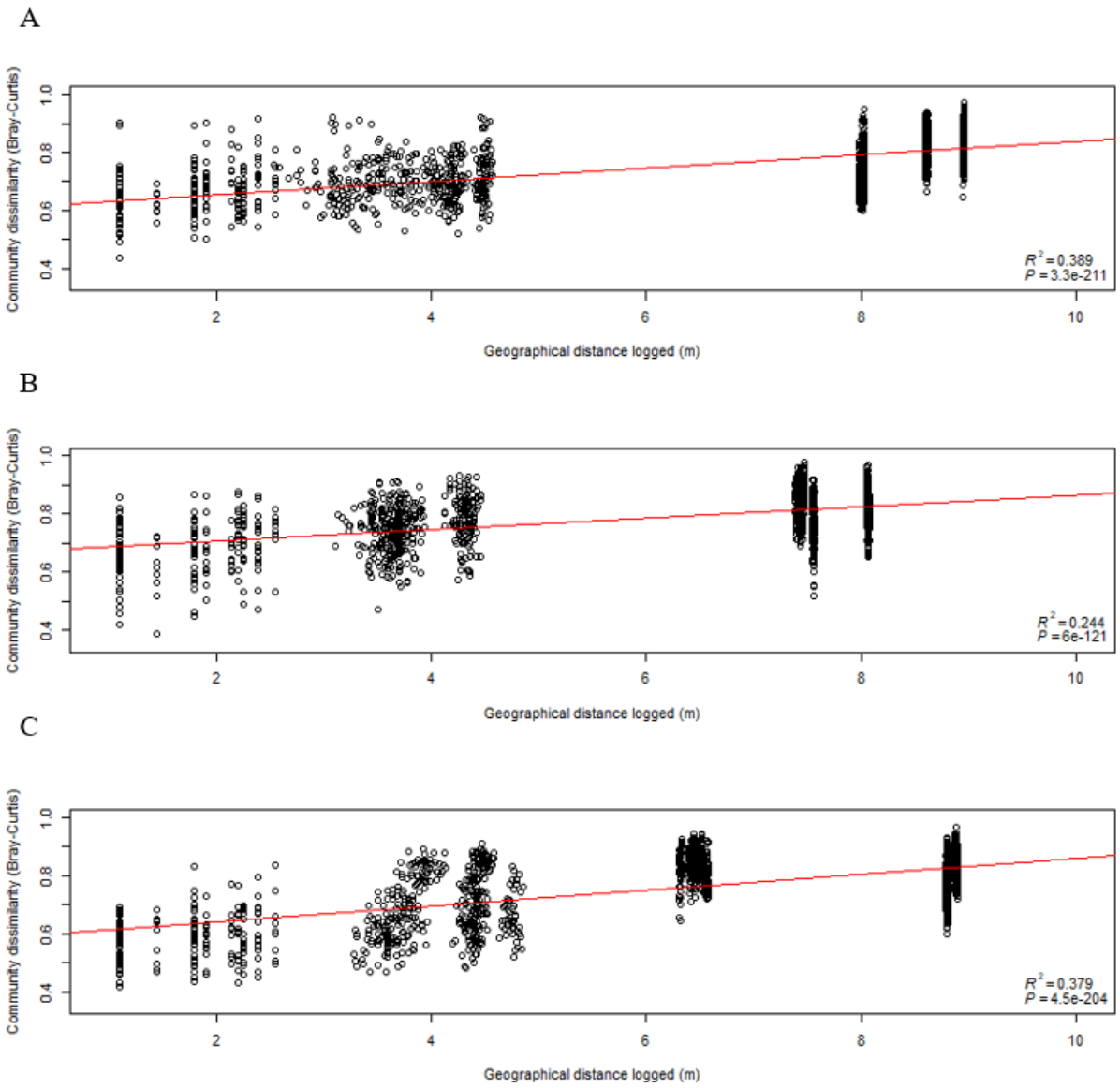

Figure 21: Eukaryotic community differentiation by distance plot for each individual region, regression line in red showing adjusted R^2 value. A) Blue Mountains region (BM), B) Budderoo National Park region (BNP), C) Upper Nepean State Conservation Area region (SCA).

4. Discussion

This thesis used a novel approach for monitoring swamp ecosystems, assessing the variation of prokaryotic and eukaryotic community structure and assemblages in response to abiotic variables and multiple spatial scales. The finding indicated that prokaryotic communities were shaped by local abiotic conditions to a greater extent, compared to eukaryotic communities where spatial scales had a larger influence on composition. The knowledge gained through this biodiversity assessment will assist government and local stakeholders to improve management programs which aim to protect these endangered ecological communities.

4.1. Community patterns

The findings of this study indicated that the ecological indices of richness, diversity and evenness were similar for prokaryotic and eukaryotic organisms across regions and sites (Figure 7, 8, 15 & 16). The benefit of analysing these univariate measurements is the establishment of baseline data that can be used in future monitoring programs for comparisons, allowing for the identification of local disturbances (Stirling & Wilsey, 2001; H. Zhang et al., 2012). However, it has been well documented that univariate metrics may be insensitive for detecting and monitoring environmental conditions, and consequently, I suggest focussing on the multivariate patterns found (Austen & Warwick, 1989).

The distribution of taxa within the prokaryotic community assemblages across regions and sites were similar (Figure 9). A large proportion of prokaryotic reads were assigned to the phyla Proteobacteria and Planctomycetes, both of which include organisms responsible for nitrogen fixation (Delmont et al., 2018). This large proportion of potential nitrogen fixing bacteria highlights the important role swamp ecosystems play in the nitrogen cycle (Bowden, 1987). The proportions of eukaryotic taxa differed slightly between regions (Figure 17), with the BM region having a larger proportion of the fungi Basidiomycota and Ascomycota compared to sites from the BNP and SCA regions. Many species within the phylum Ascomycota carry out key functional roles such as the decomposition of organic material, a key part of the carbon cycle (Leeder et al., 2011).

One of the main finding of this study was the differential patterns in composition observed between the prokaryotic and eukaryotic communities across spatial scales. In the case of prokaryotic communities, no clear differences were observed between communities obtained from different regions (Figure 10). In contrast, marked differences in eukaryotic communities were observed

between regions (Figure 18). This is likely due to the different paradigms which underpin the dispersal of these two kingdoms. In the case of prokaryotes, it has been suggested that they are potentially distributed ubiquitously throughout the environment, with the composition structure filtered by abiotic features (Delgado-Baquerizo et al., 2018; Hagström et al., 2000). Whilst eukaryotes are limited by their ability to disperse via water, air or physical movement (Dawson & Hamner, 2008).

4.2. Relationship with environmental variables

Exploring the relationship between environmental variables and community assemblages revealed that prokaryotic communities were shaped to a marginally greater extent by environmental conditions (18.37%) than eukaryotic communities (15.7%) (Figure 11 & 19). Both site area and elevation explained a relatively large proportion of the explained variation for both communities, which may indicate an effect that an environmental niche was shaping community composition within some sites. However, elevation could also be confounded by other factors such as geographical distance, rainfall and temperature (Qian & Ricklefs, 2012). For example, in the BM region, which was higher in elevation and geographically more distant from the other two regions, both prokaryotic and eukaryotic communities were distinct from the communities obtained from the other regions. Consequently, it is difficult to determine whether this was a true effect of elevation or due to the confounding influence of other environmental co-variables, including unmeasured variables.

The moisture content of sediments was also a key covariable of community composition. Both prokaryotic and eukaryotic assemblages were similar within sites with similar moisture content, regardless of the sampling location (Figure 11 & 19). This link has also been found in other studies, with soil moisture driving local variation of microbial communities (Fierer et al., 2003; Panikov, 1999; Staley & Reysenbach, 2002). As soil moisture was also highly correlated with several other abiotic conditions, the source of water, e.g. from runoff or aquifers, is a crucial component which requires management and protection from threatening processes such as mining practices (Jankowski, 2010; Krogh, 2007) and climate change (A'Bear et al., 2014; W. Zhang et al., 2005).

The diversity of bacterial communities found within sites was limited due to the acidic sediment conditions, as the species present must be adapted to low pH environments (Fierer & Jackson, 2006). While the pH at all sites were within the expected range for upland swamps (Young, 2017),

a disruption to these swamp ecosystems due to eutrophication or channelization has been shown to alter the sediment pH to a more neutral conditions, altering the relative proportions of bacteria and archaea taxa (Christiansen et al., 2019). Understanding the influence of abiotic conditions on sedimentary communities is pivotal for biomonitoring and restoration programs (Freidman & Fryirs, 2015).

4.3. Multi-scale spatial patterns

There was significant variation in prokaryotic and eukaryotic communities at each spatial level, with the greatest effect on community assemblages occurring at the site level (within ~100m) (Figure S4 and S5 in Supplementary material). The next largest source of variability was seen at the regional level (within 10kms) (Figure 13 & 21), then across regions (between 20-120kms) (Figure 12 & 20). These findings of large variation between communities at small spatial scales are consistent with research conducted by Anderson et al. (2005) on the impact of taxonomic resolution on spatial scales. While this pattern was similar for both prokaryotes and eukaryotes, the strongest relationships were found across eukaryotic communities. A study by Green et al. (2004) found similar patterns, with eukaryotic community composition decreasing in similarity with increasing spatial scales. These findings highlight the need to manage swamp ecosystems across multiple spatial scales.

4.4. Experimental limitations

Because of the large number of samples and volumes of sediment used in this study I chose to use the phosphate buffer extraction protocol by Taberlet et al. (2012); which allowed a larger amount of starting material to be processed, capturing a more accurate representation of the local communities per sample (Ranjard et al., 2003). The use of this protocol was cost effective and time saving, with extractions being carried out in the field. This approach has been widely used in eDNA studies (Evrard et al., 2019; Pansu et al., 2015), and research by Zinger et al. (2016) found the buffer method produced comparable results to the PowerMax Soil DNA Isolation Kit (MoBio Laboratories, Cambridge, UK). However, as this protocol excludes a cell lysis step, only extracellular DNA is captured, preventing a study of functionally active organisms with RNA analysis (Baldrian et al., 2012). While RNA studies capture current communities, this level of detail was not required to inform management and conservation decisions at this time, although it

would be a good avenue of exploration in the future. However, the costs and time associated with RNA work would be prohibitive for routine monitoring of these systems (Laroche et al., 2018b; Sutcliffe et al., 2019).

4.5. Implications for management and conservation

The management and protection of upland swamps and their endangered ecological communities is challenging, especially given their many threats: these including mining practices; urbanisation; eutrophication, fire and climate change. This thesis has provided a greater understanding of the influences on, and distribution of, ecologically important communities within swamps across multiple regions. As illustrated, the approach we implemented produced comprehensive ecological data which capture far more diversity than could be gained using traditional approaches. I believe this approach has the potential to provide a robust and cost-effective standardised biomonitoring program for swamp communities. Ultimately determining the overall resilience and health of these ecosystems will assist conservation managers understand the ability of these systems to withstand further stress and environmental change.

4.6. Future research directions

There are many opportunities for further analysis from the high resolution data obtained from this study. A detailed phylogenetic assessment of taxa at varying levels could provide a clearer understanding of the ecology and function of each swamp ecosystem (von Mering et al., 2007), e.g. via the implementation of bacterial functional assignment software, such as FAPROTAXA (Louca et al., 2016). Identifying indicator taxa would assist in future biomonitoring programs aiding in the management and rehabilitation of swamp ecosystems (Chariton et al., 2010; Fernandes et al., 2018; Goodsell et al., 2009). Investigating the interactions between taxa across spatial scales could also be carried out using co-occurrence network analysis (Laroche et al., 2018a). Collectively these analyses would enable us to examine the composition, function and connectivity of these endangered ecosystems.

The current study gives an insight into the complexities of swamp communities and provides key information of the biodiversity of two key taxonomic kingdoms, however, to truly understand these dynamic systems, further research across multiple temporal scales is required. This is particularly pertinent given the imminent threat of climate change.

5. Conclusion

This research has shown that the use of eDNA metabarcoding techniques was able to generate important ecological data that aids in the assessment and monitoring of these endangered ecological communities. As a novel approach for monitoring swamp ecosystems metabarcoding was able to identify important relationships between abiotic variables and community structure, highlighting the influence of local environmental variables in shaping prokaryotic communities. And furthermore, emphasised the influence of spatial scales on eukaryotic community assemblages. The high resolution biological data presented here provides the foundations for the establishment of a routine biomonitoring program for THPSS swamps within the Sydney Basin region and has the capacity to be extended to swamp communities around the globe.

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Supplementary Material

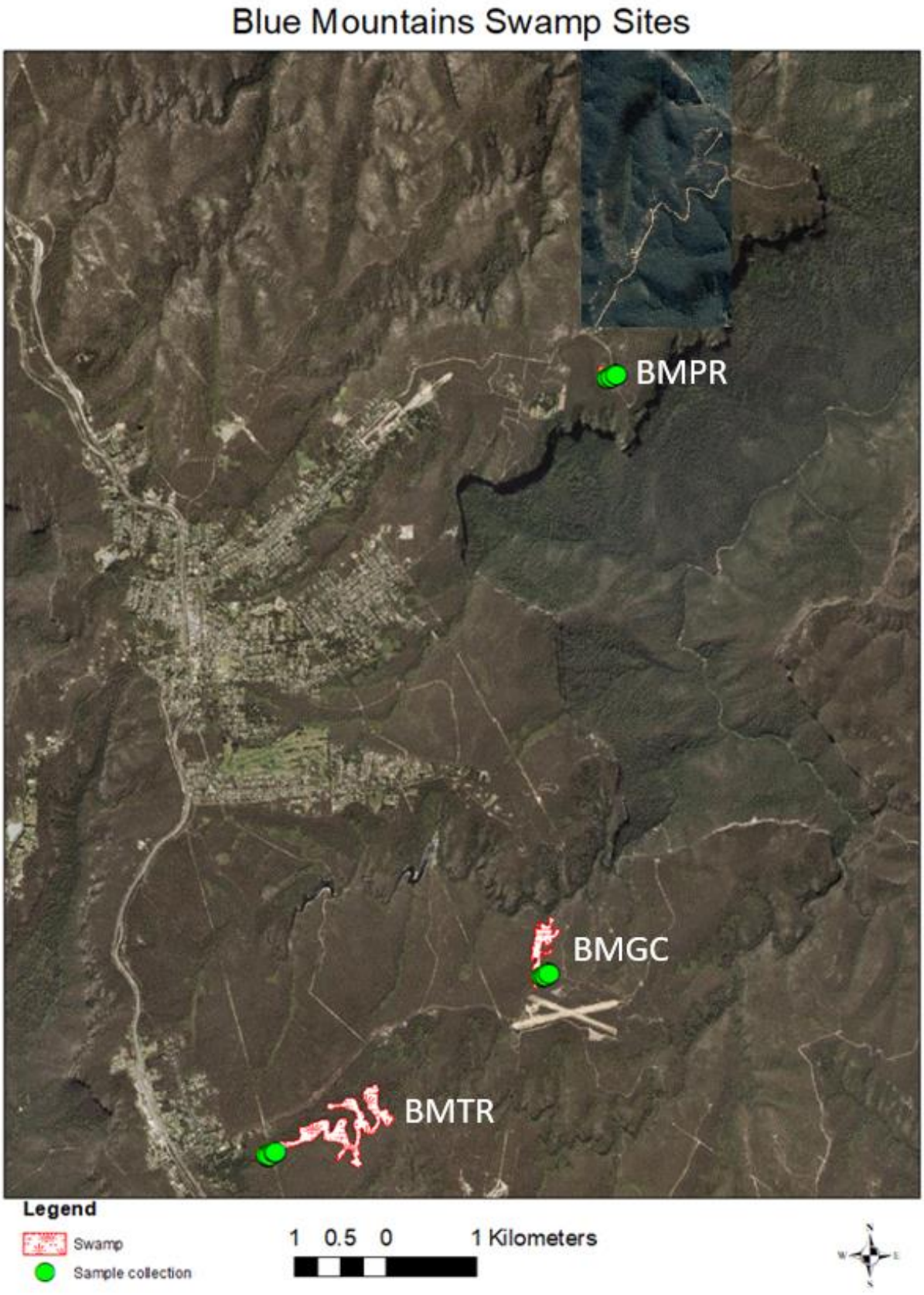

Figure S1: Blue Mountains (BM) region with swamp sites outlines in red and sample locations in green. Map was created with ArcGIS software by Esri. Data on each swamps' spatial distribution was sourced from Fryirs and Hose (2016).

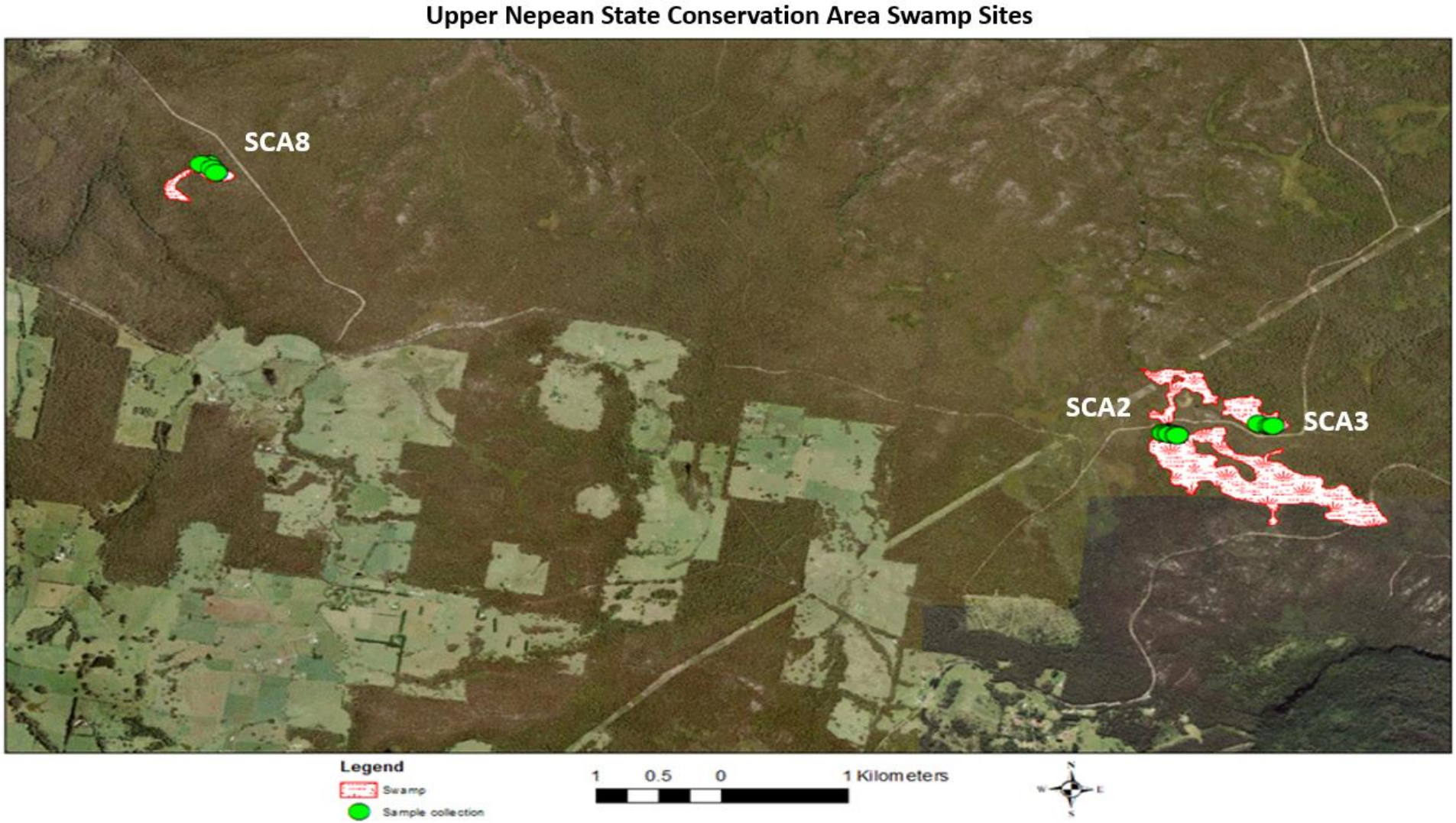

Figure S2: Upper Nepean State Conservation Area (SCA) region with swamp sites outlines in red and sample locations in green. Map was created with ArcGIS software by Esri. Data on each swamps' spatial distribution was sourced from Fryirs and Hose (2016)

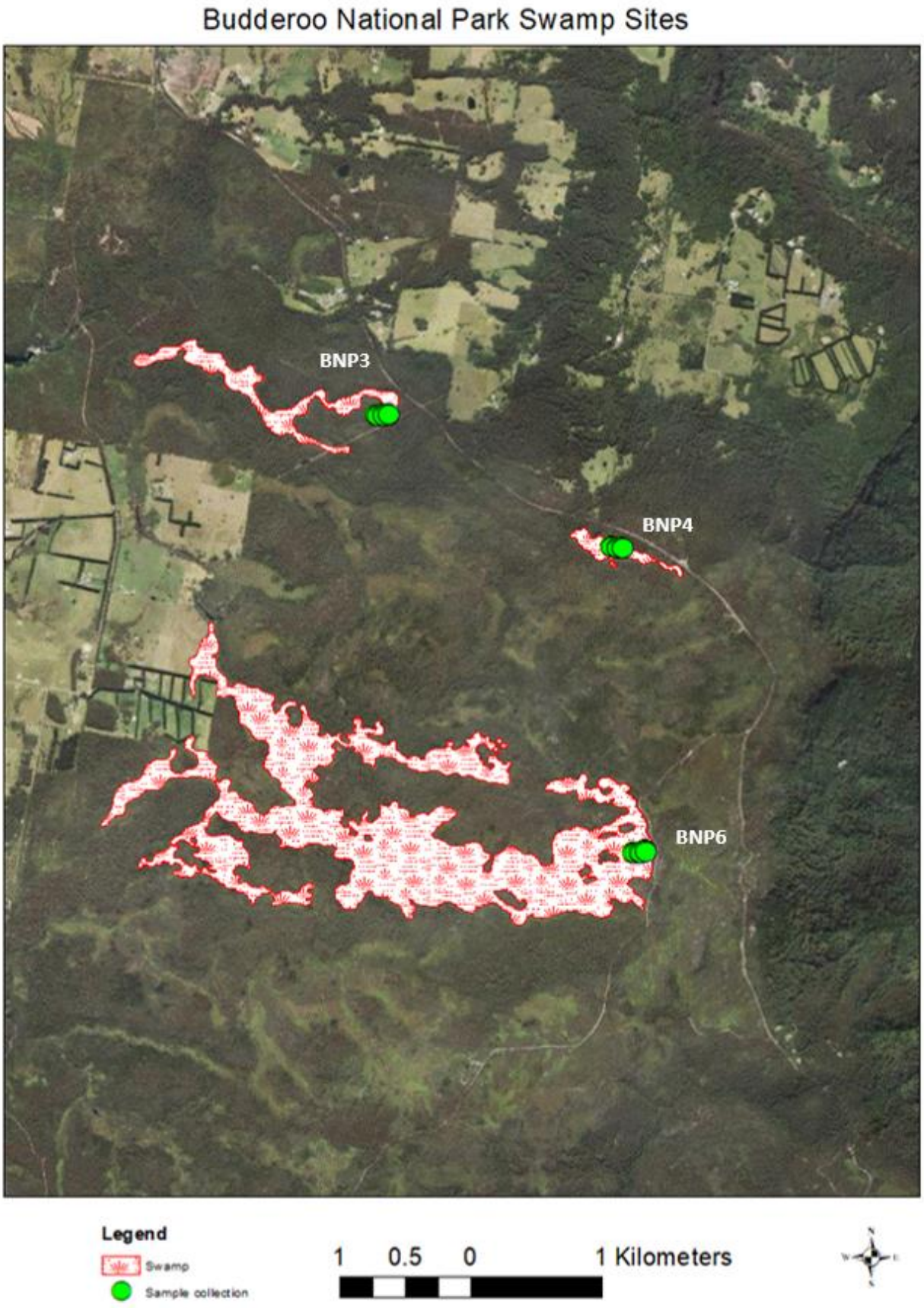

Figure S3: Budderoo National Park (BNP) region with swamp sites outlines in red and sample locations in green. Map was created with ArcGIS software by Esri. Data on each swamps' spatial distribution was sourced from Fryirs and Hose (2016).

Table S1: Positive control mixes

Three positive control mixes for 16S and 18S rDNA were created to simulate an artificial environmental community which would not be naturally found within the sample regions. The quantities of each species DNA varied between the mixtures as follows: mix 1 was an equimolar mixture, mix 2 used decreasing concentrations of samples, and mix 3 increasing concentrations of samples. DNA samples used for positive control mixes were limited, as such for Mix 3 *Carcharias Taurus* was swapped with *Negaprion brevirostris*.

Species	Initial Concentration (ng/µL)	Mix 1		Mix 2		Mix 3	
		[C]	Volume added (µL)	[C]	Volume added (µL)	[C]	Volume added (µL)
Litopenaeus vannamei	9.85	100	10.15	100	101.52	0.78	0.79
Metasequoia glyptostroboides	21.03	100	4.76	50	23.78	1.56	0.74
Saccostrea glomerata	113.50	100	0.88	25	2.20	3.13	0.27
Syngonium podophyllum	7.43	100	13.46	12.5	16.82	6.25	8.41
Liriodendron tulipifera	3.36	100	29.76	12.5	37.20	NA	NA
Lates niloticus	7.63	100	13.11	6.25	8.20	12.5	16.39
Sparisoma axillare	107.75	100	0.93	3.13	0.29	25	2.32
Carcinus maenas	8.23	100	12.15	1.56	1.90	50	20.00
Carcharias taurus	56.25	100	1.78	0.78	0.14	NA	NA
Negaprion brevirostris	0.22	NA	NA	NA	NA	100	220.00

Table S2: Prokaryotic Operational Taxonomic Unit (OTU) counts and estimates of richness with Chao1

Site	OTU count	Chao1	Chao1 Standard Error	Percentage covered (%)
BMGC	2998	4521.88	120.61	66.30
BMPR	3317	5077.08	133.69	65.33
BMTR	4280	7018.68	172.87	60.98
BNP3	3128	4834.77	131.60	64.70
BNP4	4081	6826.93	172.82	59.78
BNP6	3500	5378.98	130.73	65.07
SCA2	4278	6635.44	153.39	64.47
SCA3	3851	5894.93	138.00	65.33
SCA8	3232	5056.29	141.73	63.92

Table S3: Diversity and composition of prokaryotic communities at a region level, values based on averages per site.

Region	OTU richness	Shannon diversity (H)	Pielou's evenness (J')
BM	3532	6.91	0.847
BNP	3570	6.91	0.845
SCA	3787	6.98	0.847

Table S4: Diversity and composition of prokaryotic communities at a site level, values based on averages per plot

Site	OTU richness	Shannon diversity (H)	Pielou's evenness (J')
BMGC	1819	6.60	0.879
BMPR	2019	6.69	0.880
BMTR	2431	6.76	0.867
BNP3	1923	6.62	0.876
BNP4	2117	6.59	0.862
BNP6	1936	6.63	0.879
SCA2	2330	6.79	0.876
SCA3	2314	6.73	0.870
SCA8	2027	6.60	0.867

Table S5: Variance component analysis of prokaryotic operational taxonomic unit (OTU) communities displaying percentage of variance explained by Region, Site, Plot and Residuals.

	No. levels	Source	MS	est. var.	% of variance
Region	3	Region	2.24	0.01	0.04
Site	3	Site (Region)	1.61	0.06	0.25
Plot	3	Plot (Site (Region))	0.32	0.03	0.10
Replicates	7	Residual	0.15	0.15	0.60

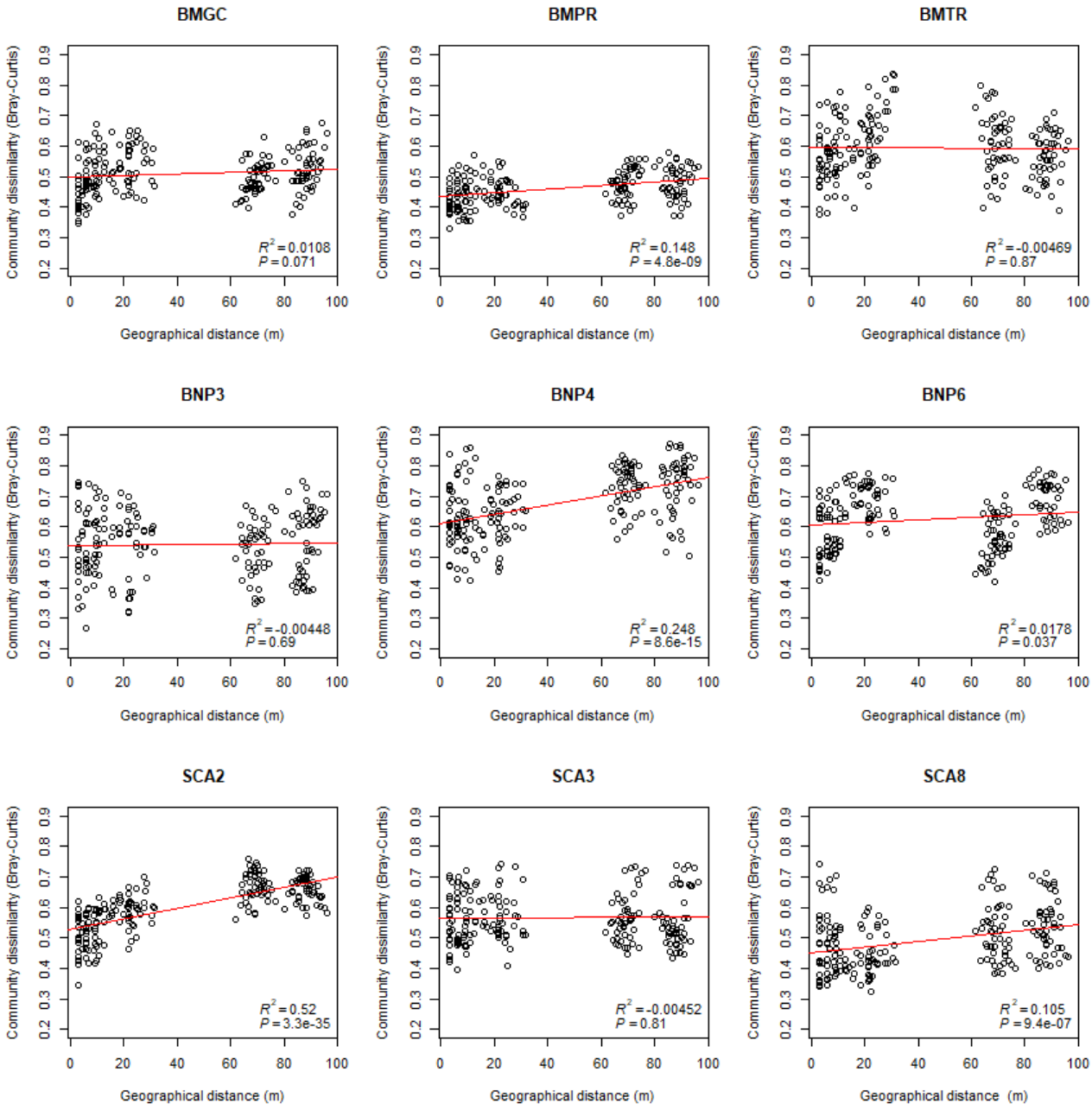

Figure S4: Distance and community dissimilarity plot of prokaryotic community assemblages within sites, regression line in red showing adjusted R^2 value.

Table S6: Eukaryotic Operational Taxonomic Unit (OTU) counts and estimates of richness with Chao1

Site	OTU count	Chao1	Chao1 Standard Error	Percentage covered (%)
BMGC	351	524.23	39.16	66.95
BMPR	346	506.92	36.70	68.26
BMTR	419	654.35	47.09	64.03
BNP3	391	556.32	35.31	70.28
BNP4	434	729.78	57.59	59.47
BNP6	400	581.62	37.59	68.77
SCA2	412	599.89	39.44	68.68
SCA3	367	518.14	34.55	70.83
SCA8	374	554.96	39.93	67.39

Table S7: Diversity and composition of eukaryotic communities at a region level, values based on averages per site.

Region	OTU richness	Shannon diversity (H)	Pielou's evenness (J')
BM	372	5.10	0.862
BNP	408	5.19	0.863
SCA	384	5.13	0.863

Table S8: Diversity and composition of eukaryotic communities at a site level, values based on averages per plot

Site	OTU richness	Shannon diversity (H)	Pielou's evenness (J')
BMGC	205	4.68	0.880
BMPR	203	4.69	0.882
BMTR	235	4.54	0.832
BNP3	227	4.68	0.863
BNP4	226	4.66	0.860
BNP6	215	4.63	0.862
SCA2	204	4.50	0.847
SCA3	219	4.67	0.866
SCA8	222	4.81	0.890

Table S9: Variance component analysis of eukaryotic operational taxonomic unit (OTU) communities displaying percentage of variance explained by Region, Site, Plot and Residuals.

	No. levels	Source	MS	est. var.	% of variance
Region	3	Region	3.07	0.02	0.05
Site	3	Site (Region)	2.01	0.07	0.20
Plot	3	Plot (Site (Region))	0.57	0.05	0.15
Replicates	7	Residual	0.21	0.21	0.60

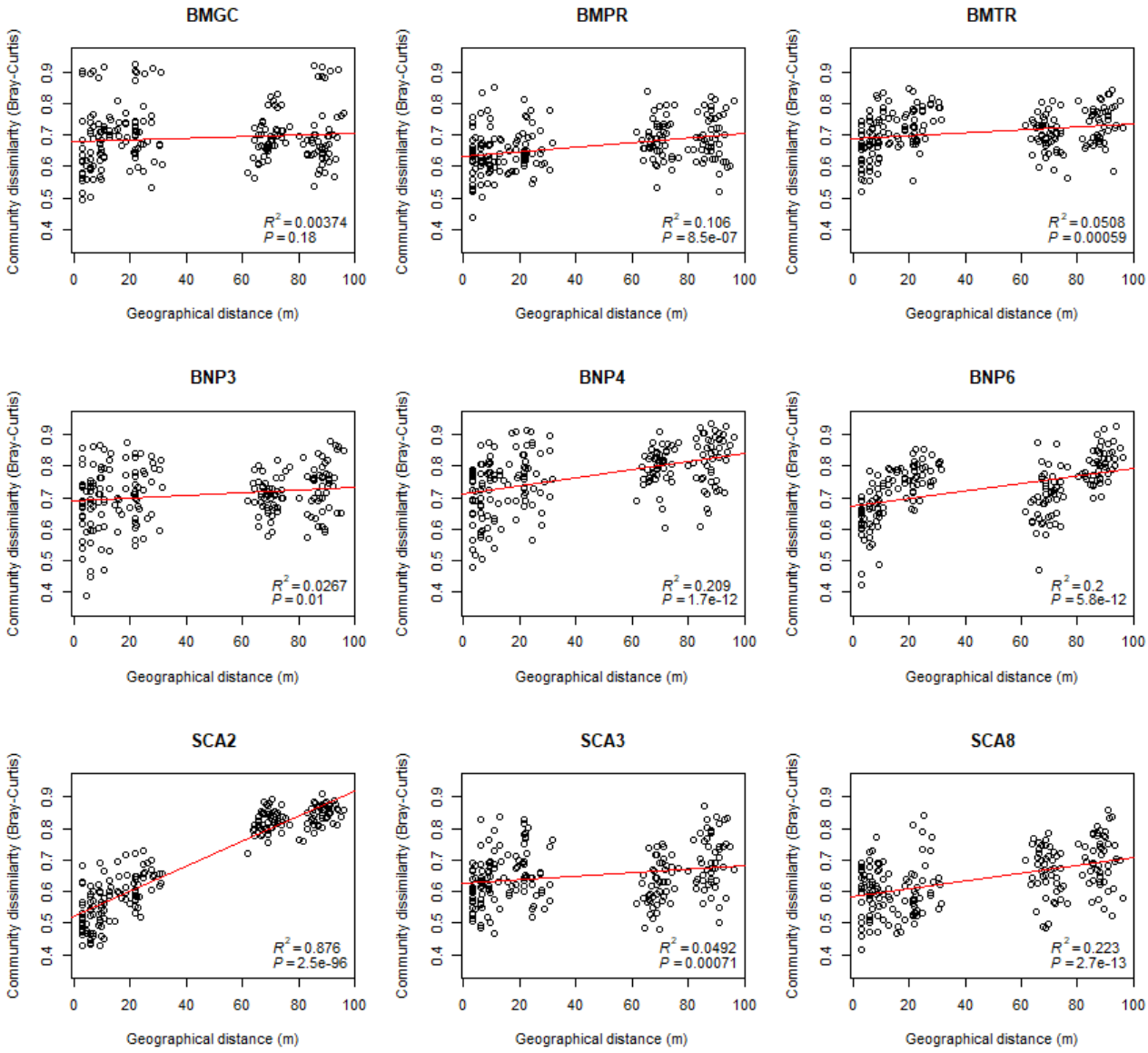

Figure S5: Distance and community dissimilarity plot of eukaryotic community assemblages within sites, regression line in red showing adjusted R^2 value.