SCHOOL OF
NATURAL
SCIENCES
*Faculty of Science
and Engineering*

MACQUARIE
University

EDNA MONITORING OF WATERBIRDS FOR THE DARLING BAAKA RIVER HEALTH PROJECT

Compiling baseline data and assessing the impacts of river condition on waterbird diversity

Citation: Davis, S., and Korbel, K.L. (2025). eDNA monitoring of waterbirds for the Darling Baaka River Health Project. Final report for the NSW Department of Climate Change, Energy, the Environment and Water. Macquarie University, March 2025.

TABLE OF CONTENTS

Abbreviations

Acronym	Full term
ALA	Atlas of Living Australia
AMM	Ammonia
BGA	Blue-Green Algae phycocyanin pigment
CHLA	Chlorophyll-a
DBRHP	Darling Baaka River Health Project
DCCEEW	NSW Department of Climate Change, Energy, the Environment and Water
DO	Dissolved Oxygen
DOC	Dissolved Organic Carbon
EAWS	Eastern Australian Waterbird Survey
eDNA	Environmental DNA
FDOM	Fluorescent Dissolved Organic Matter
FDR	False Discovery Rate
LDI	Landscape Disturbance Index
LME	Linear Mixed-Effects modelling
MFR	Macroinvertebrate Family Richness
MOTU	Molecular Operational Taxonomic Unit
MPR	Macroinvertebrate POET Richness
MSS	Macroinvertebrate SIGNAL Score
MSW	Maximum Stream Width
mtDNA	Mitochondrial DNA
NBDL	National Biodiversity DNA Library
NCBI	National Center for Biotechnology Information
NIT	Total Nitrogen
nMDS	Non-metric Multi-Dimensional Scaling
NTU	Nephelometric Turbidity Units
OXN	Oxidised Nitrogen
PCA	Principal Components Analysis
PCoA	Principal Coordinates Analysis
PCR	Polymerase Chain Reaction
PH	pH
PHO	Total Phosphorous
RCI	River Condition Index
RDA	Redundancy Analysis
RIP	Riparian condition score
SPC	Specific Conductance
TSS	Total Suspended Solids
UNSW	University of New South Wales
WLV	Water Level score
ZOO	Zooplankton health score

Executive summary

The Darling Baaka River system, along with its tributaries and floodplains, provides aquatic habitat for birds and fish, a reliable source of water for local communities, vital ecosystem services, and holds important cultural and economic value. However, the health of this river system has declined due to regulation and wetland degradation. A severe flooding event in early 2023 that led to mass fish deaths prompted the establishment of a river health monitoring program. This program, the Darling Baaka River Health Project or DBRHP (NSW Department of Climate Change, Energy, the Environment and Water, DCCEEW), aims to adapt the River Condition Index (RCI) to the unique conditions of the Darling Baaka River system by assessing key indicators of river health including fish, zooplankton and macroinvertebrate biodiversity. Macquarie University was engaged by DCCEEW to produce a report detailing the diversity of waterbirds, utilising the lower Darling Baaka River between Wilcannia and Wentworth.

Although not included in the RCI, waterbirds are also key indicators of wetland health, and many species have declined in abundance across eastern Australia due to widespread wetland degradation. Monitoring changes in waterbird species richness in response to changes in wetland health is a critical component of wetland management. Environmental DNA (eDNA) provides a complementary approach to traditional waterbird monitoring approaches (e.g., ground-based and aerial surveys), particularly for detecting rare or cryptic species or in remote areas where ground-based surveys are challenging, and baseline data is lacking.

This study aimed to conduct waterbird eDNA surveys at riverine sites across the Darling Baaka River system, to establish baseline site level data on waterbird assemblages, including threatened and/or migratory species, and assess the impact of river health on waterbird diversity. This report addresses these objectives by:

- Conducting eDNA sampling in 2024 and comparing results against historical observational records of avian species for each site.
- Characterising the environmental conditions of riverine sites where eDNA samples were collected and assessing the impact of river condition on eDNA-based waterbird community composition and diversity.
- Assessing the effectiveness of eDNA-based waterbird monitoring for consideration in future eDNA-based surveys in the Darling Baaka River system and other sites in the Murray-Darling Basin.

Key findings from this study were:

- A total of 35 bird species, including 19 waterbird species, were detected across 18 sample sites. Relatively more waterbirds were detected compared to terrestrial bird species, both overall and at the site level (average of 5-8 waterbirds detected per site compared to 0-3 terrestrial birds depending on the detection approach). There were also relatively more piscivores (fish-eating waterbirds such as pelicans and cormorants) compared to other functional groups, a pattern that was consistent across most sites.
- Five waterbird species were detected at almost every site: Australian pelican (*Pelecanus conspicillatus*), Australian wood duck (*Chenonetta jubata*), and three species of cormorants (*Phalacrocorax carbo, P. sulcirostris,* and *P. varius*).
- Measures of waterbird diversity varied across the sampling sites. As expected, the sampling sites in the Menindee Lake system (e.g., Lake Cawndilla, Site 30 and Lake Wetheral, Site 9) supported the highest number of waterbird species, while the riverine sites supported fewer species overall. Relatively more shorebirds were also detected at sites located near the Menindee Lake system (sites 9, 14, 29 and 30).
- All waterbird taxa detected with eDNA have been historically recorded across the study area based on comparison with available Atlas of Living Australia (ALA) records for the sampling sites.
- Around 24 % of variation in eDNA-based waterbird community composition was influenced by environmental variables, and measures of waterbird diversity at the species, genus and family levels were significantly negatively associated with several indices of poor water quality (higher algal biomass, dissolved organic matter and certain nutrients) and positively associated with better riparian condition scores.

Further optimisation of eDNA protocols may improve detection rates, particularly for rare or cryptic species. For sites with available ALA records, a large percentage of historically recorded waterbird species were missed with eDNA (up to 79 % depending on the detection approach). However, there was also a large percentage of eDNA-detected waterbird species that were not previously recorded at the site level (average across sites ≈ 48 %). Greater waterbird diversity may be captured by increasing the sampling effort per trip per site. As only water quality metrics were available at the sampling-trip level, more fine-scale environmental data would enable more accurate inferences of the relationship between eDNA-based waterbird diversity and river health.

Although further work is needed to refine methodologies and optimise eDNA techniques as a monitoring tool for waterbirds within the Darling Baaka River system, this study provides baseline data on waterbird assemblages that, alongside observational studies, could be used to assess river health.

INTRODUCTION

The Darling Baaka River is an inland river system that stretches for ~1,500 km from northern NSW to the Murray River. The river, along with its tributaries and floodplains, provides key habitat for supporting fish and bird species, is a reliable water source for local communities, and provides vital ecosystem services including water purification and nutrient/carbon cycling. This river system also holds deep cultural value for the Barkandji people and plays an important role in supporting agricultural and recreational activities. However, the health of the Darling Baaka River system has declined since regulation began in the 1960s, disrupting natural flow regimes. A severe flooding event in January 2023, followed by mass fish deaths in March 2023 near Menindee, highlighted the need for improved river management and enhanced monitoring.

In response to the 2023 flooding event, a river health monitoring program (the Darling Baaka River Health Project 'DBRHP') was established to develop and implement a monitoring program that adapts the River Condition Index (RCI) (DCCEEW, 2023) to the unique characteristics of the lower Darling Baaka River system. The RCI combines data on hydrological stress, water quality, biodiversity condition (fish, zooplankton, and macroinvertebrate health), landscape disturbance, riparian vegetation condition and geomorphic condition. However, the RCI does not currently incorporate data on waterbird diversity, Macquarie University was engaged to assess the waterbird diversity in the region to complement data being collected for the RCI assessment.

This work is part of the DBRHP which has been designed and delivered by the DCCEEW Science and Insights Division. The DBRHP is delivered under the EPA's Recovery Program for Water Quality Monitoring in the Darling Baaka and is funded as a Category D recovery measure under the joint Commonwealth and NSW Government Disaster Recovery Funding Arrangements.

IMPORTANCE OF WATERBIRD MONITORING

Australia's waterbirds are well-adapted to navigating the natural 'boom' and 'bust' phases of wetland productivity (Kingsford et al., 2010). For example, waterbirds that breed in large aggregations depend on flooding thresholds and water levels to initiate breeding (Brandis, 2010; Brandis & Bino, 2016; Brandis et al., 2011). Widespread wetland degradation, particularly within the highly developed parts of the Murray-Darling Basin (MDB), has led to the disruption of natural wetland dynamics and a severe decline in waterbird abundance across eastern Australia over the last 40 years (Brandis et al., 2018; Kingsford et al., 2017; Kingsford et al., 2020). Natural flow regimes have been altered by river diversions and wetland modifications, which combined have reduced breeding opportunities for waterbirds (Kingsford et al., 2010). Waterbird monitoring is typically carried out through ground-based and annual aerial surveys, although drone surveying has been utilised more recently for waterbird breeding sites (Brandis et al., 2021; Brandis et al., 2014; Francis et al., 2020; Kingsford et al., 2020). With ongoing wetland degradation and waterbird declines occurring across eastern Australia, wetland managers need robust information on the distribution of waterbird species (Fluet-Chouinard et al., 2023; Kingsford et al., 2017; Wetlands International, 2012).

MONITORING WATERBIRDS WITH ENVIRONMENTAL DNA

Environmental (e)DNA provides a non-invasive and complementary approach to traditional biodiversity assessments, by detecting taxa from the DNA shed into the environment (Taberlet et al., 2018). eDNA surveys have detected a wide diversity of waterbirds at both brackish and freshwater wetlands (Saenz-Agudelo et al., 2022; Sigsgaard et al., 2020; Zhang et al., 2023). For example, eDNA has been used to improve detection of rare and cryptic rail species (Neice & McRae, 2021), to monitor waterbird diets through detection of prey species in faecal samples (Fablet et al., 2024; Menning et al., 2022), and assess predation risks for group-nesting waterbirds (Orzechowski et al., 2019). Recently, eDNA was used to detect birds, including waterbirds, from air samples collected around ponds and estuaries (Jin et al., 2025). In Australia, eDNA has been used to detect Australian waterbirds in arid, inland wetlands, enabling the detection of up to 40% of waterbird species historically recorded through traditional ground-based surveys (Davis et al., *in* *prep*). Although further work is needed to refine eDNA protocols for optimal waterbird detection, eDNA offers a promising and complementary approach for waterbird monitoring.

PROJECT AIMS

The aims of this project were to undertake waterbird eDNA surveys at 15 riverine sites along the lower Darling Baaka River network. The data collected will be used to build a bigger picture of waterbird assemblages at riverine sites in remote areas where there is a lack of baseline information, and where extended dry and wet periods are significant drivers for species distributions. This will be achieved by:

1. Generating baseline presence/absence data for waterbird species, including threatened species, along the Darling Baaka River network from Wilcannia to Wentworth.
2. Reviewing public database records to compile a list of historical records for each site and compare with the results of the eDNA surveys.
3. Utilising environmental data on river health and condition to explore the impact of river health on the detection of individual waterbird species and overall waterbird assemblages.

PROJECT STUDY AREA

The study area for this project ranged from Wilcannia to Wentworth in south-western NSW, including the Darling Baaka River and the Great Darling Anabranch (Figure 1). This river network is characterised by semi-arid and arid climates, highly regulated flows, irregular cycles of high and low river flows and highly variable rainfall. During phase 2 of the DBRHP, DCCEEW staff targeted 18 sites to collect water samples for waterbird eDNA analysis, spanning 12 subcatchments (Tables 1-2). A total of 142 water samples were collected over 5 field trips, including 13 sites that were sampled across at least 4 trips (Tables 1-2).

Table 1. List of DCCEEW field trips and the number of sites and samples collected for the current study per trip.

ID	DCCEEW trip	Date range	No. sites	No. samples
T1	28	20/05/24 – 27/06/24	17	36
T2	29	24/07/24 – 01/08/24	7	14
T3	30	08/08/24 – 28/08/24	16	34
T4	31	06/09/24 – 24/09/24	15	30
T5	33	10/11/24 – 20/11/24	14	30

Table 2. List of site locations where eDNA samples were collected, their geographic coordinates, and the number of samples (biological replicates) and field trips (temporal replicates) per site.

Site	Lat	Long	LGA	RCI subcatchment	No. samples	No. trips
S1	-31.559	143.511	Central Darling	Lower Paroo	10	5
S2	-31.553	143.401	Central Darling	Lake Woytchugga	10	5
S4	-31.864	143.123	Central Darling	Wilcannia downstream	8	4
S5	-31.961	142.976	Central Darling	Wilcannia downstream	6	3
S6	-32.076	142.986	Central Darling	Wilcannia downstream	8	4
S7	-32.170	142.786	Central Darling	Wilcannia downstream	8	4
S9	-32.317	142.556	Central Darling	Lake Wetherell	12	5
S11	-32.358	142.461	Central Darling	Lake Wetherell	10	5
S12	-32.397	142.421	Central Darling	Lake Wetherell	10	5
S14	-32.418	142.383	Central Darling	Lake Wetherell	4	2
S17	-32.769	142.380	Central Darling	Lower Yampoola creek	8	4
S20	-33.387	142.569	Wentworth	Upstream Pooncarie	10	4
S22	-33.701	142.339	Wentworth	Downstream Pooncarie	6	3
S23	-33.963	141.957	Wentworth	Lower Darling	8	4

S24	-34.027	141.814	Wentworth	Lower Anabranch	8	4
S27	-33.269	141.792	Wentworth	Anabranch North	2	1
S29	-32.717	142.093	Unincorporated-Far West Area	Lower Redbank Creek	8	4
S30	-32.660	142.192	Unincorporated-Far West Area	Cawndilla	6	3

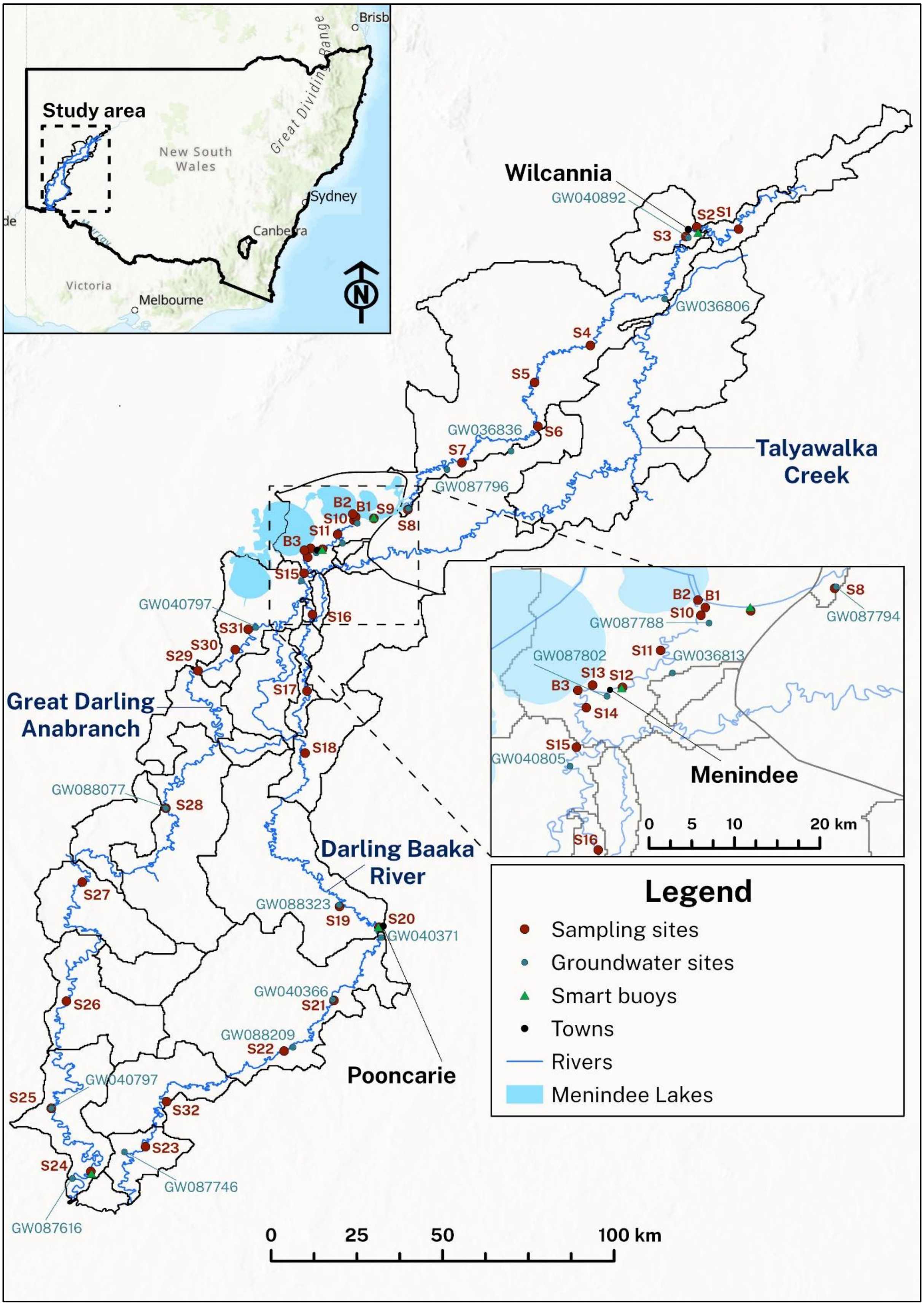

Figure 1. RCI sub-region boundaries for the lower Darling Baaka study area, showing Phase 2 sampling sites for the DBRHP.

METHODOLOGY

FIELD SAMPLING

Water samples were collected using sterilised 4 L containers, first rinsing the container with site water 10 times before collecting samples upstream. Samples were filtered in two stages by vacuum filtration on 0.45 and 0.70 µm mixed cellulose ester membranes (Pall Corporation, Port Washington, NV, USA). First, up to 1 L of water was prefiltered through a 0.7 µm syringe filter and then a 0.45 µm membrane, to aid removal of debris and algae that clog membranes. Filtering was carried out until either the full volume was filtered, or 2 hours had passed. Next, up to 200 mL of unfiltered water was added to the same 0.45 µm membrane. The amount of prefiltered (0.7 µm) and unfiltered water passed through the 0.45 µm membrane varied between samples, and in some cases no unfiltered water was added. Membranes were transported at -20°C and stored at -80°C until DNA extraction. Sterile techniques were utilised throughout, including sterilising all filtration equipment with 0.5 % sodium hypochlorite and sterilising forceps with flame. Field controls were collected by filtering 500 mL of DNA-free water alongside samples.

DNA EXTRACTION AND SEQUENCING

Filter membranes were cut into ~3 mm^2 pieces and DNA was extracted using the DNeasy Power Soil Pro Kit (Qiagen, Australia) under sterile conditions. Extraction controls (no filter or sediment) were included for each batch of DNA extractions for quality control.

An 80 bp long DNA metabarcode located in the 12S mtDNA gene region, Aves02, was chosen to target birds (F: 5'-GAAAATGTAGCCCATTTCTTCC-3', R: 5'-CATACCGCCGTCGCCAG-3') (Taberlet et al., 2018). Aves02 has good taxonomic resolution and specificity to birds (Taberlet et al., 2018). This metabarcode has previously been used to successfully detect waterbirds at inland Australian wetlands, including both abundant and rare or cryptic species (Davis et al., *in prep*). Aves02 theoretically amplifies a higher percentage of Australian waterbirds based on *in silica* PCRs (62 %) compared to other published primer sets such as Aves01 (51 %), Aves03 (32 %) and MiBird (34 %) (unpublished data).

PCR's were conducted in triplicate using 8 bp individual tagged-fusion primers (Taberlet et al., 2018). Each 96 well plate contained 12 blanks, 5-8 extraction or field controls, 3 negative controls (no template), 3 positive controls (synthetic sequences) and 70-73 samples. PCRs were carried out in a Mastercycler X50s (Eppendorf, Germany) in 20 µL reaction volumes consisting of: 10.0 µL AmplitTaq Gold 360 Master Mix (Life Technologies, Australia), 0.5 µL tagged forward fusion-primer at 0.5 µM final concentration, 0.5 µL reverse primer at 0.5µM final concentration, 7.0 µL of UltraPure™ DNase/RNase-free distilled water and 2.0 µL of DNA sample (1:10 dilution for Aves01, undiluted for Aves02). Thermocycling conditions were: 3-minute initial denaturation at 95°C, followed by 50 cycles of 1) 45-second denaturation at 95°C, 2) 45-second annealing at 58°C and 3) 15-second extension at 72°C, followed by a final extension step for 10 minutes at 72°C.

PCR products were pooled in equal volumes into a sterilised 50 mL falcon tube, purified using AMPure XP beads (Beckman-Coulter Life Sciences) and quantified on a Qubit® 3.0 Fluorometer (Thermo Fisher Scientific). The purified library was sent to the Ramaciotti Centre for Genomics (University of New South Wales, Australia) for Illumina MiSeq sequencing with NextFLEX library kits, using 2 x 150 bp (PE).

BIOINFORMATICS AND QUALITY CONTROL

Sequence data was processed by first checking read quality with *FastQC* v 0.11.9 (Andrews, 2010) and then using the Greenfield Hybrid Analysis Pipeline (*GHAP*) v 2.3 (Greenfield, 2017) to carry out demultiplexing, cleaning and classification. Processing reads with the GHAP pipeline included: 1) demultiplexing reads, allowing 2 mismatches on primers; 2) applying an Illumina base call quality score filter > 25; 3) merging paired reads; 4) dereplication; 5) trimming to remove outlier read lengths (77 – 83 bp); 6) filtering to remove potential genomic DNA, degraded sequences or chimeras; and 7) clustering with a 97 % similarity threshold to generate molecular operational taxonomic units (MOTUs).

Initial taxonomic assignments were performed with *ublast* (Usearch) in the GHAP pipeline, BLASTing MOTUs against the National Center for Biotechnology Information (NCBI) GenBank database of 12S mtDNA genes for class Aves (accessed 06/02/2025). The database was curated by dereplicating sequences and filtering to remove low-complexity k-mers, resulting in a database of 5,628 sequences. The best match with e-value < 1E-10 was retained for each MOTU, and assignment score cut-offs at each taxonomic level used the default parameters: phylum 77 %, class 80 %, order 85 %, family 90 %, genus 95 % and species 97 %. For example, taxa that matched with less than 97 % identity were dropped to genus level. MOTUs unassigned at the Kingdom level were removed.

Further filtering to minimise artefacts was performed with the R package *LULU* v 0.1.0 (Frøslev et al., 2017), merging MOTUs that are likely to be erroneous versions of more abundant MOTUs. Quality control at the PCR replicate and sample levels were carried out with the R package *metabaR* v.1.0.0 (Zinger et al., 2021) and included: 1) a tag-jump filter of 0.01 %; 2) a read depth filter to remove PCR replicates with read depth < 80, based on read counts in extraction and PCR negative controls; and 3) removal of contaminant MOTUs, identified based on extraction, PCR negative and sequencing controls (blanks). Any MOTUs with less than 10 reads across all samples were removed.

The initial NCBI classifications were manually curated to fix assignment errors. For example, taxa were dropped to genus level if there were multiple matches to Australian species with 97 % identity or higher. To more accurately classify MOTUs to species level, a second reference sequence database containing Australian waterbird 12S sequences was curated from the National Biodiversity DNA Library (NBDL). The NBDL reference database was compiled on 11/03/2025. For both classification approaches (NCBI and NBDL), the specificity index (B_s, the percentage of well-identified taxa out of all taxa amplified) was calculated at each taxonomic rank (Ficetola et al., 2010), along with the relative proportion of reads assigning to different taxa at each taxonomic rank.

STATISTICAL ANALYSES

SITE DIVERSITY AND COMMUNITY COMPOSITION

Sampling effort was assessed with MOTU accumulation curves with the function 'specaccum' from the R package *vegan* v 2.6-6.1 (Oksanen et al., 2007) (method = Coleman, expected MOTU richness, with 100 permutations). Data were normalised using a proportion-based approach, calculating relative MOTU richness per sample using the 'Wisconsin double-standardisation' method in *vegan*. Five diversity metrics were calculated including Shannon's diversity index (H), Simpson's diversity index (D), Pielou's evenness index ($J' = H/H_{max}$), family richness and genus richness. The Shannon index considers both MOTU richness and evenness and is sensitive to rare taxa, while the Simpson index reflects the dominance of the most abundant MOTUs (higher D indicates lower diversity). Differences between sites in sample-level diversity was assessed with a pairwise permutation test, using the false discovery rate (FDR) approach to correct for multiple testing.

Variation in avian community composition at the trip and site levels was assessed with a PERMANOVA using the function 'adonis2' in *vegan*, using normalised MOTU data to calculate Bray-Curtis dissimilarity indices. The PERMANOVA was run using a nested model (trips nested within sites) with 999 permutations. Next, two ordination methods were used to assess similarity between samples: a non-metric multi-dimensional scaling (nMDS) analysis (based on the Bray-Curtis dissimilarity index) and a principal coordinates analysis (PCoA).

ENVIRONMENTAL ANALYSES

A total of 21 environmental variables were included in this study (Table 3). Measures of water quality were obtained at the sampling trip level, while land use, biodiversity (macroinvertebrate and zooplankton), riparian condition, and water level measures were obtained at the site level (Table 3). Pesticides and metals were excluded, as many trips or sites were missing data for these variables and imputing missing values using the mean biased results in the PCA.

First, a principal components analysis (PCA) using only environmental data was conducted to identify variables contributing the most to variation in environmental conditions between sites. This analysis was carried at the sampling trip level, to account for variation in environmental conditions between sampling trips, and at the site level (averaging variables across sampling trips for each site).

To examine the influence of environmental variables on avian MOTU community composition, a redundancy analysis (RDA) was carried out using the R package *vegan*. In brief, RDA first conducts a principal components analysis (PCA) of the explanatory variables (environmental data) to reduce dimensionality and then performs a constrained ordination analysis to identify the top synthetic variables that best explain the variance in the response variable (MOTU data). The environmental variables listed in Table 3 were used as the explanatory variables, and relative MOTU richness, normalised with the Wisconsin double-standardisation method, was used as the response variable.

To identify environmental variables influencing the overall level of diversity among samples (Shannon diversity, family richness and genus richness), a linear mixed-effects (LME) model was run with the 'lme' function from the *lmerTest* R package v 3.1-3. Site and sampling trip were treated as random effects to account for inherent variability in the response variable (diversity) at the site and sampling trip levels unrelated to the predictor variables (environmental data). Fixed effects included all environmental variables listed in Table 3, scaled to account for differences in units and centred to reduce collinearity. Because water turbidity can cause filter clogging, which may negatively affect sample-level MOTU diversity, two additional fixed effects were included: the total amount of water filtered and filter weight per sample. Model selection was performed with the 'step' function from *lmerTest*, using the 'backwards selection' approach to first remove non-significant random effects and then non-significant fixed effects to select the optimal model (Kuznetsova et al., 2015).

Table 3. Environmental variables included in the RDA. For further details see Table A3 in Appendix A.

Category	Code	Variable	Sampling Level
Landscape disturbance	LDI	Landscape disturbance index	Subcatchment
Macroinvertebrates	MFR	Macroinvertebrate family richness	Site
Macroinvertebrates	MSS	Macroinvertebrate SIGNAL score	Site
Macroinvertebrates	MPR	Macroinvertebrate POET richness	Site
Water level	WLV	Water level score	Site
Water level	MSW	Maximum stream width (m)	Site
Riparian condition	RIP	Riparian condition score (prop)	Site
Zooplankton	ZOO	Zooplankton health score	Site
Water quality	DOC	Dissolved organic carbon (ug/L)	Trip
Water quality	FDOM	Fluorescent organic matter (QSU)	Trip
Water quality	DO	Dissolved oxygen (%)	Trip
Water quality	SPC	Specific conductance (uS/cm)	Trip
Water quality	PH	pH	Trip
Water quality	NTU	Nephelometric turbidity units	Trip
Water quality	TSS	Total suspended solids (mg/L)	Trip
Water quality	CHLA	Chlorophyll-a (ug/L)	Trip
Water quality	BGA	Blue-green algae phycocyanin (ug/L)	Trip
Water quality	AMM	Ammonia (mg/L)	Trip
Water quality	OXN	Oxidised N (mg/L)	Trip
Water quality	NIT	Total nitrogen (mg/L)	Trip
Water quality	PHO	Total phosphorous (mg/L)	Trip

SITE-LEVEL SPECIES DETECTION

Two detection approaches were used to record avian species detected in eDNA samples. In the relaxed approach, a species was considered detected in a sample if at least 1/3 PCR replicates were positive for the species. In the strict approach, at least 2/3 PCR replicates had to be positive. Species considered present at a site were checked against records extracted from the Atlas of Living Australia (ALA) database. At a site level, species detected with eDNA were compared against ALA species recorded within a 1 km radius around each site, as well as all species recorded across the entire study area within a 1km buffer of the river network. eDNA detections were also compared against ALA records for waterbirds only (orders Anseriformes (ducks and geese), Charadriiformes (shorebirds), Ciconiiformes (storks), Gruiformes (cranes and rails), Pelecaniformes (ibises, herons, spoonbills and pelicans), Podicipediformes (grebes) and Suliformes (cormorants)).

Lastly, eDNA detected species were compared against waterbird species recorded during the annual Eastern Australian Waterbird Survey (EAWS) coordinated by the University of New South Wales (UNSW) for sites located near the Menindee Lakes system (Kingsford et al., 2020). EAWS aerial surveys were carried out in October 2024 and were compared against eDNA detections for six sites, including four sites within the Lake Wetherell subcatchment (S9, S11, S12 and S14) and two sites near Lake Tandou (S29 and S30). These sites were all located within 24 km of one of the four major lakes of the Menindee Lakes system (Lake Cawndilla, Lake Menindee, Pamamaroo Lake and Lake Wetherell). This comparison was carried out at the regional rather than site-level, as EAWS sites did not overlap with the study sites.

RESULTS

QUALITY CONTROL AND SAMPLING EFFORT

Sequencing yielded a total of 12,297,109 reads. After clustering with the GHAP pipeline, filtering with the LULU and metabaR packages, removing non-avian MOTUs and carrying out taxonomic reassignment, 390,461 reads (3 % of the original sequences) across 42 avian MOTUs were retained. MetabaR filtering retained 77.23 % of PCR replicates following read depth and contamination filters. After manual curation of taxonomic assignments using the NBDL reference sequence database, avian MOTU's matched to 35 bird species, with 83.3 % of MOTUs assigning to species level.

The mean (± S.D.) volume of prefiltered water per sample (0.70 µm filters followed by 0.45 µm filters) was 326 mL (± 243 mL), the mean unfiltered volume (0.45 µm filter only) was 194 mL (± 93 mL) and the mean total volume was 416 mL (± 307 mL). Mean 0.45 µm filter weight was 0.24 g (± 0.06 g). Filter volume and weight was not significantly correlated with avian read depth or MOTU richness. There was also no significant correlation between site sample sizes and site measures of avian read depth, MOTU richness or diversity, except when the site with the smallest sample size (S27, n = 2) was included. All downstream site-based statistics excluded S27 to minimise this bias.

MOTU accumulation curves indicated that additional sampling may be required to fully capture the diversity of avian taxa present at each site (Figure 2). The maximum number of samples collected per site was 12, with typically 2 samples collected per sampling trip. Increasing the number of samples collected per sampling trip for each site may enable a greater diversity of avian taxa to be detected with eDNA.

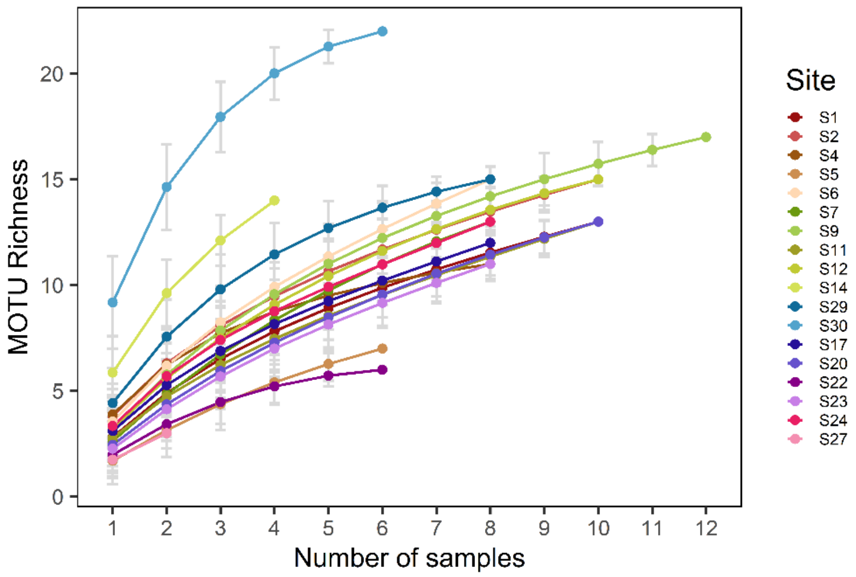

Figure 2. MOTU accumulation curves for each site, with reads pooled across PCR replicates for each sample. Most sites have shallow curves that do not reach an asymptote, indicating a gradual increase in MOTU richness with each additional sample.

SITE DIVERSITY AND COMMUNITY COMPOSITION

Across all sites, there were relatively more MOTUs and reads attributed to waterbirds compared to terrestrial taxa (Figure 3). Among waterbird taxa, relatively more reads were attributed to fish-eating birds (pelicans and cormorants) compared to other groups, a pattern that was consistent across most sites. Among all avian taxa, there were generally no significant differences between sites in sample-level MOTU diversity after correcting for multiple testing. However, for waterbird taxa, mean Shannon diversity (H) across samples was significantly higher at S30 compared to most other sites ($p < 0.05$), except for S14 and S29. Mean genus richness (for all birds and for waterbirds) and mean waterbird family richness, were also significantly higher at S30 compared to most other sites ($p < 0.05$). See Figure 4 and Appendix A, Tables A1-A2 for summaries of diversity statistics by site. Figure 5 shows a heat map of mean waterbird Shannon diversity, family richness and genus richness averaged across samples for each site.

At the sampling trip level, mean *H* across all samples and sites was significantly lower for T1 compared to T3, T4 or T5 ($p < 0.05$) and for T2 compared to T5 ($p < 0.05$) (Appendix A, Figure A1). Among waterbird taxa, T1 was significantly lower than T3 or T5 ($p < 0.05$). At the site level, diversity varied between trips and was not consistently lower at T1 or T2. For example, site S30 had the highest Shannon diversity overall, which was consistently high between sampling trips (T1, T3, T5).

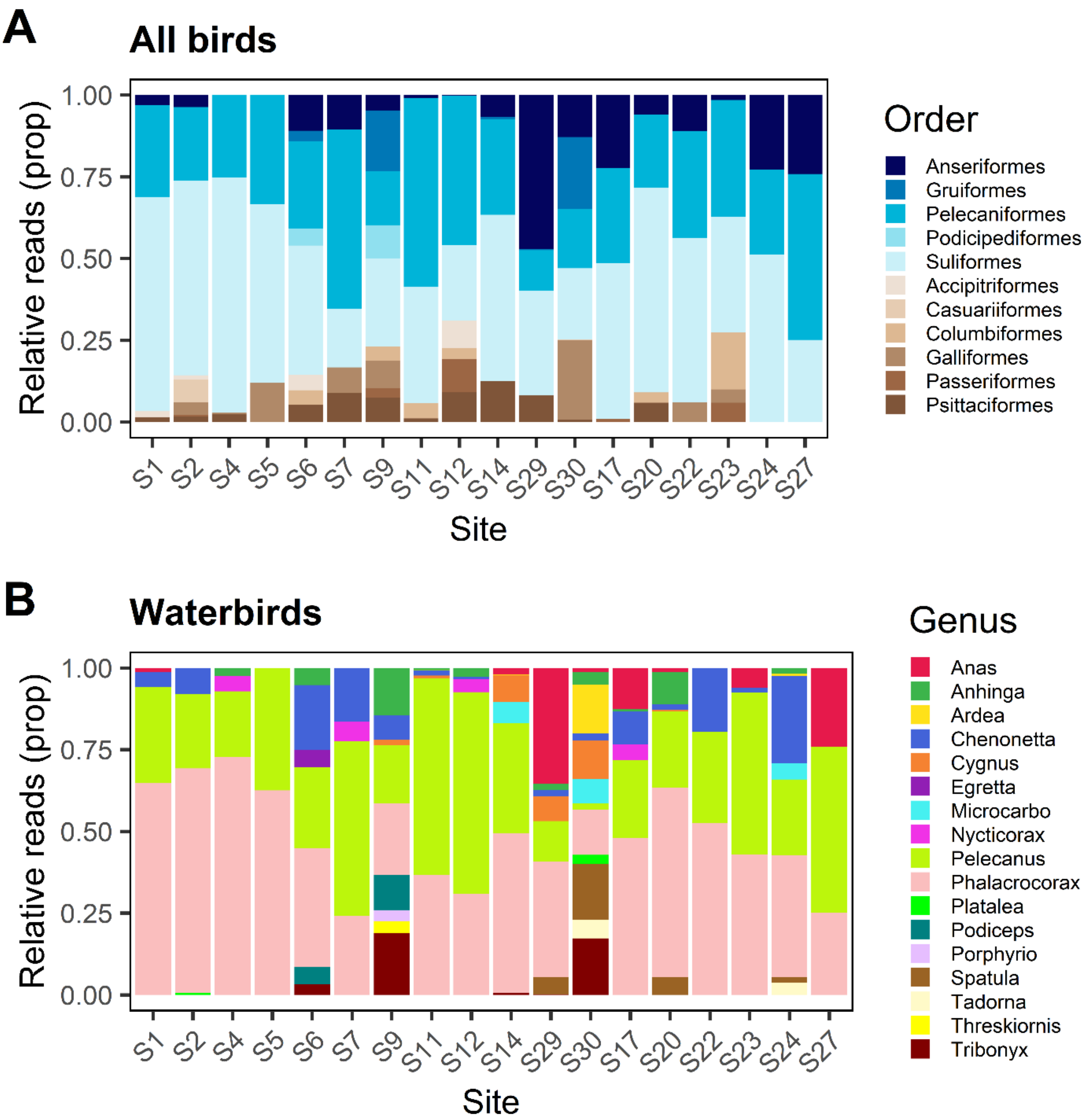

Figure 3. A) Relative abundance of reads assigned to each avian order per site (blue = aquatic taxa, brown = terrestrial taxa). B) Relative abundance of reads assigned to each waterbird genus per site. Note that site S27 only had 2 samples available. Species classifications were based on the NBDL reference sequence database.

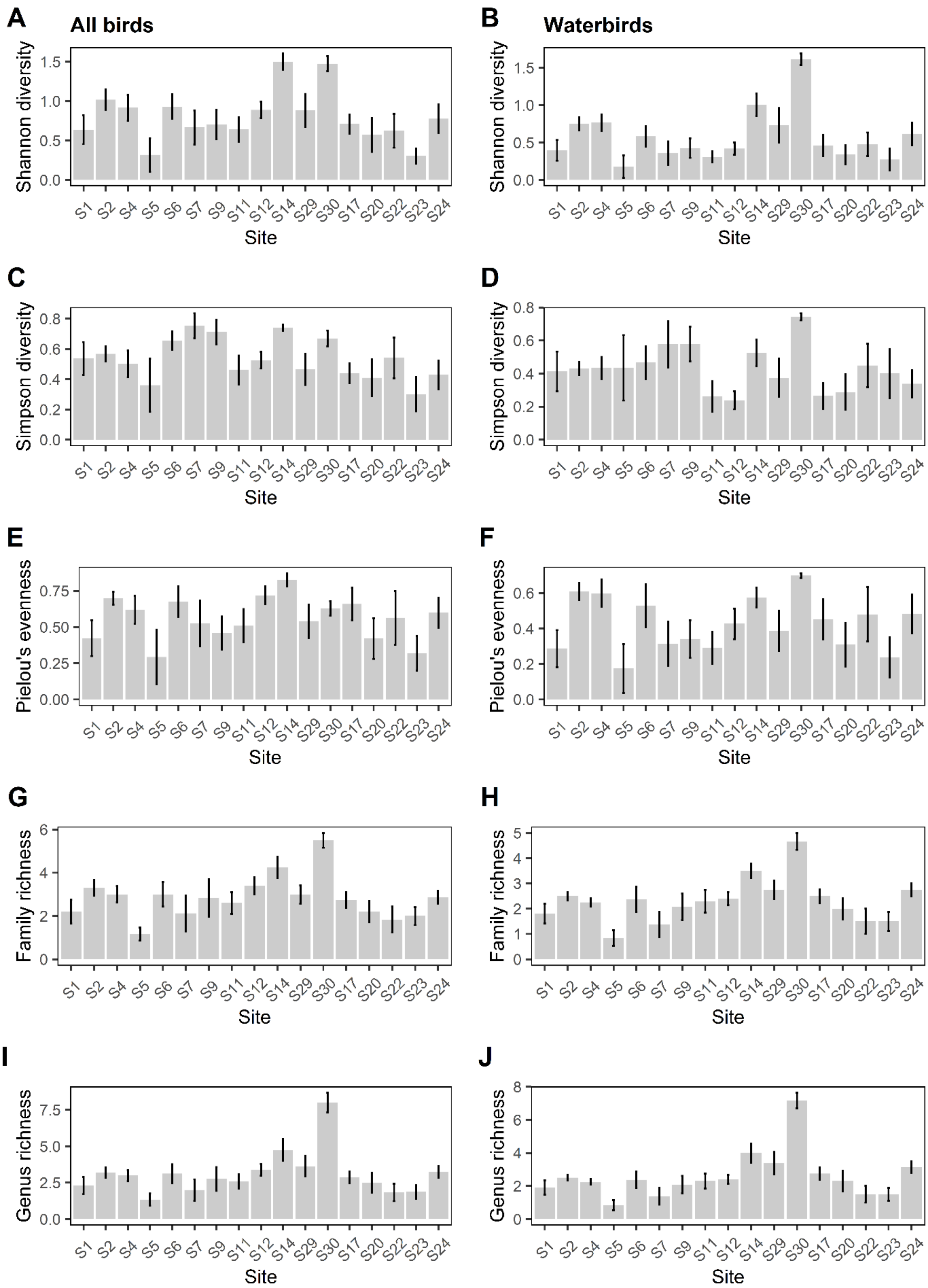

Figure 4. Mean sample-level avian MOTU diversity compared between sampling sites, for all birds (A, C, E) and waterbird taxa only (B, D, F). Error bars denote standard error. Note that higher Simpson diversity indicates lower overall diversity (greater dominance by a few taxa, fewer rare taxa). Site S27 is excluded as only 2 samples were collected. Species classifications were based on the NBDL reference sequence database.

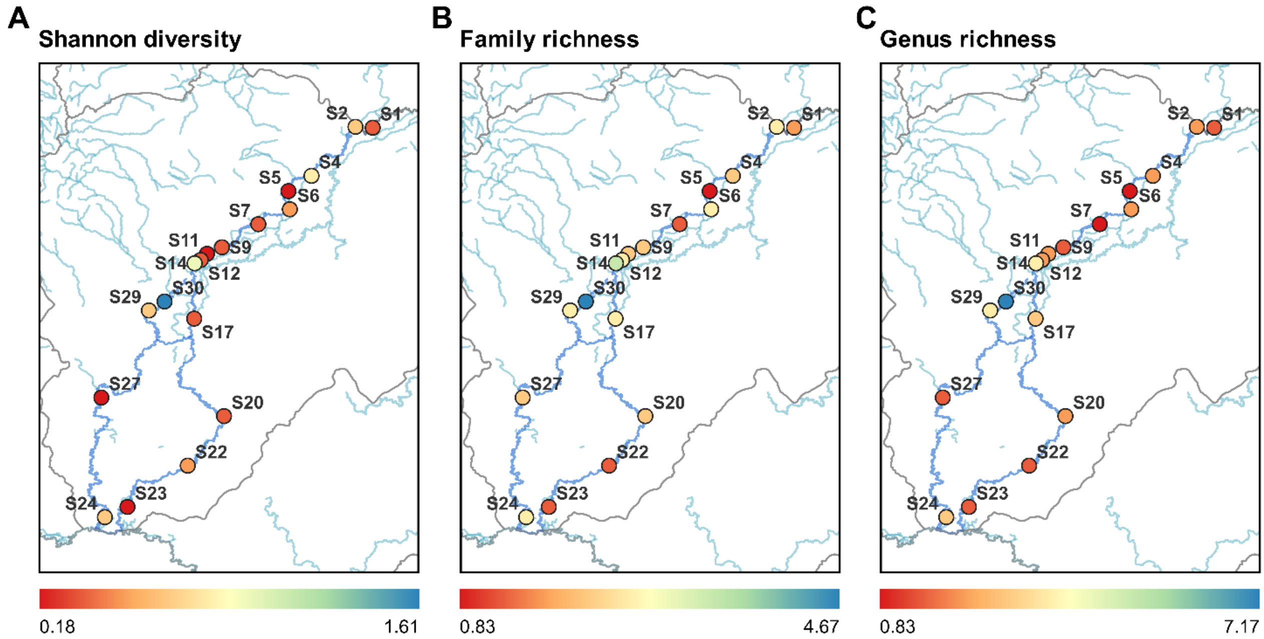

Figure 5. Heatmap of three metrics of sample-level waterbird MOTU diversity for each site (i.e., averaged across samples for each site), including A) Shannon's diversity index, B) waterbird family richness (number of unique waterbird families detected within a sample), and C) waterbird genus richness (number of unique waterbird genera detected within a sample).

The nested PERMANOVA (sampling trips nested within sites) indicated a significant effect of both sampling trip and site location on avian community composition, with sites explaining 20.4 % of the total variance ($F = 2.26$, $df = 17$, $p < 0.001$) and sampling trips within sites explaining a further 47.7 % of the total variance ($F = 1.84$, $df = 49$, $p < 0.001$). Similarly, for waterbird taxa only, sites explained 21.4 % of total variance ($F = 2.37$, $df = 17$, $p < 0.001$) and sampling trips within sites explained a further 47.3 % of total variance ($F = 1.86$, $df = 49$, $p < 0.001$). These results indicate that a substantial amount of variation is attributed to variance within sites and within trips nested within sites.

The nMDS analysis and PCoA indicated that most samples collected from within the same site were not more similar to each other than to samples from other sites, both for all avian taxa and for waterbirds alone (Figure 6 & Appendix A, Figure A2). High within-site variability may explain the lack of site-level clustering observed in the nMDS and PCoA.

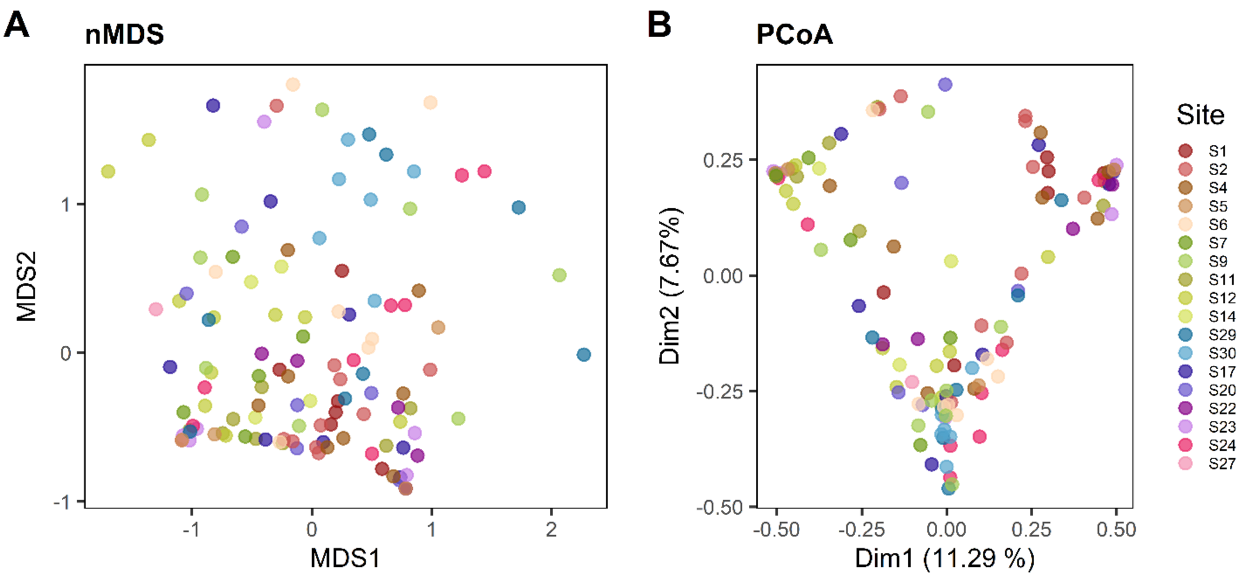

Figure 6. Similarity between samples in community composition (waterbird MOTUs only) using A) a non-metric multi-dimensional scaling (nMDS) analysis (stress = 0.088), and B) a principal coordinates analysis (PCoA), with samples coloured by site and functional zone. Both analyses used MOTU data standardised with the Wisconsin double-standardisation approach, and the Bray-Curtis dissimilarity index.

ENVIRONMENTAL ANALYSES

PCA OF ENVIRONMENTAL VARIABLES

In the PCA of environmental variables alone (sampling trip level), the top two PCs explained the most variance in environmental conditions across sites and trips (PC1 = 32.1 %, PC2 = 19.8 %) (Figure 7). PH, SPC, CHLA, FDOM, DO and MPR contributed the most to PC1, while NIT, PHO, NTU and OXN contributed the most to PC2 (see Figure 8 for site summaries of these variables). Sites located within the Central Darling LGA (sites S1 to S14) were generally characterised by higher FDOM and lower NIT, CHLA and MPR, compared to sites S17 to S30 (Figure 7). At the site level (environmental data averaged across trips for each site), PC1 and PC2 explained 44.7 % and 16.3 % of variance in environment conditions among sites (Figure 9). SPC, CHLA and NIT contributed most to PC1, while PHO, LDI and NTU contributed most to PC2. Overall, the PCA of environmental variables indicated that differences between sites were largely driven by water quality, particularly measures related to the level of salts, minerals, pollutants or organic matter present (SPC, FDOM, NTU), nutrient levels (NIT, PHO, OXN) and chlorophyll-a levels (CHLA).

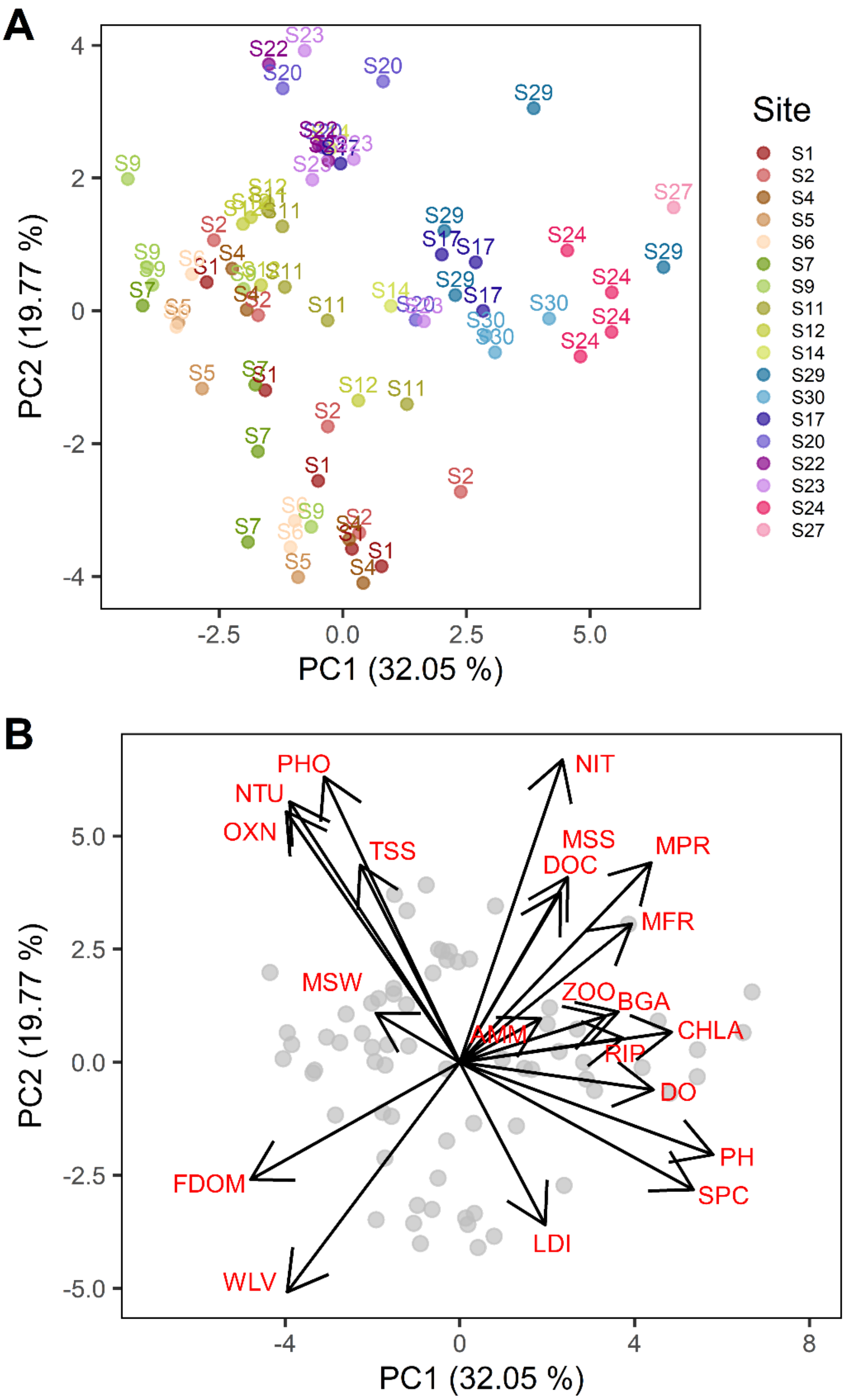

Figure 7. Principal components analysis (PCA) of environmental variables at the sampling trip level, showing A) a scatterplot of the first and second PC, with sampling trips coloured by site and functional zone; and B) a biplot mapping the relative contributions of each environmental variable (red) to PC1 and PC2, with sampling trips coloured grey. For additional information on these environmental variables, see Table 3 and Appendix A, Table A3.

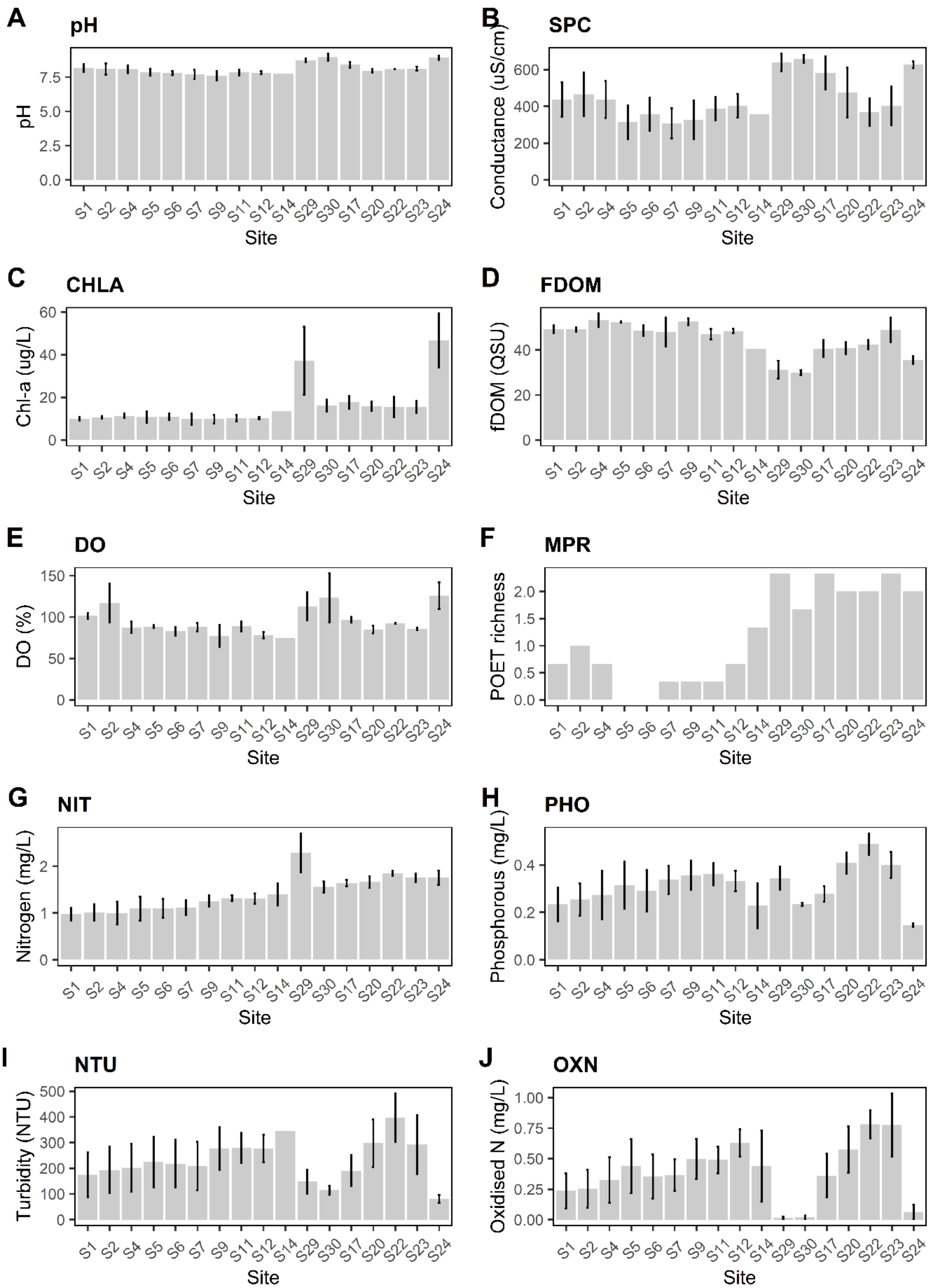

Figure 8. Mean (± S.E.) value per site (across sampling trips) for the top ten environmental variables that contributed to PC1 and/or PC2 in the PCA, for A) pH, B) specific conductance, C) concentration of chlorophyll-a pigment, D) fluorescent organic matter, E) dissolved oxygen (%), F) POET richness, G) total nitrogen, H) total phosphorous, I) nephelometric turbidity units, and J) oxidised N. For additional information on these environmental variables, see Table 3 and Appendix A, Table A3.

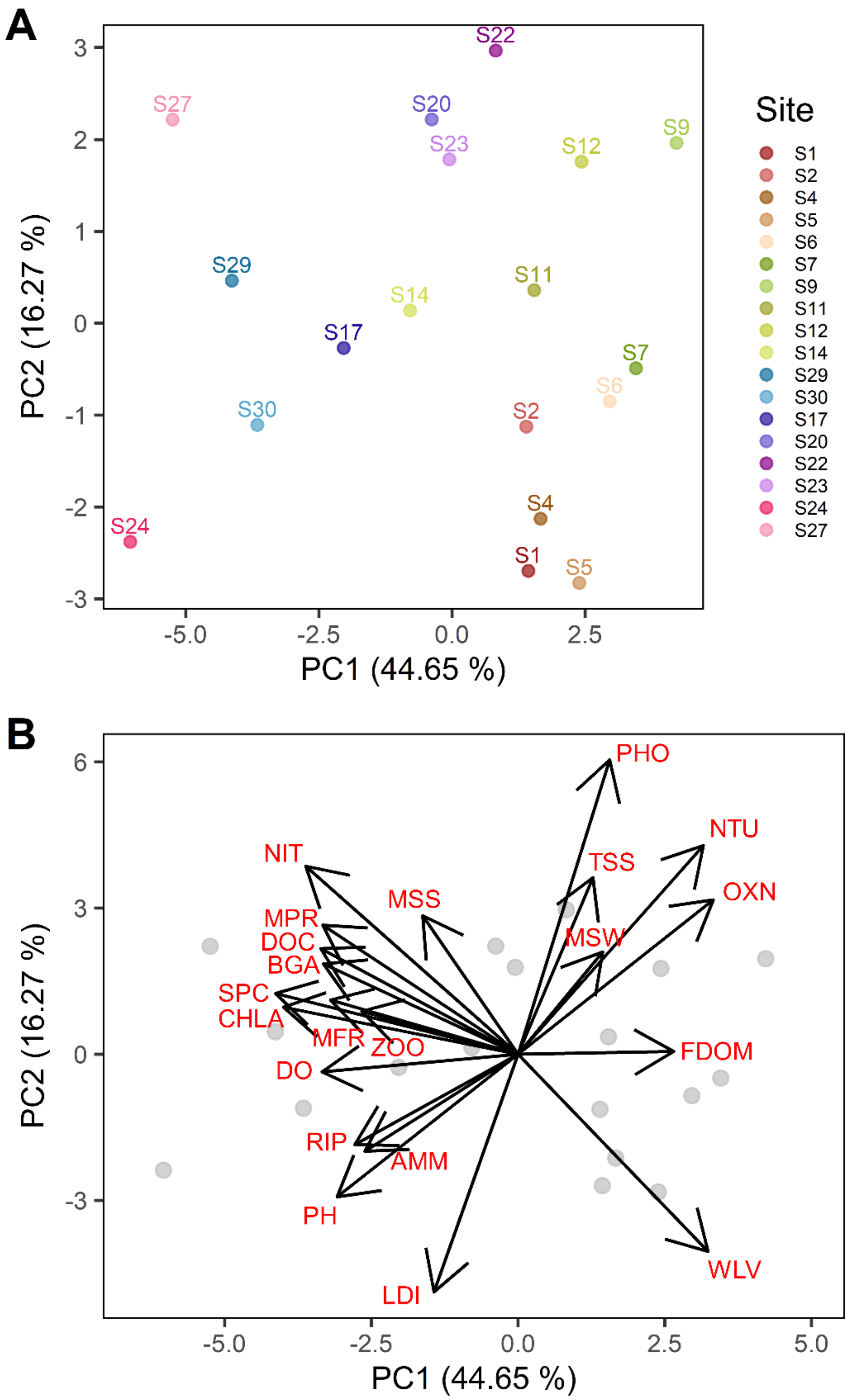

Figure 9. Principal components analysis (PCA) of environmental variables at the site level, showing A) a scatterplot of the first and second PC, with points coloured by site and functional zone; and B) a biplot mapping the relative contributions of each environmental variable (red) to PC1 and PC2, with sites coloured grey. For additional information on these environmental variables, see Table 3 and Appendix A, Table A3.

REDUNDANCY ANALYSIS

The RDA of environmental and eDNA data indicated that 20.8 % of variation in eDNA community composition was explained by the environmental variables tested, significantly more than expected by random chance based on permutation tests (F = 1.31, df = 21, p < 0.001). The remaining 76.2 % of the variation in community composition was not explained by the environmental variables tested, which could be attributed to inherent variation within sampling trips or sites, other stochastic variation, or untested environmental conditions.

Of the variation explained by environmental variables, the constrained axes (RDA1 and RDA2) explained 19.3 % and 11.5 %, respectively (Figure 10A). A total of 15 environmental variables were significantly correlated with either RDA1 or RDA2. The variables significantly contributing to RDA1 were PH, DO, FDOM, RIP, SPC, OXN, NTU, and DOC, and for RDA2 were NIT, NTU, PHO, TSS, MSS, MPR, and LDI (p < 0.05) (Figure 10A). More waterbird MOTUs were associated with the negative RD1 axis (higher pH, DO, RIP and SPC) (Figure 10B). However, this may be driven by higher overall waterbird diversity detected at site S30.

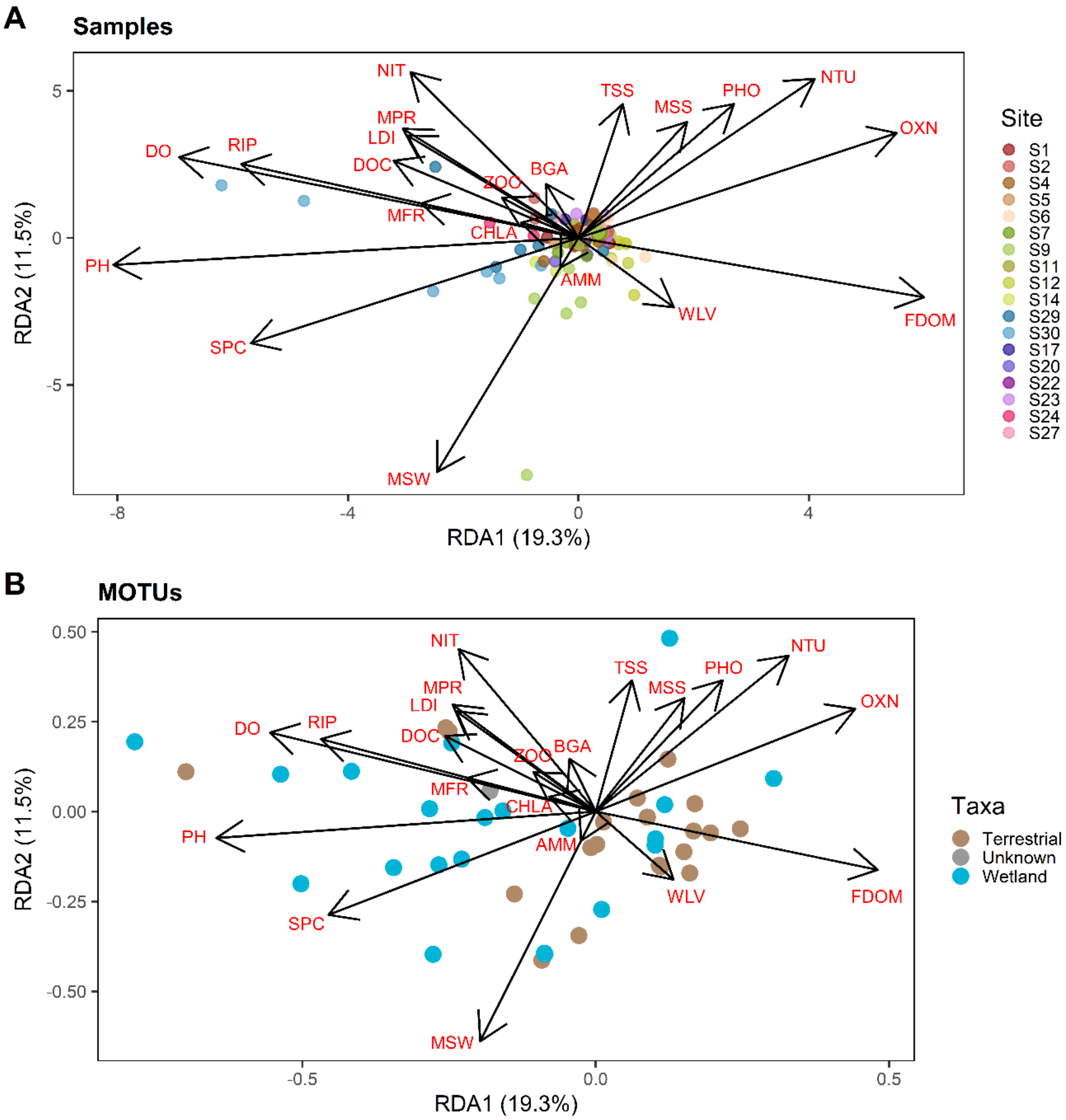

Figure 10. Redundancy analysis (RDA) using MOTU data for all birds and 21 environmental variables. A) Sample scores coloured by site, with environmental variable loadings shown in red (arrows indicates the relative contribution of each variable to RDA1 and RDA2 axes. B) MOTU scores coloured by habitat (waterbirds vs terrestrial birds). For additional information on these environmental variables, see Table 3 and Appendix A, Table A3.

Among waterbird MOTUs, 23.8 % of variation in eDNA community composition was explained by the environmental variables tested, significantly more than expected by random chance ($F = 1.53$, $df = 21$, $p < 0.001$). The constrained axes (RD1 and RD2) explained 23.8 % and 14.0 %, respectively, of the variation explained by environmental variables (Figure 11). Eight environmental variables were significantly correlated with either RDA1 or RDA2. The variables significantly contributing to RDA1 were PH, SPC, OXN, FDOM, NTU, RIP and DO, and for RD2 were DO, NTU and MPR ($p < 0.05$). Overall, the RDA indicated that a small, but significant percentage of variation in waterbird eDNA community composition was influenced primarily by water quality measures (pH, SPC, OXN, FDOM, NTU and DO), riparian condition (RIP) and macroinvertebrate POET richness (MPR).

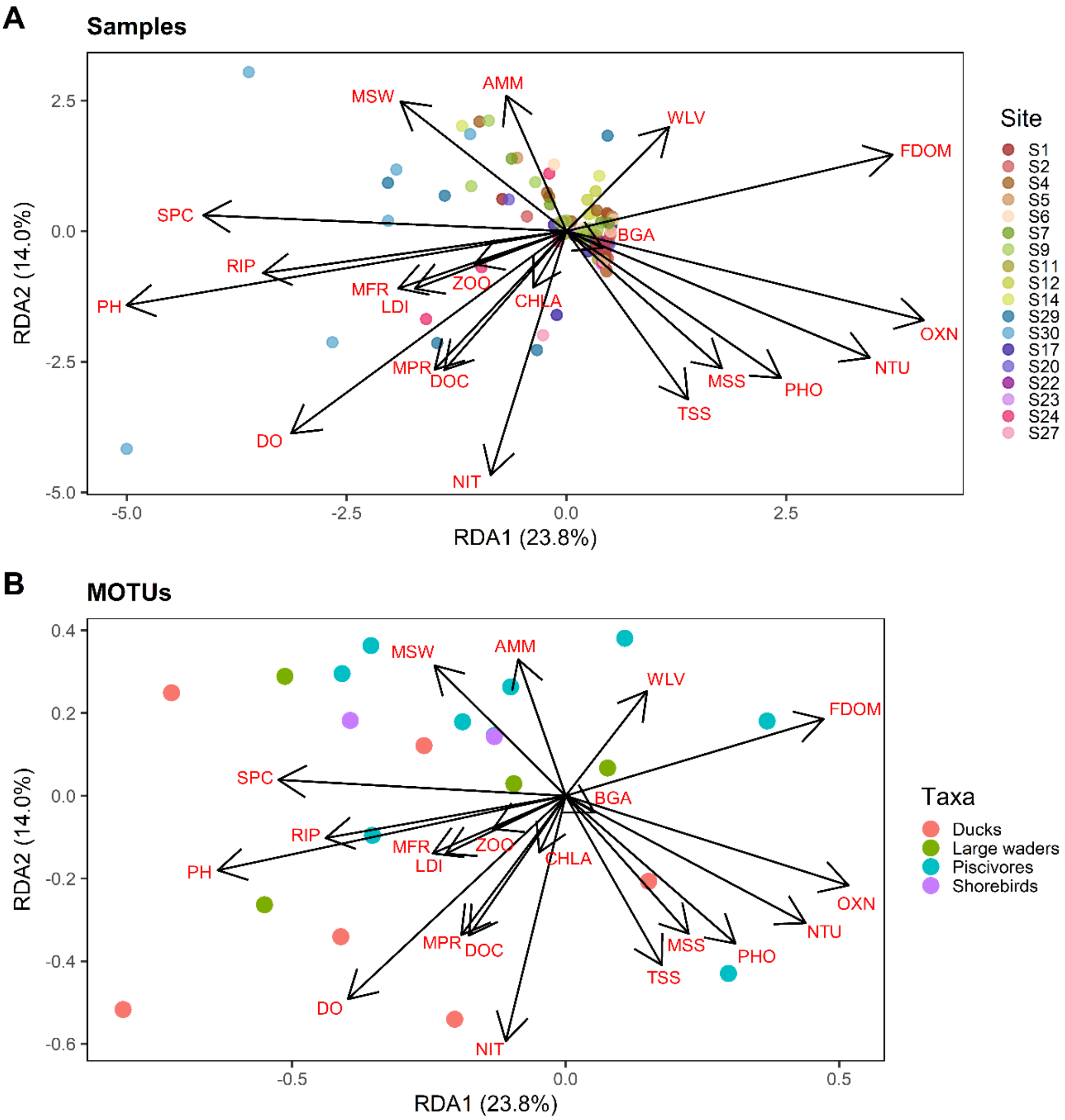

Figure 11. Redundancy analysis (RDA) using MOTU data for waterbird taxa and 21 environmental variables. A) Sample scores coloured by site, with environmental variable loadings shown in red (arrows indicates the relative contribution of each variable to RDA1 and RDA2 axes. B) MOTU scores coloured by waterbird functional group. For additional information on these environmental variables, see Table 3 and Appendix A, Table A3.

LINEAR MIXED-EFFECTS MODELLING

Nine environmental variables were identified as significant predictors of avian or waterbird MOTU diversity (Shannon diversity, family richness and/or genus richness) (Table 4). For all bird taxa, FDOM, CHLA and NIT were negatively correlated with all diversity measures. Family and genus richness were positively correlated with SPC, and genus richness was positively correlated with RIP and negatively correlated with AMM. Relative to other environmental variables, FDOM had the strongest effect on Shannon diversity, while SPC had the strongest effect on family and genus richness.

Among waterbirds, SPC and RIP were positively correlated with all diversity measures, while FDOM and NIT were negatively correlated with diversity (Table 4). Other variables that were negatively correlated with one or more waterbird diversity metric included CHLA, AMM, BGA and DOC. Relative to other environmental variables, CHLA had the strongest effect on Shannon diversity, SPC on family richness, and FDOM on genus richness. Although higher algal biomass, turbidity and dissolved organic matter can lead to filter membranes clogging, reducing DNA capture and thus potentially lowering avian diversity, filter weight and total water volume filtered were not significant predictors for any diversity metric.

Table 4. LME coefficients [a] (S.E. in brackets) for nine environmental variables that were significant predictors ($p < 0.05$) of MOTU diversity (measured as Shannon diversity, family richness or genus richness per sample), for all avian taxa and for waterbirds only. Correlations of fixed effects were all less than 0.7. For additional information on these environmental variables, see Table 3 and Appendix A, Table A3.

Predictor variable		**All birds**			**Waterbirds**		
		Shannon	**Family**	**Genus**	**Shannon**	**Family**	**Genus**
SPC	Specific conductance	n.s.	0.76 (0.15)	0.70 (0.15)	0.16 (0.04)	0.51 (0.12)	0.46 (0.14)
FDOM	Dissolved organic matter	-0.30 (0.06)	-0.50 (0.18)	-0.65 (0.20)	-0.14 (0.06)	-0.44 (0.15)	-0.62 (0.19)
CHLA	Chlorophyll-a	-0.19 (0.06)	-0.47 (0.16)	-0.73 (0.17)	-0.21 (0.05)	-0.35 (0.13)	n.s.
NIT	Total nitrogen	-0.15 (0.06)	-0.43 (0.16)	-0.44 (0.17)	-0.16 (0.05)	-0.27 (0.12)	-0.35 (0.15)
AMM	Ammonia	-0.13 (0.04)	n.s.	-0.47 (0.13)	-0.12 (0.04)	n.s.	-0.27 (0.12)
RIP	Riparian condition	n.s.	n.s.	0.67 (0.23)	0.18 (0.07)	0.27 (0.11)	0.54 (0.19)
BGA	Blue-green algae phycocyanin	n.s.	n.s.	n.s.	n.s.	n.s.	-0.48 (0.13)
DOC	Dissolved organic carbon	n.s.	n.s.	n.s.	0.09 (0.04)	n.s.	n.s.
NTU	Turbidity	-0.21 (0.05)	n.s.	n.s.	n.s.	n.s.	n.s.

[a] Predictor variables were scaled and centred prior to analysis, meaning each LME coefficient represents the expected increase (or decrease) in the response variable for every one standard deviation increase in the predictor variable, while holding all other predictors at their mean rather than zero.

SITE-LEVEL SPECIES DETECTION

COMPARISON OF REFERENCE SEQUENCE DATABASES

Species classifications were improved by using the NBDL reference sequence database compared to the NCBI database. Using the NBDL database, more MOTUs were classified to species level (83.3 %) and fewer classified only to family (4.7 %) or class (0 %) levels compared to the NCBI database (species = 65.1 %, family = 23.5 % and class = 2.3 %). A total of 29 bird species were detected using the NCBI database while 35 bird species were detected using the NBDL database (Table 5). Further details are provided in Appendix A. Comparisons between detection approaches and ALA records below were based on NBDL species classifications.

RELAXED DETECTION

With relaxed detection (at least 1/3 PCR replicates had a positive detection), up to 18 bird species were detected within a site across all samples (mean = 10.3, S.D. = 3.7) (Table 5 & Appendix A, Table A4). Five species were detected at almost every site: Australian pelican (*Pelecanus conspicillatus*), Australian wood duck (*Chenonetta jubata*), and three species of cormorants (*Phalacrocorax carbo, P. sulcirostris* and *P. varius*). Sites with the highest number of bird species recorded were S30 (n = 18), S6 (n = 14), S9 (n = 15) and S12 (n = 13). S9 and S30 also had the highest number of waterbird species recorded (n = 15 and 11, respectively). Although terrestrial birds comprised 46 % of bird species recorded, the mean (± S.D.) number of terrestrial species recorded per site was only 2.6 (± 1.6) compared to waterbirds (7.7 ± 2.9). Relatively more terrestrial bird species were detected in the northern region of the study area (sites S1 to S12) and relatively more shorebirds were detected at sites located near the Menindee Lakes system (sites S9, S14, S29 and S30) (Figure 12A).

STRICT DETECTION

With strict detection (at least 2/3 PCR replicates had a positive detection), up to 14 bird species were detected within a site across all samples (mean = 5.3, S.D. = 2.7) (Table 5 & Appendix A, Table A5). Dominant taxa detected were similar to the relaxed detection approach, but many less abundant taxa were lost. Sites with the highest number of bird species recorded were S30 (n = 14), S9 (n = 8) and S29 (n = 8). Terrestrial birds comprised 30 % of bird species recorded and the mean number of terrestrial species recorded per site was 0.4 (± 0.6) compared to 4.8 (± 2.3) for waterbirds. More species from the functional groups ‘large waders’ and ‘shorebirds’, as well as terrestrial birds, were lost with strict detection (Figure 12B).

Table 5. Avian species detected with eDNA, sorted by total number of eDNA reads per species. ‘Sites’ gives the number of sites where this species was considered ‘detected’ under a relaxed detection approach (at least 1/3 PCR replicates was positive) and a strict detection approach (at least 2/3 PCR replicates were positive). Species classifications were based on the NBDL reference sequence database. Due to limited availability of sub-species level sequence data, species listed in bold may also represent contamination of domesticated species DNA from nearby properties [ab].

Scientific name	Common name	Habitat	Distribution	Reads	Sites	
					Relaxed	Strict
Phalacrocorax carbo	Great cormorant	Wetland	Australian	68,999	17	17
Meleagris gallopavo [a]	**Wild turkey**	**Terrestrial**	**Introduced**	**54,433**	**2**	**1**
Pelecanus conspicillatus	Australian pelican	Wetland	Endemic	50,869	18	18
Phalacrocorax sulcirostris	Little black cormorant	Wetland	Australian	22,704	18	16
Psephotus haematonotus	Red-rumped parrot	Terrestrial	Endemic	22,011	9	3
Tribonyx ventralis	Black-tailed native hen	Wetland	Endemic	17,513	5	2
Cygnus atratus	Black swan	Wetland	Endemic	16,368	7	5
Chenonetta jubata	Australian wood duck	Wetland	Endemic	13,429	14	6
Phalacrocorax varius	Great pied cormorant	Wetland	Australian	12,003	16	11
Ardea alba modesta	Eastern great egret	Wetland	Australian	11,298	4	1
Gallus gallus [b]	**Red junglefowl**	**Terrestrial**	**Introduced**	**8,617**	**9**	**0**
Anhinga novaehollandiae	Australasian darter	Wetland	Australian	6,692	10	5
Eolophus roseicapilla	Galah	Terrestrial	Endemic	4,556	4	0
Anas superciliosa	Pacific black duck	Wetland	Australian	4,294	8	0
Threskiornis moluccus	Australian white ibis	Wetland	Australian	4,049	1	0

Scientific name	Common name	Habitat	Distribution	Reads	Sites Relaxed	Strict
Platycercus elegans	Crimson rosella	Terrestrial	Endemic	3,968	1	0
Ocyphaps lophotes	Crested pigeon	Terrestrial	Endemic	3,897	3	1
Melopsittacus undulatus	Budgerigar	Terrestrial	Endemic	3,828	2	1
Haliastur sphenurus	Whistling kit	Terrestrial	Australian	3,367	4	0
Nycticorax caledonicus	Nankeen night heron	Wetland	Australian	2,787	4	0
Egretta novaehollandiae	White-faced heron	Wetland	Australian	2,619	1	0
Milvus migrans	Black kite	Terrestrial	Australian	2,445	4	1
Nymphicus hollandicus	Cockatiel	Terrestrial	Endemic	2,318	1	0
Microcarbo melanoleucos	Little pied cormorant	Wetland	Australian	1,223	3	1
Tadorna tadornoides	Australian shelduck	Wetland	Endemic	1,025	2	1
Platalea flavipes	Yellow-billed spoonbill	Wetland	Endemic	835	2	1
Geopelia placida	Peaceful dove	Terrestrial	Australian	370	2	0
Petrochelidon ariel	Fairy martin	Terrestrial	Endemic	213	1	0
Sturnus vulgaris	Common starling	Terrestrial	Introduced	201	1	1
Calyptorhynchus banksii	Red-tailed black cockatoo	Terrestrial	Endemic	190	1	0
Podiceps cristatus	Great crested grebe	Wetland	Australian	182	3	1
Spatula rhynchotis	Australasian shoveler	Wetland	Australian	84	4	2
Phaps chalcoptera	Common bronzewing	Terrestrial	Endemic	44	1	0
Dromaius novaehollandiae	Emu	Terrestrial	Endemic	32	2	0
Porphyrio porphyrio melanotus	Australasian purple swamphen	Wetland	Australian	10	1	0

[a] The MOTU assigned to *Meleagris gallopavo* could not be classified to sub-species level and thus may represent wild introduced turkey, which have not been recorded in the study area based on the Atlas of Living Australia or alternatively, contamination of domesticated turkey (*M. g. domesticus*) DNA from nearby properties.
[b] The MOTU assigned to *Gallus gallus* could not be classified to sub-species level and thus may represent wild introduced red junglefowl, which have been recorded in south-western NSW based on the Atlas of Living Australia or alternatively, contamination of domesticated chicken (*G. g. domesticus*) DNA from nearby properties.

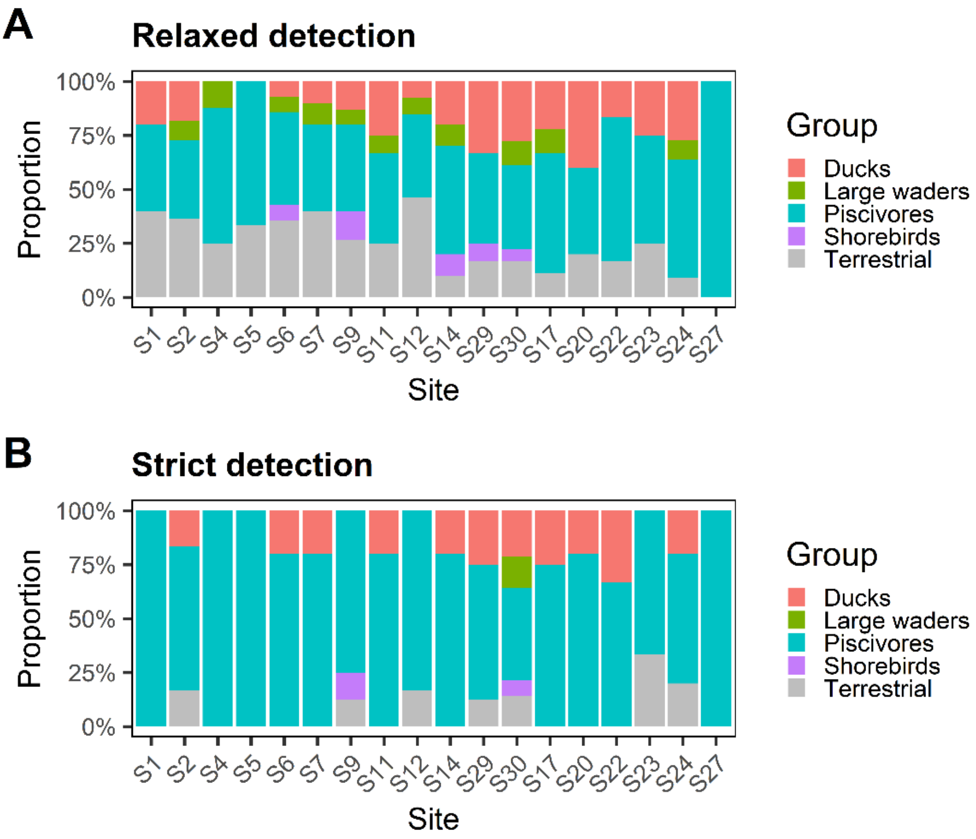

Figure 12. The relative proportion of species detected based on their functional group (ducks, large waders, piscivores, shorebirds and terrestrial birds), compared between A) a relaxed detection approach (at least 1/3 PCR replicates had to be positive for a species) and B) a strict detection approach (at least 2/3 PCR replicates had to be positive for a species).

COMPARISON WITH ALA RECORDS

All taxa detected with eDNA have been historically recorded across the study area based on ALA records, except for *Meleagris gallopavo* (wild turkey or domesticated turkey *M. g. domesticus*). Compared against site-specific ALA records, there was wide variation in the number of ALA recorded species that were detected with eDNA. Five sites lacked site-level ALA records (S5, S7, S11, S27, and S30), and another seven sites lacked recent records from the last three years, 2022, 2023, and 2024 (S2, S4, S6, S22, S23, S24, and S29) (Tables 6-7). In most cases, eDNA missed a large portion of avian taxa recorded at each site but also captured additional species not previously recorded. For sites with historical ALA records available, an average of 83.0 % (S.D. = 26.3) of historically recorded taxa were missed by eDNA; and an average of 55.5 % (S.D. = 37.1) of eDNA detected taxa were not previously recorded, based on a relaxed detection approach (Table 6). With strict detection, the average percentage of historically recorded species missed by eDNA rose to 87.4 % (S.D. = 27.1). When compared against recent ALA records (last three years), an average of 74.6 % (S.D. = 18.3) of ALA recorded species were missed by eDNA; and an average of 76.2 % (S.D. = 16.3) of eDNA detected species were not previously recorded, based on relaxed detection. With strict detection, the average percentage of ALA recorded species missed by eDNA rose to 90.5 % (S.D. = 11.6)

Table 6. Comparison between avian species detected with eDNA and all historical Atlas of Living Australia (ALA) records for each site (defined by 1 km buffer around each site). Matched = number of species that were historically recorded and detected with eDNA, missing = number of species that were historically recorded but missed with eDNA, and extra = species detected with eDNA but not historically recorded. Species classifications were based on the NBDL reference sequence database.

	Relaxed detection					Strict detection				
Site	**eDNA**	**ALA**	**Matched**	**Missing**	**Extra**	**eDNA**	**ALA**	**Matched**	**Missing**	**Extra**
S1	10	22	2	20	8	4	22	0	22	4
S2	11	15	2	13	9	6	15	1	14	5
S4	8	1	0	1	8	4	1	0	1	4
S5	6	0	0	0	6	3	0	0	0	3
S6	14	1	1	0	13	5	1	1	0	4
S7	10	0	0	0	10	5	0	0	0	5
S9	15	28	9	19	6	8	28	7	21	1
S11	12	0	0	0	12	5	0	0	0	5
S12	13	104	12	92	1	6	104	6	98	0
S14	10	81	9	72	1	5	81	4	77	1
S17	9	2	0	2	9	4	2	0	2	4
S20	10	91	8	83	2	5	91	4	87	1
S22	6	1	0	1	6	3	1	0	1	3
S23	8	57	5	52	3	3	57	2	55	1
S24	11	84	8	76	3	5	84	4	80	1
S27	2	0	0	0	2	2	0	0	0	2
S29	12	54	9	45	3	8	54	5	49	3
S30	18	0	0	0	18	14	0	0	0	14

Table 7. Comparison between avian taxa detected with eDNA and recent (last 3 years) Atlas of Living Australia (ALA) records for each site (defined by 1 km buffer around each site). Matched = number of species that were historically recorded and detected with eDNA, missing = number of species that were historically recorded but missed with eDNA, and extra = species detected with eDNA but not historically recorded. Species classifications were based on the NBDL reference sequence database.

	Relaxed detection					Strict detection				
Site	**eDNA**	**ALA**	**Matched**	**Missing**	**Extra**	**eDNA**	**ALA**	**Matched**	**Missing**	**Extra**
S1	10	2	1	1	9	4	2	0	2	4
S2	11	0	0	0	11	6	0	0	0	6
S4	8	0	0	0	8	4	0	0	0	4
S5	6	0	0	0	6	3	0	0	0	3
S6	14	0	0	0	14	5	0	0	0	5
S7	10	0	0	0	10	5	0	0	0	5
S9	15	18	6	12	9	8	18	4	14	4
S11	12	0	0	0	12	5	0	0	0	5
S12	13	8	3	5	10	6	8	2	6	4
S14	10	24	3	21	7	5	24	0	24	5
S17	9	2	0	2	9	4	2	0	2	4
S20	10	21	4	17	6	5	21	2	19	3
S22	6	0	0	0	6	3	0	0	0	3
S23	8	0	0	0	8	3	0	0	0	3
S24	11	0	0	0	11	5	0	0	0	5
S27	2	0	0	0	2	2	0	0	0	2
S29	12	0	0	0	12	8	0	0	0	8
S30	18	0	0	0	18	14	0	0	0	14

Among waterbird taxa, an average of 69.0 % (S.D. = 28.1) of historically recorded waterbird species were missed by eDNA; and an average of 47.9 % (S.D. = 40.2) of eDNA detected waterbird species were not previously recorded, based on a relaxed detection approach (Table 8). With strict detection, the average percentage of historically recorded waterbird species missed by eDNA rose to 78.6 % (S.D. = 28.0). Only four sites had records of waterbird species for the past three years (sites S9, S12, S14, and S20). For these sites, an average of 53.1 % (S.D. = 15.0) of recently recorded waterbird species were missed by eDNA; and an average of 64.3 % (S.D. = 14.0) of eDNA detected waterbird species were not previously recorded, based on a relaxed detection approach (Table 9). With strict detection, the average percentage of recently recorded waterbird species missed by eDNA rose to 75.7 % (S.D. = 20.5).

Table 8. Comparison between waterbird species detected with eDNA and all historical Atlas of Living Australia (ALA) waterbird records for each site (defined by 1 km buffer around each site). Matched = number of species that were historically recorded and detected with eDNA, missing = number of species that were historically recorded but missed with eDNA, and extra = species detected with eDNA but not historically recorded. Species classifications were based on the NBDL reference sequence database.

	Relaxed detection					**Strict detection**				
Site	**eDNA**	**ALA**	**Matched**	**Missing**	**Extra**	**eDNA**	**ALA**	**Matched**	**Missing**	**Extra**
S1	6	1	0	1	6	4	1	0	1	4
S2	7	2	0	2	7	5	2	0	2	5
S4	6	0	0	0	6	4	0	0	0	4
S5	4	0	0	0	4	3	0	0	0	3
S6	9	1	1	0	8	5	1	1	0	4
S7	6	0	0	0	6	5	0	0	0	5
S9	11	24	9	15	2	7	24	7	17	0
S11	9	0	0	0	9	5	0	0	0	5
S12	7	29	7	22	0	5	29	5	24	0
S14	9	19	8	11	1	5	19	4	15	1
S17	8	0	0	0	8	4	0	0	0	4
S20	8	17	6	11	2	5	17	4	13	1
S22	5	1	0	1	5	3	1	0	1	3
S23	6	12	4	8	2	2	12	1	11	1
S24	10	20	7	13	3	4	20	3	17	1
S27	2	0	0	0	2	2	0	0	0	2
S29	10	24	8	16	2	7	24	5	19	2
S30	15	0	0	0	15	12	0	0	0	12

Table 9. Comparison between waterbird species detected with eDNA and recent (last 3 years) Atlas of Living Australia (ALA) waterbird records for each site (defined by 1 km buffer around each site). Matched = number of species that were historically recorded and detected with eDNA, missing = number of species that were historically recorded but missed with eDNA, and extra = species detected with eDNA but not historically recorded. Species classifications were based on the NBDL reference sequence database.

	Relaxed detection					**Strict detection**				
Site	**eDNA**	**ALA**	**Matched**	**Missing**	**Extra**	**eDNA**	**ALA**	**Matched**	**Missing**	**Extra**
S1	6	0	0	0	6	4	0	0	0	4
S2	7	0	0	0	7	5	0	0	0	5
S4	6	0	0	0	6	4	0	0	0	4
S5	4	0	0	0	4	3	0	0	0	3
S6	9	0	0	0	9	5	0	0	0	5
S7	6	0	0	0	6	5	0	0	0	5
S9	11	18	6	12	5	7	18	4	14	3
S11	9	0	0	0	9	5	0	0	0	5
S12	7	4	2	2	5	5	4	2	2	3
S14	9	3	2	1	7	5	3	0	3	5
S17	8	0	0	0	8	4	0	0	0	4
S20	8	8	3	5	5	5	8	2	6	3
S22	5	0	0	0	5	3	0	0	0	3
S23	6	0	0	0	6	2	0	0	0	2
S24	10	0	0	0	10	4	0	0	0	4
S27	2	0	0	0	2	2	0	0	0	2
S29	10	0	0	0	10	7	0	0	0	7
S30	15	0	0	0	15	12	0	0	0	12

COMPARISON WITH AERIAL SURVEY DATA

During the October 2024 aerial survey of the Menindee Lakes system, 35 unique waterbird taxa were recorded, including 31 classified to species level. Most waterbird species detected with eDNA at nearby sites (S9, S11, S12, S14, S29, and S30) were recorded in the annual aerial survey, except for Australasian purple swamphen (*Porphyrio porphyrio melanotus*), eastern great egret (*Ardea alba modesta*) and nankeen night heron (*Nycticorax caledonicus*). Due to limitations with consistently identifying some waterbirds from aerial surveys; small grebes, small egrets, terns and migratory wading birds are not classified to species level in the EAWS (Kingsford et al., 2020). As such, birds in the *Ardea* genus were recorded in the October 2024 survey but were not classified to species level. With the relaxed eDNA detection approach, an average of 69.9 % (S.D. = 8.8) of EAWS recorded waterbirds were missed by eDNA and 8.7 % (S.D. = 5.0) of eDNA detected species were not recorded in the EAWS. With strict detection, the percentage of EAWS recorded waterbirds missed by eDNA rose to 78.5 % (S.D. = 7.5).

KEY FINDINGS

EDNA-BASED WATERBIRD DIVERSITY AND COMMUNITY COMPOSITION

Overall, 35 avian species were detected using eDNA, 34 of which have previously been recorded within the study area based on historical ALA records. Five abundant waterbird species were detected at almost every site based on a relaxed detection approach: Australian pelican, Australian wood duck, and three species of cormorants. As expected for riverine sites, fish-eating species (piscivores) were the most detected taxa across sites. Other common waterbird species included Australasian darter (*Anhinga novaehollandiae*), Pacific black duck (*Anas superciliosa*) and black swan (*Cygnus atratus*).

More bird species, including more waterbirds, were detected at sites 6, 9, 12, and 30. Among waterbirds, mean sample-level diversity at the species, genus and family levels were significantly higher at site 30, followed by site 14. Across all samples, waterbird genus richness was highest at sites 9 and 30. These sites, except for site 6, are all located within 15km of one of the four major lakes of the Menindee Lake system. For example, site 30, located around 3 km from Kangaroo Lake, captured a wide range of waterbird functional groups across 13 genera, including ducks, piscivores, wading birds and shorebirds. Relatively more shorebirds were captured at sites located near the Menindee Lake system (sites 9, 14, 29 and 30). In contrast, sites 1, 4, 5, 7, 22, 23 and 27 detected fewer than six waterbird species across four genera (based on relaxed detection).

For sites with available ALA records, a large percentage of observationally recorded species were missed with eDNA. However, there was also a large percentage of eDNA detected species that were not previously recorded at each site, even though these species have been recorded elsewhere within the study area. For example, at site 9, eDNA missed 12 species recently recorded in the ALA database (including abundant ducks and cormorants) but detected an additional five (including common species such as Australian pelican and the cryptic species Australasian purple swamphen). Overall, more than half of the sites lacked or had sparse historical waterbird records and only four sites had recent records (last 3 years). In addition to ground-based and aerial surveys, and with further optimization for riverine ecosystems, eDNA may provide a complementary approach for site-level waterbird monitoring across the Darling Baaka River system.

IMPACT OF RIVER HEALTH ON WATERBIRD DIVERSITY AND COMMUNITY COMPOSITION

The results of the environmental analyses indicated that a small but significant percentage of variation in eDNA-based waterbird community composition was influenced by environmental variables, particularly water quality measures, riparian condition and macroinvertebrate POET richness. Further, eDNA-based estimates of waterbird diversity (Shannon diversity, family and genus richness) were significantly negatively associated with several indices of poorer water quality (higher algal biomass, dissolved organic matter and certain nutrients) and positively associated with better riparian condition scores and higher specific conductance. For example, sites 14, 30 and 24 had the highest riparian condition scores, and sites 14 and 30 also had the highest sample-level estimates of waterbird diversity. Higher specific conductance, which typically indicates higher concentrations of salts, minerals and/or pollution, may be correlated with another untested environmental variable that positively influences waterbird diversity. Overall, these results suggest river health influences waterbird diversity and community composition within the Darling Baaka River system, although additional fine-scale environmental data at the sampling-trip level is needed to more accurately assess this relationship.

STUDY LIMITATIONS

Further optimisation of eDNA protocols may improve species detection rates, particularly for rare or cryptic species. In particular, greater avian diversity may be captured by increasing the sampling effort per site. Another potential limitation was that a substantial number of eDNA reads were attributed to fish rather than birds prior to filtering (Appendix A), which may have lowered avian diversity overall. In wetlands with abundant breeding waterbirds, the Aves02 barcode performs well and has minimal amplification of fish (Davis et al., *in prep*). However, it may be less optimal for riverine sites with lower overall waterbird abundance. Using fish blocking primers, multi-barcode amplification or exploring the use of air eDNA samples to detect birds (e.g., Jin et al., 2025) may help resolve this issue.

Lastly, while many environmental variables were based on data collected during the same sampling trip as eDNA samples (water quality metrics), others were only obtained at a site level (i.e., single index for the site during the entire sampling period). Incorporating sampling trip level estimates of other variables, such as water levels, flow rates and changes in other vertebrate taxa diversity (e.g., fish) would enable more accurate comparisons between eDNA based waterbird diversity and the changing dynamics of riverine sites over time.

CONCLUSIONS

Australian waterbirds are well adapted to navigating the changing mosaic of wetland productivity and resource availability across the landscape; and are important indicators of wetland health. With widespread and ongoing wetland degradation, it is vital to monitor changes in waterbird assemblages in response to changes in wetland health and condition. eDNA offers a complementary approach to traditional ground or aerial-based waterbird monitoring, particularly in remote areas where baseline data on waterbird assemblages are missing.

This study aimed to establish baseline presence/absence data for waterbirds within the Darling Baaka River system using eDNA data and examine how river health influences eDNA based waterbird diversity. This was achieved through the analysis and results presented in this report and the species lists provided in Appendix A. While further work is needed to refine methodologies and address the challenges and limitations identified in this report, this study provided the first eDNA based assessment of avian assemblages across the Darling Baaka River system and how river health may impact waterbird diversity and community composition.

ACKNOWLEDGMENTS

The project was run and lead by NSW DCCEEW staff under the DBRHP, funded through the EPA natural disaster recovery funding, in collaboration with Anthony Chariton (Macquarie University). Sampling was undertaken by NSW DCCEEW, Water, Wetlands and Coastal Science Branch staff: Kathryn Korbel, Ben Humphreys, Bella Campbell, Jaydon King, Lachlan Hill, Zacchary Larkin. eDNA extractions were completed by Kathryn Korbel, Kurt Fahey, Kitty McKnight and Jaydon King. PCR and library preparation were completed by Christine Chivas. Additional environmental data were provided by Remi Rouquette, Megan Powell, Yoshi Kobayashi, Sophie Hewitt, Shawn Ryan, Guyo Gufu, Sharon Bowen, Anna Helfensdorfer, and Sarah O'Hea Miller (NSW DCCEEW). Waterbird species records were extracted from the Australian Living Atlas (ALA) for each sampling site by Chris Hasselerharm (NSW DCCEEW) and from the October 2024 annual Eastern Australian Waterbird Survey (EAWS) for the Menindee Lakes system (provided by John Porter, UNSW). The project leaders would also like to thank CSIRO's National Biodiversity DNA Library, and partners DCCEEW, Bioplatforms Australia and the Australian National Wildlife Collection, for making sequence reference data generated under that partnership available for this analysis. The findings presented in this report are based on a pre-release version of these reference data, by agreement with the NBDL, to support reporting to DCCEEW. A draft version of this report was reviewed by Kathryn Korbel, Jennifer Spencer and Yasmin Bokhari Friberg (NSW DCCEEW).

APPENDIX A: FURTHER DETAILS ON ANALYSIS AND RESULTS

Non-specific amplification

Prior to further filtering, more MOTUs were attributed to Aves than any other class (77.4 %), but fewer reads were attributed to Aves (13.2 %). Pre-filter reads were predominantly attributed to one MOTU assigned to the Actinopterygii class (bony fish). When excluding this MOTU, Aves comprised 69.3 % of reads.

Taxonomic resolution

Using the NCBI reference sequence database, out of 43 avian MOTUs, 9.3 % only matched to genus level, 23.5 % to family level and 2.3 % to class level. The specificity index at each taxonomic rank was 65.1 % for species, 74.4 % for genus and 97.7 % for family. The 4 MOTUs that were only assigned to genus level belonged to Corvus (crows and ravens), Spatula (shovelers), Phalacrocorax (cormorants) and Anus (ducks). For example, one MOTU matched with 98% identity to a rare vagrant (*Spatula clypeata,* Northern shoveler) likely due to the Australian species (*Spatula rhynchotis,* Australasian shoveler) being missing from the database. The 12 MOTUs that only assigned to family level belonged to Accipitridae (raptors), Anatidae (ducks and geese), Cacatuidae (cockatoos), Hirundinidae (swallows and martins), Meliphagidae (honeyeaters), Monarchidae (flycatchers and magpie-larks), Phalacrocoracidae (cormorants and shags), Phasianidae (pheasants, chickens, etc.), Psittacidae (Parrots), and Threskiornithidae (ibises and spoonbills).

Using the NBDL reference sequence database, 11.9 % of avian MOTUs only matched to genus level, 4.8 % to family level and 0 % to class level. The specificity index at each taxonomic rank was 83.3 % for species, 95.2 % for genus and 100 % for family. The 5 MOTUs that were only assigned to genus level belonged to Corvus (crows and ravens), Anus (ducks) and Cacatua (cockatoos and corellas). MOTUs assigning to Spatula and Phalacrocorax previously unclassified at the species level using the NCBI database were successfully classified with the NBDL database. However, a MOTU previously classified as Western corella (*Cacatua pastinator*) using the NCBI database was dropped to genus level using the NBDL database owing to multiple matches at the species level with a more complete reference database. The 2 MOTUs that were only classified at the family level belonged to Meliphagidae (honeyeaters) and Monarchidae (flycatchers and magpie-larks).

Site-level statistics

Table A1. Site-level statistics, showing the total number of avian reads and MOTUs, the mean number of MOTUs per sample, and three diversity indices (with standard error, S.E.).

Site	n	Nb Reads	Nb MOTU	Sample MOTUs		Shannon diversity		Pielou's Evenness		Simpson diversity	
				Mean	S.E.	Mean	S.E.	Mean	S.E.	Mean	S.E.
Avian MOTUs											
S1	10	17804	13	3.3	0.8	0.67	0.19	0.42	0.12	0.55	0.11
S2	10	20793	15	4.8	0.7	1.04	0.14	0.70	0.04	0.57	0.05
S4	8	9724	11	5.0	0.8	0.98	0.18	0.59	0.09	0.51	0.09
S5	6	754	7	1.8	0.9	0.36	0.25	0.30	0.19	0.37	0.18
S6	8	18398	15	4.1	0.9	0.96	0.16	0.69	0.11	0.67	0.06
S7	8	8421	13	2.9	1.2	0.71	0.24	0.53	0.16	0.76	0.08
S9	12	35163	17	3.6	1.0	0.71	0.19	0.45	0.11	0.71	0.08
S11	10	6328	13	3.4	0.7	0.66	0.16	0.51	0.12	0.47	0.10
S12	10	25309	15	3.8	0.5	0.89	0.11	0.72	0.06	0.53	0.05
S14	4	11021	14	7.3	1.2	1.55	0.11	0.81	0.05	0.75	0.02
S17	8	12806	12	3.8	0.6	0.73	0.12	0.63	0.11	0.45	0.07
S20	10	13031	13	2.8	0.9	0.58	0.22	0.43	0.14	0.41	0.12
S22	6	4861	6	2.3	0.7	0.63	0.22	0.56	0.19	0.54	0.13
S23	8	13565	11	2.5	0.7	0.27	0.09	0.30	0.12	0.28	0.11
S24	8	13283	13	4.0	0.7	0.79	0.19	0.60	0.10	0.43	0.09
S27	2	6555	3	2.0	1.0	0.55	0.55	0.50	0.50	0.33	0.33
S29	8	62642	15	5.3	1.2	0.86	0.20	0.53	0.11	0.44	0.10
S30	6	110000	22	11.8	1.1	1.49	0.12	0.62	0.06	0.67	0.06
Waterbird MOTUs											
S1	10	17081	9	2.9	0.7	0.47	0.16	0.32	0.11	0.45	0.12
S2	10	18410	8	3.9	0.5	0.77	0.10	0.61	0.05	0.44	0.04
S4	8	9276	8	4.3	0.6	0.92	0.15	0.63	0.08	0.49	0.08
S5	6	709	5	1.3	0.6	0.21	0.18	0.17	0.13	0.44	0.20
S6	8	13150	10	3.4	0.8	0.62	0.15	0.55	0.12	0.49	0.10

Site	n	Nb Reads	Nb MOTU	Sample MOTUs		Shannon diversity		Pielou's Evenness		Simpson diversity	
				Mean	S.E.	Mean	S.E.	Mean	S.E.	Mean	S.E.
S7	8	4837	8	2.1	0.9	0.41	0.19	0.33	0.13	0.61	0.14
S9	12	22354	12	2.8	0.7	0.45	0.13	0.35	0.11	0.59	0.10
S11	10	5268	9	3.0	0.7	0.35	0.09	0.31	0.09	0.29	0.09
S12	10	12247	7	2.8	0.4	0.42	0.08	0.43	0.09	0.24	0.05
S14	4	8474	12	6.3	0.9	1.05	0.14	0.57	0.04	0.54	0.08
S17	8	12768	10	3.5	0.5	0.50	0.15	0.45	0.11	0.28	0.08
S20	10	8950	10	2.5	0.8	0.33	0.14	0.31	0.13	0.29	0.11
S22	6	4804	5	2.0	0.5	0.48	0.16	0.48	0.15	0.45	0.13
S23	8	11033	7	1.9	0.6	0.21	0.15	0.15	0.10	0.36	0.16
S24	8	13241	12	3.9	0.6	0.63	0.16	0.48	0.11	0.34	0.08
S27	2	6555	3	2.0	1.0	0.19	0.19	0.17	0.17	0.10	0.10
S29	8	45094	12	4.9	1.1	0.73	0.26	0.38	0.12	0.35	0.13
S30	6	53707	18	10.7	0.8	1.70	0.07	0.72	0.01	0.76	0.02

Table A2. The number of unique families and genera detected per site for all avian taxa and for waterbirds or terrestrial taxa only, including MOTUs not identified to species level. Species classifications were based on the NBDL reference sequence database.

Site	Unique families			Unique genera		
	All birds	Waterbirds	Terrestrial	All birds	Waterbirds	Terrestrial
S1	7	3	4	8	4	4
S2	10	4	6	10	5	5
S4	7	4	3	7	4	3
S5	3	2	1	4	2	2
S6	11	7	4	12	7	5
S7	9	4	5	8	4	4
S9	12	7	5	13	9	4
S11	8	5	3	10	7	3
S12	10	5	5	13	5	8
S14	6	5	1	8	7	1
S17	7	5	2	8	7	1
S20	6	4	2	9	7	2
S22	4	3	1	4	3	1
S23	6	3	3	6	4	2
S24	6	5	1	10	9	1
S27	3	3	0	3	3	0
S29	7	5	2	10	8	2
S30	11	8	3	16	13	3

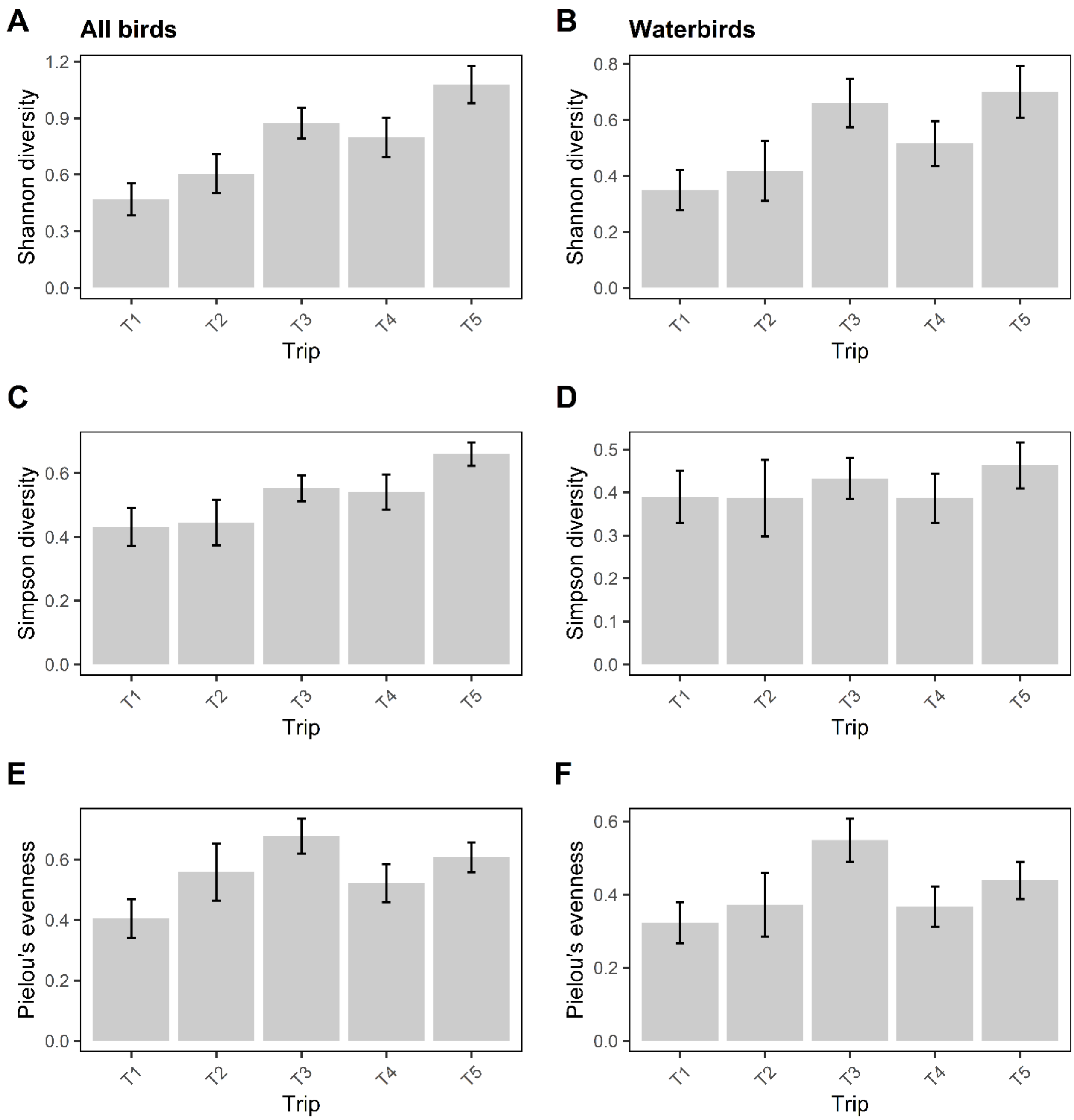

Figure A1. Mean MOTU diversity across samples for each sampling trip (error bars denote standard error) for three diversity metrics. T1 = DCCEEW trip 28, T2 = 29, T3 = 30, T4 = 31, T5 = 33.

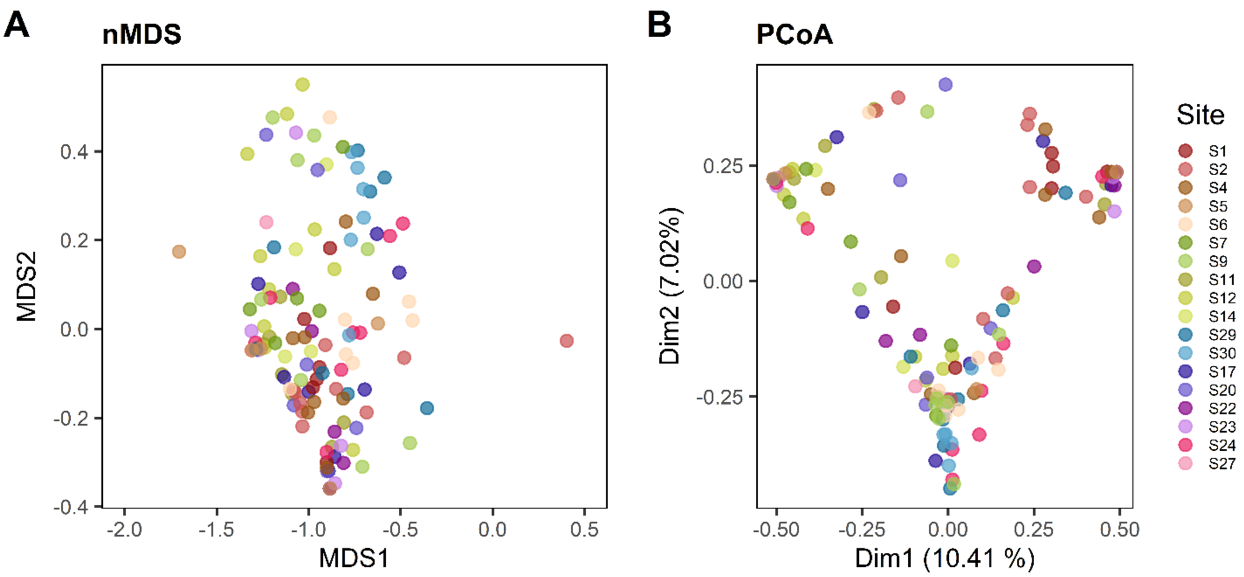

Figure A2. Similarity between samples in community composition (all avian MOTUs) based on a non-metric multi-dimensional scaling (nMDS) analysis, with samples coloured by site and functional zone (stress = 0.088) and using the Bray-Curtis dissimilarity index.

Table A3. Environmental variables included in the PCA, RDA, and LME analyses.

Code	Variable	Unit	Details
Subcatchment level variables			
LDI	Landscape disturbance index	Index	Index based on land use scores, infrastructure scores and land cover changes for 2024.
Site level variables			
MFR	Macroinvertebrate family richness	Count	Total no. macroinvertebrate families present, averaged across 3 sample replicates per site.
MSS	Macroinvertebrate SIGNAL score	Index	Average SIGNAL score per site (sum of SIGNAL scores divided by family richness), averaged across 3 sample replicates per site.
MPR	Macroinvertebrate POET richness	Count	Total no. POET macroinvertebrate families present, averaged across 3 sample replicates per site.
WLV	Water level score	Index	Rank of water level from: 0 = No flow, 1 = Low, 2 = Moderate, 3 = High or 4 = Flood.
MSW	Maximum stream width	Meters	Maximum stream width in meters.
RIP	Riparian condition	Index	Index based on scores for riparian habitat, cover, natives, debris and features; as a proportion of the total score possible.
ZOO	Zooplankton health	Index	Overall zooplankton health status based on the overall zooplankton community health score (ZCHS). Averaged across 2 temporal replicates (May/June and Oct/Nov). Ranges from 1-3.
Sampling trip level variables			
DOC	Dissolved organic carbon	µg/L	The fraction of organic carbon that passes through a filter (dissolved).
FDOM	Fluorescent organic matter	QSU	The fraction of dissolved organic matter that emits fluorescence when exposed to specific wavelengths of light, in quinine sulphate units (QSU).
DO	Dissolved oxygen	%	The percentage of oxygen saturation in water relative to the maximum amount it can hold at a given temperature.
SPC	Specific conductance	µS/cm	Measure of water's capacity to conduct electricity based on the presence of ions (salts, minerals, pollutants).
PH	pH	pH	Acidity or alkalinity of water.
NTU	Nephelometric turbidity units	NTU	Measure of water clarity, how much light is scattered by suspended particles.

Code	Variable	Unit	Details
TSS	Total suspended solids	mg/L	Organic + inorganic suspended solids.
CHLA	Chlorophyll-a	µg/L	Concentration of chlorophyll-a (from plants, photosynthetic algae and/or cyanobacteria).
BGA	Blue-green algae phycocyanin	µg/L	Concentration of phycocyanin pigment (from cyanobacteria).
AMM	Ammonia	mg/L	Ammonia, NH_3
OXN	Oxidised N	mg/L	Oxidised nitrogen (nitrate, nitrite and nitrous oxide).
NIT	Total nitrogen	mg/L	Organic nitrogen + ammonia + oxidised nitrogen.
PHO	Total phosphorous	mg/L	Total phosphorus.

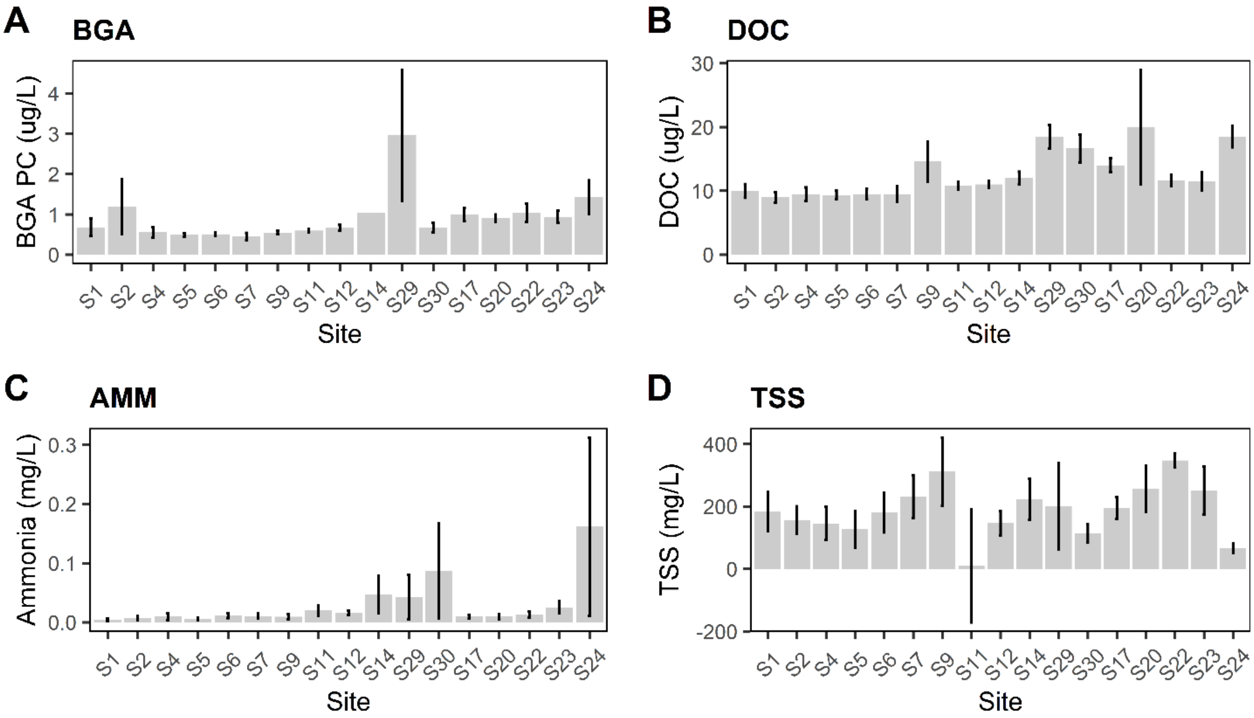

Figure A3. Mean (± S.E.) value per site (averaged across sampling trips) for additional environmental variables examined in the PCA, including A) the concentration of blue-green algae (BGA) pigment phycocyanin, B) dissolved organic carbon (DOC), C) ammonia and D) total suspended solids (TSS).

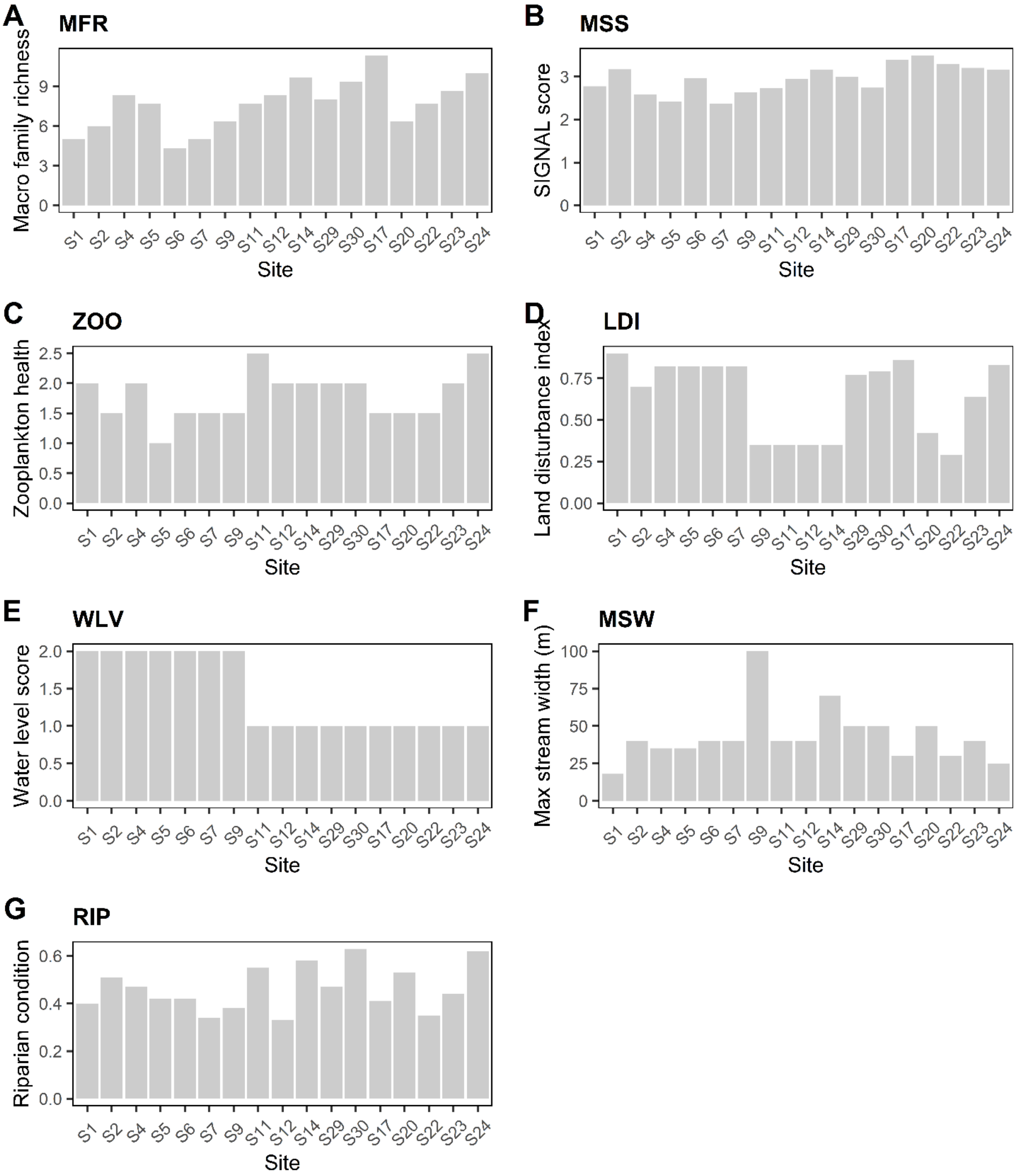

Figure A4. Scores per site for additional environmental variables examined in the PCA, including A) macroinvertebrate family richness, B) macroinvertebrate SIGNAL score, C) score of overall zooplankton health, D) landscape disturbance index at the sub-catchment level, E) water level score, F) maximum stream width and G) riparian condition index. Error bars are not shown as these metrics lacked trip-level data.

Table A4. Summary of bird species detected per site (number of samples where the species was considered 'detected') using a relaxed detection approach (at least 1/3 PCR replicates was positive for the species). Species classifications were based on the NBDL reference sequence database.

Species	S1	S2	S4	S5	S6	S7	S9	S11	S12	S14	S17	S20	S22	S23	S24	S27	S29	S30
Waterbirds																		
Anas superciliosa	1	1	0	0	0	0	0	1	0	1	0	2	0	1	0	0	2	2
Anhinga novaehollandiae	0	0	1	0	1	0	5	2	3	0	3	3	0	0	1	0	3	3
Ardea alba modesta	0	0	0	0	0	0	0	1	0	1	0	0	0	0	1	0	0	7
Chenonetta jubata	3	5	0	0	5	5	3	1	2	0	3	2	5	1	7	0	2	3
Cygnus atratus	0	0	0	0	0	0	1	2	0	6	1	3	0	0	0	0	4	15
Egretta novaehollandiae	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Microcarbo melanoleucos	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	5
Nycticorax caledonicus	0	0	1	0	0	1	0	0	2	0	1	0	0	0	0	0	0	0
Pelecanus conspicillatus	13	19	14	3	12	11	8	16	24	7	11	8	6	9	10	3	7	17
Phalacrocorax carbo	16	24	15	5	13	6	11	13	13	8	12	11	9	9	12	0	11	16
Phalacrocorax sulcirostris	8	12	9	3	6	5	6	6	9	12	5	4	1	3	12	2	9	18
Phalacrocorax varius	5	11	9	1	8	6	7	5	6	5	1	0	2	1	3	0	8	13
Platalea flavipes	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3
Podiceps cristatus	0	0	0	0	1	0	2	0	0	0	0	0	0	0	0	0	0	1
Porphyrio porphyrio melanotus	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
Spatula rhynchotis	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	3	8
Tadorna tadornoides	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	5
Threskiornis moluccus	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
Tribonyx ventralis	0	0	0	0	1	0	6	0	0	1	0	0	0	0	0	0	1	6
Terrestrial birds																		
Calyptorhynchus banksii	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
Dromaius novaehollandiae	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
Eolophus roseicapilla	1	0	0	0	1	0	0	0	1	0	1	0	0	0	0	0	0	0
Gallus gallus	0	1	3	2	1	1	3	1	0	0	0	0	2	2	0	0	0	0
Geopelia placida	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0
Haliastur sphenurus	1	1	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0
Meleagris gallopavo	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	6
Melopsittacus undulatus	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0
Milvus migrans	0	2	0	0	2	1	0	0	2	0	0	0	0	0	0	0	0	0
Nymphicus hollandicus	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
Ocyphaps lophotes	0	0	0	0	0	0	0	1	1	0	0	0	0	3	0	0	0	0
Petrochelidon ariel	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0
Phaps chalcoptera	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
Platycercus elegans	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
Psephotus haematonotus	1	0	2	0	0	2	3	1	0	3	0	0	0	0	2	0	1	4
Sturnus vulgaris	0	0	0	0	0	0	0	0	3	0	0	0	0	0	0	0	0	0

Table A5. Summary of bird species detected per site (number of samples where the species was considered 'detected') using a strict detection approach (at least 2/3 PCR replicates were positive for the species). Species classifications were based on the NBDL reference sequence database.

Species	S1	S2	S4	S5	S6	S7	S9	S11	S12	S14	S17	S20	S22	S23	S24	S27	S29	S30
Waterbirds																		
Anas superciliosa	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Anhinga novaehollandiae	0	0	0	0	0	0	2	0	1	0	0	1	0	0	0	0	1	1
Ardea alba modesta	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2
Chenonetta jubata	0	1	0	0	2	2	0	0	0	0	1	0	2	0	2	0	0	0
Cygnus atratus	0	0	0	0	0	0	0	1	0	2	0	1	0	0	0	0	1	6
Egretta novaehollandiae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Microcarbo melanoleucos	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
Nycticorax caledonicus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Pelecanus conspicillatus	4	6	4	1	4	4	2	6	8	2	3	2	2	4	3	1	2	6
Phalacrocorax carbo	5	8	6	1	4	2	4	5	4	3	4	3	4	3	4	0	4	6
Phalacrocorax sulcirostris	3	4	3	1	3	2	2	2	3	4	1	2	0	0	5	1	3	6
Phalacrocorax varius	2	4	3	0	2	2	2	2	2	2	0	0	0	0	0	0	2	5
Platalea flavipes	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
Podiceps cristatus	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
Porphyrio porphyrio melanotus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Spatula rhynchotis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	3
Tadorna tadornoides	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2
Threskiornis moluccus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Tribonyx ventralis	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	2
Terrestrial birds																		
Calyptorhynchus banksii	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Dromaius novaehollandiae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Eolophus roseicapilla	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Gallus gallus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Geopelia placida	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Haliastur sphenurus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Meleagris gallopavo	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2
Melopsittacus undulatus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
Milvus migrans	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Nymphicus hollandicus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Ocyphaps lophotes	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
Petrochelidon ariel	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Phaps chalcoptera	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Platycercus elegans	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Psephotus haematonotus	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	1
Sturnus vulgaris	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0

Table A6. Comparison between avian taxa detected with eDNA and all historical Atlas of Living Australia (ALA) records for the study area (defined by a 1 km buffer around the river network). Matched = the number of species that were historically recorded and detected with eDNA, missing = the number of species that were historically recorded but missed with eDNA, and extra = the number of species detected with eDNA but not historically recorded. Species classifications were based on the NBDL reference sequence database.

	Relaxed detection				**Strict detection**			
Site	eDNA	Matched	Missing	Extra	eDNA	Matched	Missing	Extra
S1	10	10	266	0	4	4	272	0
S2	11	11	265	0	6	6	270	0
S4	8	8	268	0	4	4	272	0
S5	6	5	271	1	3	3	273	0
S6	14	14	262	0	5	5	271	0
S7	10	10	266	0	5	5	271	0
S9	15	14	262	1	8	8	268	0
S11	12	12	264	0	5	5	271	0
S12	13	13	263	0	6	6	270	0
S14	10	10	266	0	5	5	271	0
S17	9	9	267	0	4	4	272	0
S20	10	10	266	0	5	5	271	0
S22	6	6	270	0	3	3	273	0
S23	8	8	268	0	3	3	273	0
S24	11	11	265	0	5	5	271	0
S27	2	2	274	0	2	2	274	0
S29	12	12	264	0	8	8	268	0
S30	18	17	259	1	14	13	263	1

Bibliography

Andrews, S. (2010). FastQC: a quality control tool for high throughput sequence data. In: Babraham Bioinformatics, Babraham Institute, Cambridge, United Kingdom.
Brandis, K. (2010). Colonial waterbird breeding in Australia: wetlands, water requirements and environmental flows. *University of NSW, Sydney*.
Brandis, K., & Bino, G. (2016). *A review of the relationship between flow and waterbird ecology in the Condamine-Balonne and Barwon-Darling River. Final report to the Murray-Darling Basin Authority*. Murray-Darling Basin Authority.
Brandis, K., Bino, G., & Kingsford, R. (2021). More than just a trend: integrating population viability models to improve conservation management of colonial waterbirds. *Environmental Management*, *68*(4), 468-476.
Brandis, K., Bino, G., Spencer, J., Ramp, D., & Kingsford, R. (2018). Decline in colonial waterbird breeding highlights loss of Ramsar wetland function. *Biological Conservation*, *225*, 22-30.
Brandis, K., Kingsford, R., Ren, S., & Ramp, D. (2011). Crisis water management and ibis breeding at Narran Lakes in arid Australia. *Environmental Management*, *48*(3), 489-498.
Brandis, K. J., Koeltzow, N., Ryall, S., & Ramp, D. (2014). Assessing the use of camera traps to measure reproductive success in Straw-necked Ibis breeding colonies. *Australian Field Ornithology*, *31*(2), 99-106.
DCCEEW. (2023). *River Condition Index (RCI)*. https://data.nsw.gov.au/data/dataset/river-condition-index-rci
Fablet, L., Pellerin, A., Zarzoso-Lacoste, D., Dubut, V., & Walch, L. (2024). Metabarcoding reveals waterbird diet in a French Ramsar wetland: implications for ecosystem management. *Knowledge & Management of Aquatic Ecosystems*(425), 9.
Ficetola, G. F., Coissac, E., Zundel, S., Riaz, T., Shehzad, W., Bessière, J., Taberlet, P., & Pompanon, F. (2010). An in silico approach for the evaluation of DNA barcodes. *BMC genomics*, *11*(1), 1-10.
Fluet-Chouinard, E., Stocker, B. D., Zhang, Z., Malhotra, A., Melton, J. R., Poulter, B., Kaplan, J. O., Goldewijk, K. K., Siebert, S., & Minayeva, T. (2023). Extensive global wetland loss over the past three centuries. *Nature*, *614*(7947), 281-286.
Francis, R. J., Lyons, M. B., Kingsford, R. T., & Brandis, K. J. (2020). Counting mixed breeding aggregations of animal species using drones: lessons from waterbirds on semi-automation. *Remote Sensing*, *12*(7), 1185.
Frøslev, T. G., Kjøller, R., Bruun, H. H., Ejrnæs, R., Brunbjerg, A. K., Pietroni, C., & Hansen, A. J. (2017). Algorithm for post-clustering curation of DNA amplicon data yields reliable biodiversity estimates. *Nature communications*, *8*(1), 1-11.
Greenfield, P. (2017). Greenfield hybrid analysis pipeline (GHAP). v1. *Canberra, ACT: CSIRO*.
Jin, T., Zhang, S., Ye, X., Chen, J., & Liu, X. J. (2025). A Preliminary Application of Bird Surveys Using Environmental DNA Technology: in Futian Mangrove Nature Reserve. *bioRxiv*, 2025.2001. 2013.632664.
Kingsford, R., Roshier, D., & Porter, J. (2010). Australian waterbirds–time and space travellers in dynamic desert landscapes. *Marine and Freshwater Research*, *61*(8), 875-884.
Kingsford, R. T., Bino, G., & Porter, J. L. (2017). Continental impacts of water development on waterbirds, contrasting two Australian river basins: Global implications for sustainable water use. *Global Change Biology*, *23*(11), 4958-4969.
Kingsford, R. T., Porter, J. L., Brandis, K. J., & Ryall, S. (2020). Aerial surveys of waterbirds in Australia. *Scientific data*, *7*(1), 1-6.
Kuznetsova, A., Christensen, R. H., Bavay, C., & Brockhoff, P. B. (2015). Automated mixed ANOVA modeling of sensory and consumer data. *FOOD QUALITY AND PREFERENCE*, *40*, 31-38.
Menning, D. M., Uher-Koch, B. D., Flamme, M. J., Simmons, T., Schmutz, J. A., & Talbot, S. L. (2022). eDNA Metabarcoding Analyses of Diet in Yellow-Billed Loons of Northern Alaska. *Waterbirds*, *45*(2), 159-166. https://doi.org/10.1675/063.045.0206
Neice, A. A., & McRae, S. B. (2021). An eDNA diagnostic test to detect a rare, secretive marsh bird. *GLOBAL ECOLOGY AND CONSERVATION*, *27*, e01529.
Oksanen, J., Kindt, R., Legendre, P., O'Hara, B., Stevens, M. H. H., Oksanen, M. J., & Suggests, M. (2007). The vegan package. *Community ecology package*, *10*(631-637), 719.

Orzechowski, S. C., Frederick, P. C., Dorazio, R. M., & Hunter, M. E. (2019). Environmental DNA sampling reveals high occupancy rates of invasive Burmese pythons at wading bird breeding aggregations in the central Everglades. *PLoS ONE*, *14*(4), e0213943.
Saenz-Agudelo, P., Delrieu-Trottin, E., DiBattista, J. D., Martínez-Rincon, D., Morales-González, S., Pontigo, F., Ramírez, P., Silva, A., Soto, M., & Correa, C. (2022). Monitoring vertebrate biodiversity of a protected coastal wetland using eDNA metabarcoding [Article]. *Environmental DNA*, *4*(1), 77-92. https://doi.org/10.1002/edn3.200
Sigsgaard, E. E., Torquato, F., Frøslev, T. G., Moore, A. B. M., Sørensen, J. M., Range, P., Ben-Hamadou, R., Bach, S. S., Møller, P. R., & Thomsen, P. F. (2020). Using vertebrate environmental DNA from seawater in biomonitoring of marine habitats [Article]. *Conservation biology*, *34*(3), 697-710. https://doi.org/10.1111/cobi.13437
Taberlet, P., Bonin, A., Zinger, L., & Coissac, E. (2018). *Environmental DNA: For biodiversity research and monitoring*. Oxford University Press.
Wetlands International. (2012). *Waterbird Population Estimates, Fifth Edition. Summary Report*.
Zhang, S., Zhao, J. D., & Yao, M. (2023). Urban landscape-level biodiversity assessments of aquatic and terrestrial vertebrates environmental DNA. *Journal of environmental management*, *340*, Article 117971. https://doi.org/10.1016/j.jenvman.2023.117971
Zinger, L., Lionnet, C., Benoiston, A. S., Donald, J., Mercier, C., & Boyer, F. (2021). metabaR: an R package for the evaluation and improvement of DNA metabarcoding data quality. *Methods in Ecology and Evolution*, *12*(4), 586-592.

MACQUARIE University

Macquarie University is a vibrant hub of intellectual thinkers, all working towards a brighter future for our communities and our planet.

A PLACE OF INSPIRATION

Macquarie is uniquely located in the heart of Australia's largest high-tech precinct, a thriving locale which is predicted to double in size in the next 20 years to become the fourth largest CBD in Australia.

Our campus spans 126 hectares, with open green space that gives our community the freedom to think and grow. We are home to fantastic facilities with excellent transport links to the city and suburbs, supported by an on-campus train station.

RENOWNED FOR EXCELLENCE

We are ranked among the top two per cent of universities in the world, and with a 5-star QS rating, we are renowned for producing graduates that are among the most sought after professionals in the world.

A PROUD TRADITION OF DISCOVERY

Our enviable research efforts are brought to life by renowned researchers whose audacious solutions to issues of global significance are benefiting the world we live in.

BUILDING SUCCESSFUL GRADUATES

Our pioneering approach to teaching and learning is built around a connected learning community: our students are considered partners and co-creators in their learning experience.

FIND OUT MORE

Macquarie University NSW 2109 Australia
T: +61 (2) 9850 7111
mq.edu.au
ABN 90 952 801 237
CRICOS Provider 00002J