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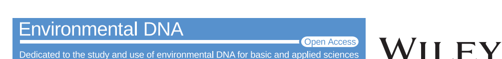

Developing an eDNA approach for wetland biomonitoring: Insights on technical and conventional approaches

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Abstract

Wetlands are ecologically and culturally significant ecosystems that are experiencing biodiversity declines globally. Biomonitoring techniques that use environmental DNA (eDNA) to detect and monitor biodiversity are well established in lake, riverine, and marine ecosystems. However, their use in wetlands requires further development due to the presence of sediments that block eDNA filters to limit water filtration, alongside a lack of standardized methodology. In this study, we examined eDNA dynamics to understand spatiotemporal biodiversity patterns in an Aotearoa New Zealand wetland and to optimize their application to wetland-specific challenges. We sampled four sites across Opuatia Wetland at three time points during an austral spring. We conducted conventional taxonomic surveys, tested three different filter sizes (1.2 μm, 5 μm, and semi-quantitative dacron filters), and assessed our ability to detect foreign DNA (from kea; *Nestor notabilis*) at different time points and distances post-release. We found significant differences in DNA sequence composition across time and space, and when using different sized filters. eDNA data generally complemented (versus replaced) conventional survey and identification methods, with certain species only detected by one method or the other. Taxonomic resolution of conventional sampling and identification methods often exceeded that of eDNA. Foreign DNA was detectable 10 m from its release point for up to 1 week post-release. Our results provide new considerations for future eDNA research in wetland environments, where rapid biomonitoring techniques are needed to support conservation and preservation.

KEYWORDS
conservation, conventional surveys, DNA degradation, environmental DNA, method optimization, New Zealand, wetlands

1 | INTRODUCTION

Despite the ecological and cultural significance of wetlands, they are among the most threatened ecosystems in the world (Davidson, 2014; Fluet-Chouinard et al., 2023), and have diminished in global extent by 3.4 million km^2 since 1700 (Fluet-Chouinard et al., 2023). As well as factors like land-use change and peat extraction (Van Asselen et al., 2013), wetlands are under

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pressure from invasive plants and animals (Kingsford et al., 2016), which have both economic (Diagne et al., 2021) and environmental impacts (Doherty et al., 2016). Thus, monitoring biodiversity and invasive species incursions is an important component of wetland restoration and preservation.

Environmental DNA (eDNA; genetic material released by organisms into the environment) is a rapid, noninvasive biomonitoring technique that has surged in popularity over the past decade owing to its relative ease of use for a range of applications (Barnes & Turner, 2016; Beng & Corlett, 2020; Harper et al., 2019; Saenz-Agudelo et al., 2021; Stat et al., 2017). However, environmental factors such as pH, temperature, solar radiation, and microbial activity are known to impact eDNA integrity under laboratory (Barnes et al., 2014; Dejean et al., 2011; Goldberg et al., 2013; Strickler et al., 2015), riverine (Nukazawa et al., 2018; Pont et al., 2018), and marine (Ely et al., 2021; Murakami et al., 2019) conditions. The movement of eDNA through ecosystems can also be complex, depending on a range of hydrological and abiotic factors (Harrison et al., 2019). Moreover, experimental design aspects, such as the volume of water filtered (i.e., using different filter pore sizes) can significantly impact biodiversity detection (Tsuji et al., 2019; Turner et al., 2014). While these technical aspects of eDNA sampling are well-understood in riverine and marine environments (e.g., Jeunen et al., 2022; Pont et al., 2018; Smith et al., 2023), and best practice eDNA sampling guidelines are frequently becoming available (e.g., De Brauwer et al., 2022; Minamoto et al., 2021), the use of eDNA in wetland environments requires further development.

A common challenge affecting eDNA use in wetlands is that high sediment volumes can block eDNA filters, limiting the volume of water that can be processed (Goldberg et al., 2016). To date, active sampling has been shown to outperform passive sampling in a controlled environment that mimicked a natural wetland (Nordstrom et al., 2023). Meanwhile, increasing the filter pore size (from 0.45 to 6 μm) in a high-particulate wetland system increased the volume of water filtered (Goldberg et al., 2018). To the best of our knowledge, no research has to date examined eDNA decay rates in wetlands, with limited studies investigating the changing patterns of biodiversity detection across time and space in these environments (e.g., Coleman et al., 2023; Saenz-Agudelo et al., 2021).

A second area of required research is the determination of relationships between eDNA and conventional biomonitoring methods in a wetland context. Various conventional methods (such as netting, visual surveys, and sound monitoring) are currently employed in wetlands, but these are typically labor- and time-intensive and thus can be expensive (Beng & Corlett, 2020; Hervé et al., 2022). They often also require specific taxonomic expertise (Leese et al., 2018), and may involve capturing macroorganisms, with sometimes lethal collections (Saenz-Agudelo et al., 2021). Compared to conventional methods, eDNA has been shown to detect greater biodiversity for fish in stream and river environments (e.g., Boivin-Delisle et al., 2020; David et al., 2021; Pont et al., 2018). However, the converse can also be true. For example, conventional methods outperformed eDNA for detecting semi-aquatic watersnake species in a lentic environment (Rose et al., 2019), and inconsistency between the two methods was found for amphibians in headwater streams (Takahara et al., 2020).

In Aotearoa New Zealand, substantial losses in wetland extent (~2.4 million ha historically to a current extent of about ~250,000 ha; Ausseil et al., 2011) have reduced biodiversity and impacted the intrinsic relationship between Māori (Indigenous People of Aotearoa) and their repo (wetlands) (Taura et al., 2021). Invasive species are widespread in wetlands nationwide, where they have devastating impacts – altering ecosystem composition, structure, and function (e.g., *Salix cinerea*, gray willow; Griffiths et al., 2018). We chose Opuatia Wetland (Waikato region) to focus our research. Our objectives were to examine: (1) how eDNA disperses and persists locally; how eDNA detection of biodiversity varies: (2) with different filter sizes; (3) between four spatially-distant sites; and (4) during three time points over an austral spring; and (5) how eDNA compares to floristic and faunistic conventional biomonitoring methods (Figure 1).

2 | MATERIALS AND METHODS

2.1 | Site description

Opuatia Wetland (950 ha) is located in the lower Waikato River catchment, north of Lake Whangape, in Te Ika-a-Māui/the North Island of Aotearoa New Zealand (Figure 2). Hills border the wetland to the north, east, and south, and these are primarily used for crops and agriculture (Barnes et al., 2001; Browne & Campbell, 2005). The overall wetland is a mosaic of fen, swamp, and marsh components (Browne & Campbell, 2005; Reeves, 2011; Waikato Regional Council, n.d.). It features a restiad peat bog—which ranks among the five significant restiad peat bogs remaining in the Waikato Region and is thus considered a site of high value and national importance (Singers, 2019; Waikato Regional Council, n.d.)—and a wide range of hydrological characteristics. The water table is close to or above the surface level and is relatively stable (Browne & Campbell, 2005)—which serves as a general indicator of a healthy wetland (Reeves, 2011)—though water levels are lower during late summer and autumn (February to April) (Browne & Campbell, 2005). A causeway separates sites at the northern edge of the wetland (i.e., SS1 and SS2; Figure 2), with a culvert underneath that prevents winter flooding (Reeves, 2011). All sites feature continuous free water, with the wetland discharging westward via Opuatia Stream into the Waikato River (Barnes et al., 2001; Singers, 2019).

Opuatia Wetland holds ecological importance and also bears special significance to Horahora Marae (social cultural center/village). Horahora Marae is descended from the great voyaging waka (canoe) Tainui and the tribes of Ngāti Pou and Waikato-Tainui. The Waikato River is their tūpana (ancestor) and is a source of their tribal identity (Deed of Settlement in Relation to the Waikato River, 2009).

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FIGURE 1 A graphical diagram depicting the various experiments and surveys conducted to address the objectives of the study.

2.2 | Site selection, characteristics, and sampling periods

Four sampling locations ("SS1" to "SS4") were chosen based on their spatial distance from each other, water availability, and site access, with sampling occurring during spring 2022 (i.e., the start of September to early December)—the period of highest species activity (i.e., breeding season). Wetland types at each location were classified following the Johnson and Gerbeaux (2004) classification system, with SS1 corresponding to a swamp and SS2-SS4 corresponding to marsh. SS1 contained a mix of indigenous and exotic herbaceous wetland vegetation (Reeves, 2011), and SS2 and SS3 were dominated by introduced crack willow (*Salix fragilis*) and gray willow (*S. cinerea*), with a sparse mixture of native and introduced species beneath this canopy. Crack willow similarly dominated SS4.

Water quality parameters were measured at each site during each sampling event, including pH (Eutech pH Testr 30), water temperature, dissolved oxygen, conductivity, and salinity (YSI Prosolo). Daily weather was recorded for the entire wetland from AccuWeather, Churchill, Waikato (https://www.accuweather.com/en/nz/churchill/1078713/weather-forecast/1078713; Table S1) from approximately 1 week prior to sampling until the final eDNA sample was collected. A rough estimate of water level fluctuations was performed during each site visit using a graduated bamboo stick anchored at each site at the beginning of spring. The initial water level was noted and then subsequently recorded during each visit to determine the difference from the initial reading.

2.3 | eDNA

2.3.1 | eDNA sampling

All eDNA samples were collected using kits provided by commercial eDNA testing laboratory Wilderlab New Zealand Ltd (Wellington, New Zealand). Five to six replicates were collected at each site, including a control sample (1 L distilled water; a total of 15 samples) (Table 1), using a composite sampling strategy following decontamination of all equipment with a 10% bleach solution. In brief, this involved using a pole with an attached jug to collect surface water from various points across the site (with the pole extended away from the body and the collector not entering the direct collection area in order to avoid contamination), which was then pooled into a bucket and left for 10–15 min to allow any sediment to settle. Different volumes of water, dependent on filter size (see below) and sedimentation load, were then pushed through a filter designed to trap DNA using a syringe, with a caulking gun used to assist filtration as required. Following this, 60 cc of air was passed through the filter to remove excess water and 300 μL of a DNA/RNA shield

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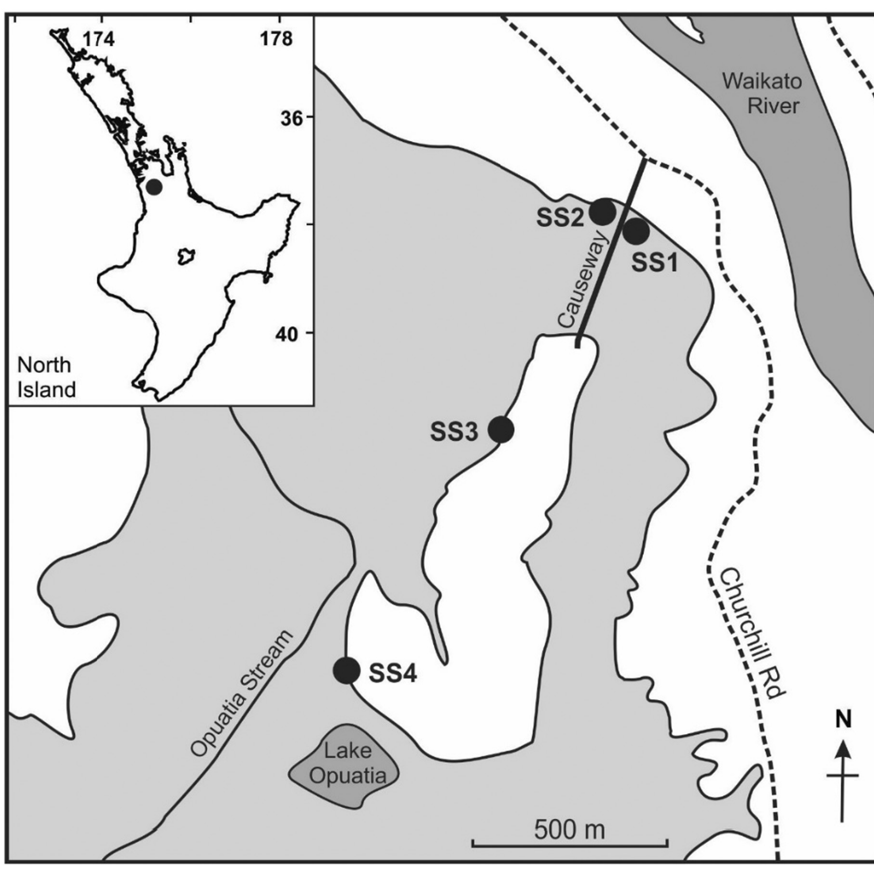

FIGURE 2 Location of Opuatia Wetland (light gray) and study sample sites (inset: location of Opuatia in Aotearoa New Zealand's North Island). Dark gray represents water bodies, and white denotes pasture. SS1 and SS4 are approximately 1.4 km apart.

preservative was injected. Preserved filters were then sent to Wilderlab for eDNA analysis.

2.3.2 | Technical experiments

Dispersal distance and residence time experiment

To determine the residence time and dispersal distance of DNA at the wetland, DNA from kea (*Nestor notabilis*) fecal samples (since kea is not naturally found in the North Island; Tennyson et al., 2014) was released on 16 September 2022. A karakia (Māori prayer) was performed prior to the DNA release and sampling in accordance with tikanga (Māori customs).

Hamilton Zoo staff collected 140 g of kea excrement from the zoo's colony between 6 and 14 September 2022, from sand/mulch and directly from perches immediately after defecation (i.e., following feeding) and stored this at −20°C until release. The excrement was then diluted in 1.5 L of water (boiled and cooled before use to minimize and remove contaminants) to form a slurry. Three replicates were collected from the slurry (85–167 mL) to quantify the kea DNA sequence counts prior to release.

SS1 was chosen as the slurry release site based on the wetland's hydrology. To first measure water flow at the site, we performed a simple experiment, anchoring two bamboo sticks 1 m apart in the flooded wetland area and placing oranges at one end (Figure S1). The oranges did not move throughout the day, indicating a very low flow rate, which we used to determine the distances from the release points and timing of our eDNA sample collections post-fecal slurry release (i.e., we assumed that the kea DNA would dissipate at a slow rate and, for example, did not collect eDNA samples at the 25 m line prior to 24 h due to this).

The kea DNA slurry (total volume = 600 mL) was released at four points within the release area—a horizontal transect approximately 5 m in width (Figure S1). eDNA sampling was performed following composite sampling strategy described above, using 5 μm filters at distances of 1, 10, and 25 m away from the release point for six time points post-release (1, 5, 24, 48 h, 1, and 3 weeks) (Table 2; Figure S1). Samples were collected before release (i.e., start of spring collections) to confirm that kea and/or the genus *Nestor* was not already present at Opuatia. Sample collection involved walking on either side of the transect to minimize disturbance within the transect area and followed the composite sampling strategy outlined above.

In situ residence time experiment

To determine the in situ residence time of DNA at its release point in the wetland (i.e., versus dispersal distance post-release; 'Dispersal distance and residence time experiment' Section), DNA from kea fecal samples was released at SS1 on 4 September 2023. As for the dispersal distance experiments above, Hamilton Zoo staff collected 140 g of kea excrement from the zoo's colony (from mid to late August 2023), and this was diluted in 1.5 L of water to form a slurry. Weather was again recorded for the entire wetland from 1 week before slurry release until 16 September 2023, as described

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TABLE 1 (A) Summary table of sample collection information for eDNA experiments at Opuatia Wetland; and (B) water quality measurements, time of recording, and water level fluctuations.

(A)

Site	Latitude	Longitude	Collection date	Spring period	Filter size (μm)	Replicates	Volume filtered (mL)	No. of controls
SS1	−37.422857	175.071516	08/09/2022	Start	1.2	5	100–205	1
					5	5	800–1000	1
					Dacron	6	10,000–38,000	0
			20/10/2022	Middle	1.2	5	150–170	1
					5	6	250–450	1
					Dacron	6	21,000–37,000	0
			02/12/2022	End	1.2	5	205–360	1
					5	6	260–600	1
					Dacron	6	1000–65,750	0
SS2	−37.422580	175.071082	07/09/2022	Start	5	5	490–1000	1
	−37.422380	175.071010	19/10/2022	Middle	5	5	510–1000	1
	−37.422580	175.071082	29/11/2022	End	5	6	250–640	1
SS3	−37.427204	175.068028	07/09/2022	Start	5	5	1000	1
	−37.426990	175.067830	19/10/2022	Middle	5	5	1000	1
	−37.427204	175.068028	29/11/2022	End	5	5	870–1000	1
SS4	−37.432995	175.063563	07/09/2022	Start	5	5	1000	1
	−37.433088	175.063192	19/10/2022	Middle	5	5	1000	1
	−37.432995	175.063563	29/11/2022	End	5	5	700–1000	1

(B)

Site	Collection date	Water temperature (°C)	pH	DO (%)	DO (mg/L)	Conductivity (SPC-μS/cm)	Salinity (SAL-ppt)	Time	Water level difference from initial (cm)[a]
SS1	08/09/2022	11.5	6.9	44.1	4.69	101.3	0.00	09:03	N/A
	20/10/2022	13.8	6.5	2.9	0.30	147.3	0.07	08:30	−18
	02/12/2022	18.3	6.9	5.3	0.49	178.3	0.08	09:05	+26
SS2	07/09/2022	16.3	6.5	85.3	8.33	104.6	0.00	14:00	N/A
	19/10/2022	21.3	6.2	98.3	8.71	173.6	0.08	13:10	No water
	29/11/2022	21	6.8	10.1	0.90	189.0	0.09	13:07	+33
SS3	07/09/2022	17.8	7.3	75.7	7.18	141.9	0.00	12:24	N/A
	19/10/2022	14.5	6.6	15.2	1.51	238.8	0.11	09:03	No water
	29/11/2022	19.4	6.9	18.4	1.70	242.2	0.11	11:21	+42
SS4	07/09/2022	13.1	9.7	65.4	6.73	136.9	0.00	10:26	N/A
	19/10/2022	15.6	6.5	11.1	1.10	234.0	0.11	10:52	No water
	29/11/2022	18	6.9	10.5	1.00	235.6	0.11	09:28	+40

[a]Initial water level at the start of spring: SS1 at 60 cm; SS2 at 70 cm; SS3 at 40 cm; SS4 at 40 cm.

above (Table S1). Prior to release, water flow was measured following the orange experiment described above; after 30 s, the oranges moved approximately 0.75 m, indicating a flow rate of 0.025 m/s.

The kea slurry was released steadily into the wetland at a single point. eDNA sampling was repeatedly performed at the release site at five time points (2, 4, 6, 9, and 12 days post-release) using a sampling pole with an attached jug to collect surface water from the release point into a bucket, with replicate eDNA samples then collected using 5 μm filters.

Filter size comparisons

To determine the optimum filter size for wetland eDNA sampling, we compared 1.2, 5 μm, and qualitative dacron (Aqua One Micro Pad; relatively more coarse than the 1.2 and 5 μm filters) filters at SS1 (Table 1). The standard volume processed for the 1.2 and 5 μm filters was 1 L and was 100 L for the dacron filter, where an electric pump was used to actively push water through the filter. However, due to turbidity, not all samples met these standard volumes. Thus, water was filtered up to the given limit or until it

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TABLE 2 Summary of the dispersal distance and in situ residence time experiments, including sampling information, water quality measurements and their time of recording, and water level fluctuations.

Collection date	Transect distance (m)	Time post-release	Replicates	Volume filtered (mL)	No. of controls	Water temperature (°C)	pH	DO (%)	DO (mg/L)	Conductivity (SPC-µS/cm)	Conductivity (SPC-mS/cm)	Salinity (SAL-ppt)	Time	Water level difference from initial (cm)[a]
Dispersal distance and residence time														
16/09/2022	1	1 h	6	400–650	1	12.2	6.5	17.2	1.90	—	43.0	0.10	09:00	−16
	10		5	1000										
	1	5 h	6	300–844	1	16.3	5.8	57.0	5.80	—	37.1	0.00	14:30	
	10		6	370–850										
17/09/2022	1	24 h	6	356–800	1	12.4	6.5	14.0	1.52	—	78.7	0.10	09:26	−17
	10		6	650–1000										
	25		6	390–805										
18/09/2022	1	48 h	6	310–600	1	13.1	6.0	16.4	1.80	—	44.9	0.10	09:23	−17.5
	10		6	457–950										
	25		6	200–400										
23/09/2022	1	1 week	6	305–720	1	14.5	6.5	21.5	1.90	150.7	—	0.07	09:02	−15
	10		6	550–910		14.4	6.3	17.4	1.56	164.4	—	0.08	09:04	
	25		6	350–570		14.3	6.6	15.6	1.48	163.9	—	0.08	09:06	
7/10/2022	1	3 weeks	6	400–650	1	14.1	6.3	3.7	0.33	167.2	132.7	0.08	09:37	+45
	10		6	268–820		14.8	6.4	11.1	1.06	164.9	132.7	0.08	10:03	
	25		6	300–850		14.6	6.4	8.1	0.76	165.7	132.8	0.08	10:08	
In situ residence time														
06/09/2023	N/A	Day 2	3	300–700	N/A	15.4	6.7	34.3	3.43	153.3	—	0.07	10:02	N/A
08/09/2023	N/A	Day 4	3	230–710		12.8	6.6	19.7	2.08	160.2	—	0.08	09:45	N/A
10/09/2023	N/A	Day 6	3	255–550		12.9	6.8	16.9	1.79	168.0	—	0.08	09:48	N/A
13/09/2023	N/A	Day 9	3	450–550		13.2	6.8	23.0	2.41	153.9	—	0.07	09:41	N/A
16/09/2023	N/A	Day 12	3	400–555		13.1	6.9	23.0	2.42	148.9	—	0.07	09:47	N/A

[a]Initial water level at the start of spring at SS1 was 60 cm.

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became clogged for each sample. Five to six replicates were performed for each filter size.

2.3.3 | Biodiversity assessment

Spatial and temporal variation aspects

Spatial and temporal variation across Opuatia Wetland were examined using the 5 μm filter at sites SS1-SS4 at the beginning, middle, and end of spring sampling periods (Table 1). The sampling methodology followed the composite sampling strategy outlined above. In the middle of spring, the water level dropped entirely below ground level in the original sampling areas for SS2-SS4, necessitating minor shifts in sampling locations to where water was accessible as close as possible to each original site (SS2 was 12.8 m NNW of the original sampling area, SS3 was 13 m to the NW, and SS4 was 11.5 m to the WSW; see Table 1 for GPS coordinates).

2.3.4 | Environmental DNA analysis

DNA extraction and amplification procedures followed the protocols outlined in Wilkinson (2023). All stages (e.g., sample extraction, PCR preparation, PCR cleanup) were conducted at separate, dedicated workstations, including an isolated “post-PCR” room. Prior to use, all work surfaces were sanitized using fresh household hypochlorite bleach diluted at a ratio of 1:10, and nitrile gloves were worn to prevent contamination.

Lysates were obtained from the sampled filters and stored at −20°C until DNA extraction. DNA extraction and purification used 200 μL of each sample lysate, with these processed into a GD141 cartridge on the Genolution Nextractor NX-48S system using the standard extraction settings. Subsequent steps involving DNA quality/quantity analysis, adapter-fusion, indexing, and amplification were conducted as part of a single-step quantitative PCR process using an Applied Biosystems QuantStudio 1 qPCR instrument. DNA extracts were amplified using eight fusion-tag mitochondrial and nuclear rRNA assays designed for detecting vertebrate, invertebrate, plant, microeukaryote, and microbial DNA (see Table S2 for primer details). Fusion tag primers included Illumina P5 and P7 adapter sequences, an Illumina TruSeq™ sequencing primer bind site (forward primer only), unique 8 bp index sequences, and locus-specific primers. PCR reactions were carried out in duplicate, with each reaction containing 5 μL SensiFAST 1 × LoRox SYBR Mix (Bioline), 0.25 μL forward primer (10 μM), 0.25 μL reverse primer (10 μM), 0.5 μL BSA (10 mg mL^{-1}, Sigma Aldrich), 2 μL deionized water and 2 μL template DNA. qPCR cycling conditions included initial denaturation of 3 min at 95°C, and then 40 cycles of 5 s at 95°C, 10 s at the appropriate annealing temperature (see Wilkinson et al., 2024), and 15 s at 72°C. DNA quality and quantity were confirmed using qPCR. DNA libraries were pooled at equimolar concentration, cleaned, and double-end size selected using AMPure XP magnetic beads. Sequencing libraries (at a final concentration of 50 pM in sterile DNAse/RNAse-free water) used the same locus-specific primers (Table S2) and an Illumina iSeq 100 instrument (150 PE). A negative control reaction containing 2 μL of deionized water in place of the template DNA was included with each sequencing run. See Wilkinson (2023) for further details.

The iSeq 100 output sequence fastq files were de-multiplexed in R (R Core Team, 2021) using the insect package (v.1.4.0; Wilkinson et al., 2018) and trimmed sequences were filtered to generate an amplicon sequence variant (ASVs) table using the DADA2 package (Callahan et al., 2016). For validation purposes, an eDNA-based macroinvertebrate community index (MCI) was calculated for each sample, initially entailing the identification of each ASV to the lowest possible taxonomic rank. The taxon assignment followed a standard four-step classification process and used a very conservative 99% threshold for the SINTAX algorithm (which is used to generate count tables with taxonomic assignments; Edgar, 2016) to reduce the misclassification and overclassification rate (this allowed one mismatch for sequences more than 50 bp in length, essentially equating to a minimum 98% identity for species level assignment; see Wilkinson et al., 2024 for further details). Following taxon assignment, any DNA identified as having human origin was removed for privacy reasons (Wilkinson et al., 2024).

2.4 | Conventional surveys

Conventional survey methods, including zooplankton sampling, gee-minnow trapping, 5-min bird counts, and botanical surveying, were conducted for comparison with eDNA methods. Among these, the 5-min bird count and botanical surveys were carried out by Waikato Regional Council staff members. All surveys were conducted after eDNA collection to prevent contamination of the latter. Zooplankton samples and gee-minnow traps were carried out at the start (7–9 September 2022), middle (19–20 October 2022), and end of spring (29–30 November 2022), while the botanical survey was performed in the middle of spring. The 5-min bird count was conducted at the middle and end of spring on October 21, 2022 and December 5, 2022, respectively, between 10:30 am and 11:30 am.

2.4.1 | Zooplankton surveys

To collect the zooplankton samples, up to 9 L of surface water was passed through a 40 μm mesh at SS2-SS4. Material retained on the mesh was then washed with filtered tap water into a sample container and preserved with ethanol (>50% final concentration). Samples were enumerated in 5 mL aliquots until a minimum of 300 individuals, or the entire sample was counted. Individuals were identified to species level wherever possible using standard taxonomic keys (e.g., Chapman et al., 2011; Shiel, 1995).

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2.4.2 | Gee-minnow traps

A total of 12 gee-minnow traps (4 mm mesh) were deployed at SS3 and SS4. Traps were positioned along the shoreline at 3 pm on the day of deployment, with an air pocket at the top due to anoxic conditions, and left overnight before collection the following morning at 9 am (i.e., deployed for 18 h, total). The water level dropped overnight for all deployments; therefore, the total maximum fishing time was uncertain. All species caught in the traps were identified and recorded, as were species observed outside the traps during the trapping period. While the primary function of a gee-minnow trap is to sample small fish species (Lake, 2013), other taxonomic groups were also recorded (e.g., arthropods and annelids).

2.4.3 | Five-minute bird counts

Birds were identified to species level using sight or sound over a 5-min duration and counted within an approximately 300 m radius of the observer. SS1 and SS2 were examined together due to their close proximity. Environmental conditions (e.g., wind) were noted as they interfere with sound perception. Audio playbacks (a standard detection method for secretive wetland birds; Dowding, 2012; Williams, 2021) were performed three times for matuku/Australasian bittern (*Botaurus poiciloptilus*; presumed to be present at Opuatia Wetland) and pūweto/spotless crake (*Zapornia tabuensis*; potential resident)—as these species are secretive and territorial.

2.4.4 | Botany survey

Ground surveys were conducted in the immediate vicinity of each eDNA sample collection area at SS2-SS4, covering an area of approximately 25 × 10 m for a duration of 45 min. A list was compiled for all plants present within the survey area, with identification at the genus and species level where possible.

2.5 | Data analyses

As well as general overviews of the entire dataset as relevant, we give special focus to key wetland species of interest, including birds, fish, and plants for our various analyses (as described below). These species are integral components of wetland condition indicators (Clarkson et al., 2004) and contribute to the cultural food basket and materials for traditional Māori practices (Harmsworth, 2021). To first evaluate the consistency of DNA sequence counts among each set of eDNA sample replicates, an Analysis of Variance was performed using the aov function in R v.2023.06.1 + 524 (R Core Team, 2023); this showed no significant differences among replicates for each experiment (Table S3), thus replicates were combined as relevant for further analysis.

2.5.1 | Technical experiments

Dataset characteristics

The entire eDNA dataset (excluding the residence time samples) was examined to understand the proportion of sequences that were obtained for each taxonomic rank, as well as the degree of missing data in the eDNA reference database.

DNA residence time experiments

To examine the rate of DNA degradation, both the dispersal distance and in situ residence time eDNA datasets were searched for the presence of kea (at either species *N. notabilis* or genus *Nestor* levels) at each sampling time point (pre- and post-excrement release) and transect distance (for the dispersal distance dataset).

Filter size comparison

Nonmetric multidimensional scaling (nMDS) plots were generated to visualize differences among DNA sequence composition across filter sizes at the start, middle, and end of spring for the entire eDNA dataset and at species and genus-level—as well as just for the key wetland species of interest (birds, fish, and plants)—using the metaMDS function from the vegan v.2.6-4 package (Oksanen et al., 2022) in R. We used vegan::metaMDS with default parameters including Bray–Curtis dissimilarity with autotransform = FALSE. Subsequently, a permutational multivariate analysis of variance (PERMANOVA) and permutational multivariate analysis of dispersion (PERMDISP) was performed using the adonis function from vegan to examine statistical support for differentiation among the various groupings.

To understand the filtering capacity for each filter size, we calculated the average volume of water processed (mL) across each eDNA sample replicate for the three temporal points.

Finally, to understand how species richness changed with the number of replicates taken for each filter size, species accumulation curves were generated from the entire eDNA dataset using the specaccum function from vegan. To understand the effects of filter size more thoroughly, species richness was also plotted as the proportion of each taxonomic group against the total number of groups detected.

2.5.2 | Biodiversity assessment

Spatial variation

To understand how biodiversity changed across space at Opuatia Wetland, nMDS analyses examining eDNA patterns across the various sites for the entire eDNA dataset and at species and genus-level—as well as just for the key wetland species of interest (birds, fish, and plants)—were performed using nMDS plots, PERMANOVA, and PERMDISP. Only the 5 μm filter results at SS1 were used when comparing spatial variation. To understand how species richness changed with the number of sites sampled, species accumulation curves were generated from the entire eDNA dataset using the specaccum function in vegan. Species richness was also plotted as the proportion of

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each taxonomic group against the total number of groups detected to further understand the effects of spatial variation.

Temporal variation

To understand how biodiversity changed across time at Opuatia Wetland, further nMDS analyses examining eDNA patterns across the three temporal points (beginning, middle, and end of spring) for the entire eDNA dataset and at species and genus level—as well as just for the key wetland species of interest (birds, fish, and plants)—were performed using nMDS plots, PERMANOVA, and PERMDISP. Species richness was also plotted as the proportion of each taxonomic group against the total number of groups detected to further understand the effects of temporal variation.

2.5.3 | Conventional surveys compared to eDNA

Species lists and Venn diagrams were generated to compare the agreement between conventional survey and eDNA results across all sites at the start, middle, and end of spring by identifying any taxa that were present in one dataset but not the other. For the Venn diagrams, cases where the same method detected an organism at both genus and species level, only the latter was counted.

3 | RESULTS

3.1 | Technical experiments

3.1.1 | Dataset characteristics

All field controls detected DNA sequences, some of which were identified as species that are known to inhabit Opuatia Wetland. These controls were filtered on site; thus, a potential contaminant source may have been airborne DNA. However, the overall degree of potential contamination was low (0.6–2.8%). Over 5300 (55.8%) sequences in the entire dataset (excluding the residence time samples) were unable to be identified to any taxonomic rank. Only 7.1% and 3.0% (970 total sequences) of all sequences could be classified according to genus and species levels, respectively (Figure 3). See Table S4 for the full dataset of amplicon sequence variants (ASV).

3.1.2 | DNA residence time experiments

Kea DNA was not detected in samples collected before release, suggesting it is unlikely that kea is present at Opuatia and that there was no cross-contamination. The total number of kea DNA sequence counts from the three replicates taken from the fecal slurry (i.e., before release in the wetland) was 76,145, corresponding to 43,479 *N. notabilis* species-level counts and 32,666 counts at the *Nestor* genus level.

The strongest detections of kea DNA to species level in the wetland occurred 1 m from the release point at 1 h (17,543), 5 h (975), and 24 h (233), and 10 m from the release point at 5 h (226). Thus, the species DNA detection count at the 1 m, 1 h point had more than halved compared to the level in the starting slurry, while the genus DNA sequence count had more than doubled (Table 3). Overall, we were able to detect kea DNA to the 10 m line for up to 1 week post-release. However, as time progressed, the taxonomic resolution became dominated by genus versus species level detections. Kea DNA was never detected at the 25 m transect (Table 3). The weather was sunny, partly cloudy, with moderate wind for 2 days prior to the experiment up to 48 h post-release, while occasional showers and low to moderate winds occurred on Days 3–5, and heavy rainfall occurred the night before the 1-week post-release date (Table S1). It rained on six of the remaining days before the 3-week experiment end date.

For the in situ residence time experiment, kea DNA was only detected at 2 days post-release, with a DNA sequence count of nine at the *Nestor* genus level and zero counts detected at the species level. In comparison to the dispersal distance residence time experiment, slight water flow was observed, and light showers occurred for 6 days over the course of the experiment.

3.1.3 | Filter size comparison

nMDS analysis of the entire dataset revealed significant differences in DNA sequence composition among the 1.2, 5 μm, and dacron filters, as well as differences related to the time of sampling (Figure 4a). For example, eDNA results for the different filter sizes clustered separately at the start ($F_{2,13}=4.757$; $p=0.001$; PERMANOVA; $F_{2,23}=4.650$; $p=0.021$; PERMDISP), middle ($F_{2,14}=4.525$; $p=0.001$; PERMANOVA; $F_{2,14}=8.187$; $p=0.008$; PERMDISP), and end ($F_{2,14}=2.570$; $p=0.001$; PERMANOVA; $F_{2,14}=1.545$; $p=0.248$; PERMDISP) of spring. These patterns were driven by statistically significant pairwise comparisons between 1.2 μm versus 5 μm filters at the end of spring, 1.2 μm versus dacron filters at the start and middle of spring, and 5 μm versus dacron filters at the start and middle of spring (Table S5). These patterns were consistent with nMDS analysis of DNA sequence composition at the species and genus levels, as well as for the focused analysis on key species of interest (Figure S2; Table S5).

The average volume filtered across the three filter sizes at all temporal points fell short of the standard volume (Table 4). For example, the dacron filter only processed a quarter of the standard volume. Overall, the dacron filter processed the highest average volume of water (27.8 L; middle of spring), followed by the 5 μm filter (960 mL; start of spring), and the 1.2 μm filter (267 mL; end of spring).

Species accumulation curves from the entire dataset at all three temporal points consistently showed that as the number of replicates increased, so did the species richness for all three filter sizes (Figure 4b, Figure S3). The dacron filter (which filters the greatest volume) obtained the highest mean species richness for all three temporal points, followed by the 5 μm filter and then the 1.2 μm filter, except at the end of spring, where the 1.2 μm filter obtained a slightly higher mean species richness ($n=1834$) compared to the 5 μm filter ($n=1727$) at five replicates. See Figure S4 for details of

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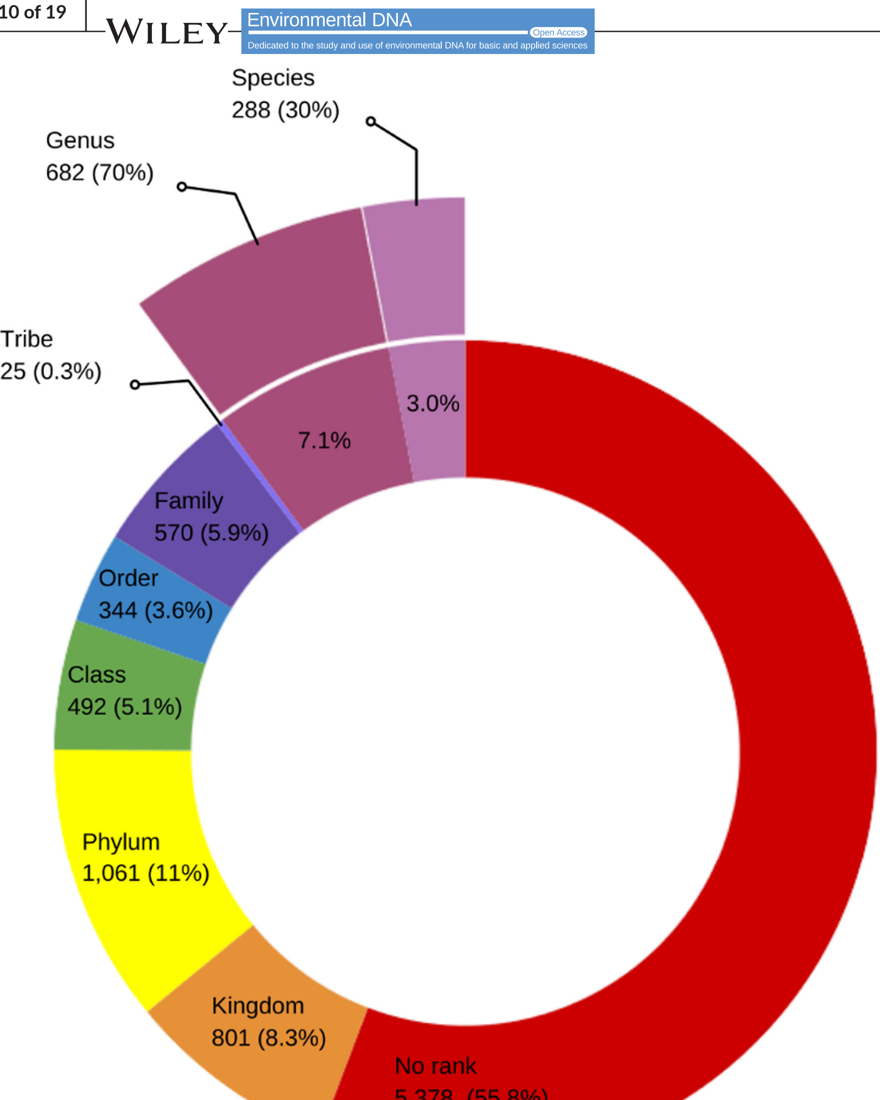

FIGURE 3 The proportion of sequences derived from all sites and filter sizes for each taxonomic rank for the entire dataset, excluding the residence time samples (inner ring), and the proportion of sequences exclusively obtained at the species and genus levels (outer ring).

TABLE 3 The residence time experiment DNA sequence count, where the first value is the DNA sequence count at the species level and the subsequent value at the genus level.

Distance from release point	Time since release						
	Pre-release	1 h	5 h	24 h	48 h	1 week	3 weeks
1 m	43,479; 32,666	17,543; 69,898	975; 31,738	233; 7632	22; 4037	0; 113	ND
10 m		0; 133	226; 8556	0; 32	0; 78	62; 63	ND
25 m		—	—	ND	ND	ND	ND

Abbreviations: —, not tested; ND, not detected.

each taxonomic group as a proportion of the total number of groups detected for each filter.

3.2 | Biodiversity assessments

3.2.1 | Spatial variation

nMDS analysis of the entire dataset revealed differences in DNA sequence composition across the four sites (Figure 5a), with statistically significant differences observed at the start ($F_{3,16}=10.368$; $p=0.001$; PERMANOVA; $F_{3,16}=3.401$; $p=0.042$; PERMDISP), middle ($F_{3,17}=8.820$; $p=0.001$; PERMANOVA; $F_{3,17}=14.471$; $p=0.001$; PERMDISP), and end ($F_{3,18}=7.190$; $p=0.001$; PERMANOVA; $F_{3,18}=1.957$; $p=0.166$; PERMDISP) of spring. Statistically significant pairwise comparisons were detected for SS2 versus SS3 and SS2 versus SS4 at the start and middle of spring, for SS1 versus SS3 at the middle and end of spring, and for SS1 versus SS4 at the middle of spring (Table S6). These patterns were consistent with nMDS analysis of DNA sequence composition at the species and genus levels, as

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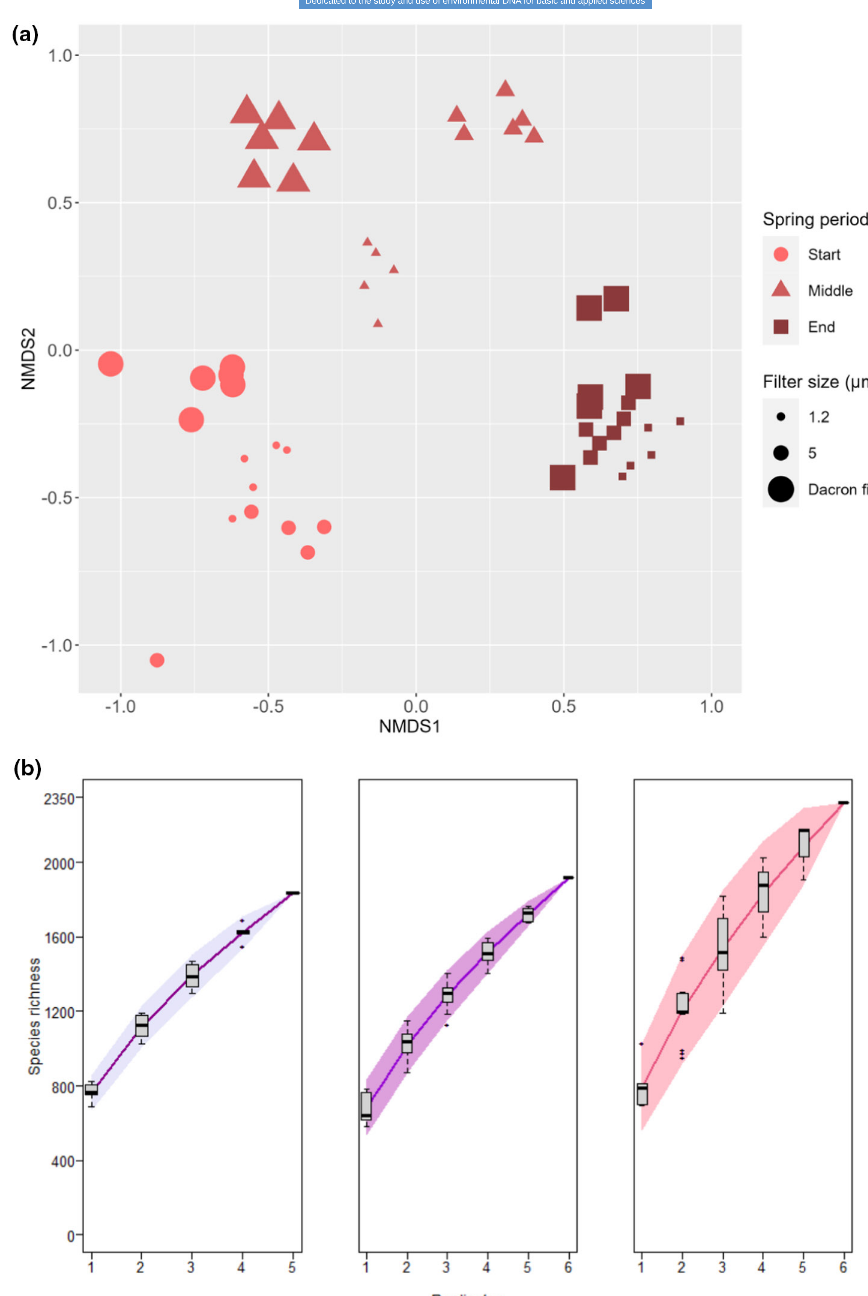

FIGURE 4 Filter comparison: (a) nMDS plot comparing the difference among DNA sequences between the 1.2 μm, 5 μm, and dacron filters at the start, middle, and end of spring from the entire dataset; and (b) Species accumulation curves overlaid with boxplots of species richness (absolute number of species detected) at the end of spring for the 1.2 μm (left), 5 μm (middle), and dacron (right) filters. The solid line indicates the random sampling model of species accumulation provided from the data and the shaded area represents the 95% confidence interval.

TABLE 4 The average volume filtered (mL) for each filter size at all temporal periods.

Filter size	Start	Middle	End
1.2 μm[a]	161	155	267
5 μm[a]	960	350	494
Dacron[b]	19,200	27,800	24,800

[a]Standard volume = 1000 mL.
[b]Standard volume = 100,000 mL.

well as for the focused analysis on key species of interest (Figure S5; Table S6).

Species accumulation curves from the entire dataset at all three temporal points consistently showed that as the number of sites increased, so did the species richness (Figure 5b, Figure S6). The highest mean species richness was recorded at the end of spring ($n=4756$), followed by the middle ($n=3667$) and the start of spring ($n=3398$).

3.2.2 | Temporal variation

Consistent with spatial results, nMDS analysis of the entire dataset revealed significant differences in DNA sequence composition between the three temporal spring sampling points at SS1 ($F_{2,14}=11.620$; $p=0.001$; PERMANOVA; $F_{2,14}=7.826$; $p=0.004$; PERMDISP), SS2 ($F_{2,13}=13.887$; $p=0.001$; PERMANOVA; $F_{2,13}=3.841$; $p=0.051$; PERMDISP), SS3 ($F_{2,12}=11.141$; $p=0.001$; PERMANOVA; $F_{2,12}=8.527$; $p=0.008$; PERMDISP), and SS4 ($F_{2,12}=9.012$; $p=0.001$; PERMANOVA; $F_{2,12}=7.829$; $p=0.010$; PERMDISP) (Figure 5a). Statistically significant pairwise

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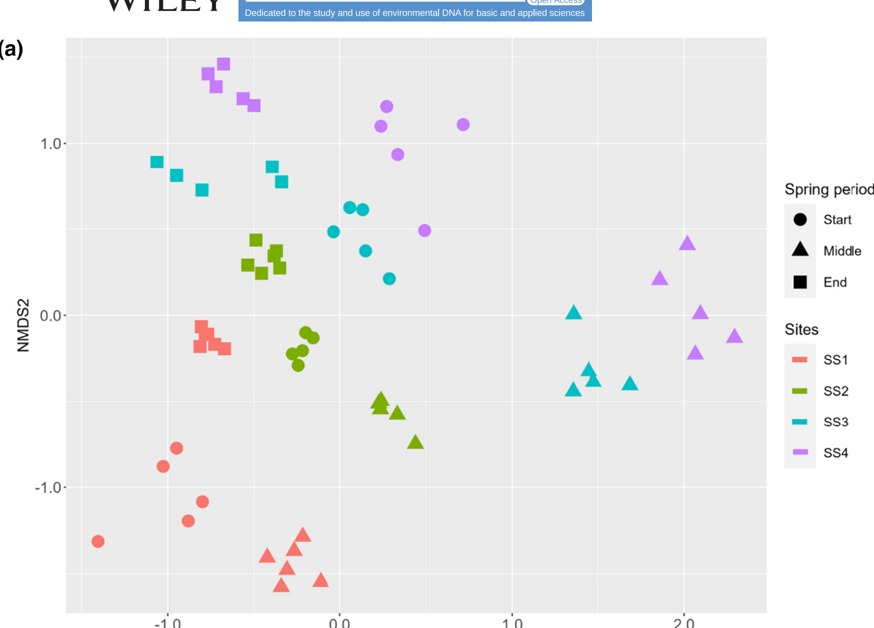

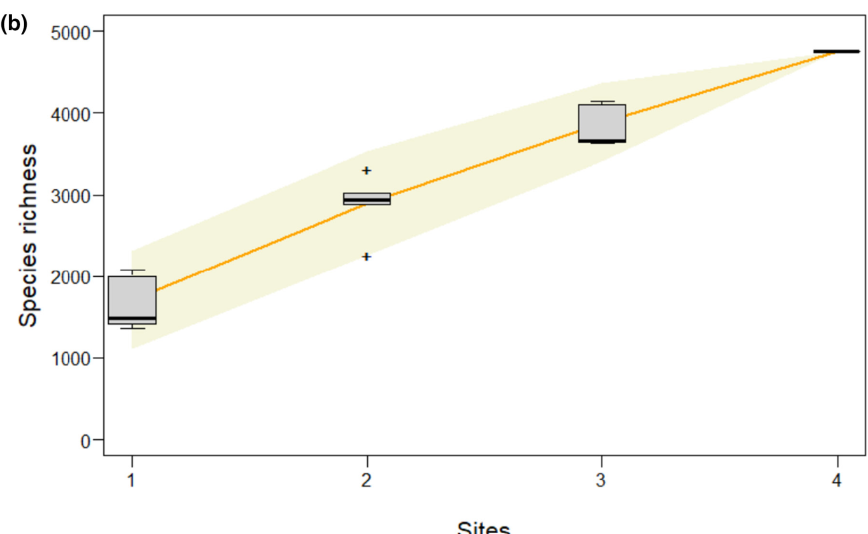

FIGURE 5 Spatial variation: (a) nMDS plot comparing the difference among DNA sequences across SS1-SS4 at the start, middle, and end of spring from the entire dataset; and (b) Species accumulation curves overlaid with boxplots (absolute number of species detected) at the end of spring for the number of sampling sites. The solid line indicates the random sampling model of species accumulation provided from the data and the shaded area represents the 95% confidence interval.

comparisons were detected for the start versus middle of spring at SS3 and SS4, start versus end of spring at SS1 and SS2, and middle versus end of spring at SS1, SS3, and SS4 (Table S7). As for the previous comparisons, patterns were consistent with nMDS analysis of DNA sequence composition at the species and genus levels, as well as for the focused analysis on key species of interest (Figure S5; Table S7). See Figure S7 for details of each taxonomic group as a proportion of the total number of groups detected for each temporal/spatial comparison.

3.3 | Conventional surveys compared to eDNA

3.3.1 | Zooplankton and eDNA surveys

The zooplankton surveys detected a higher number of unique taxa than the eDNA surveys across all three temporal points (e.g., 27 versus five unique taxa detections at the end of spring for the zooplankton versus eDNA surveys, respectively; Figure 6). Exceptions were *Acanthocyclops robustus* and *Synchaeta pectinata*, which were detected in both surveys at the end of spring at SS2-SS4, and SS2, respectively (Figure S8; Table S8). Several organisms were detected in the eDNA data at the genus level, but further identified to the species level using the conventional zooplankton survey methods (e.g., *Brachionus* sp. in the eDNA dataset was identified as *Brachionus angularis* or *Brachionus quadridentatus* by conventional survey; Figure S8). Notably, the rotifer *Parencentrum plicatum* was identified in the zooplankton survey at the start of spring, representing a new record for Aotearoa New Zealand; however, it was not detected in the eDNA results (Figure S8; Table S8). Conversely, eDNA sampling detected *Eubosmina coregoni* at SS2, SS3, and SS4 in the middle and/or end of spring—a potentially new record of a non-native species for the country; however, it was not detected in the zooplankton survey.

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FIGURE 6 Venn diagrams demonstrating unique and shared species detection from eDNA and conventional ("Conv.") surveys across all sites at the start, middle, and end of spring. These comparisons involve species and genus level detections, with the different icons representing the different conventional methods (green = zooplankton surveys; lime green = gee-minnow traps; yellow = bird-counts; purple = botany surveys). Asterisks indicate cases where one method detected an organism at a genus level, while the other resolved to the species level.

3.3.2 | Gee-minnow and eDNA surveys

Contrasting the zooplankton results, there were fewer unique detections (range 2–9) per survey method for the eDNA versus gee-minnow traps (Figure 6). In particular, all fish species captured in the gee-minnow traps at the end of spring were also detected in the eDNA data (Figure S9). However, several fish species were detected using eDNA that were not captured in the gee-minnow traps (e.g., brown bullhead catfish *Ameiurus nebulosus*, goldfish *Carassius auratus*, common bully *Gobiomorphus cotidianus*), while the traps caught invertebrates (water spider *Dolomedes* sp., diving beetle *Rhantus suturalis*) that were not detected using eDNA (Figure S9; Tables S9–S11).

3.3.3 | Bird counts and eDNA surveys

The 5-min bird counts identified four and five detections that overlapped with the eDNA surveys at the middle and end of spring, respectively (Figure 6), with both methods predominantly detecting different species (up to 12 unique species in the bird counts and up to seven in the eDNA; Figure 6) (Figure S10, Tables S12 and S13). Notably, *Botaurus poiciloptilus* (matuku/Australasian bittern)—a critically threatened native bird—responded to an audio playback of its birdsong at SS1, but was not detected in the eDNA data.

3.3.4 | Botany and eDNA surveys

The botany versus eDNA surveys had the largest detection of shared taxa across the whole dataset ($n = 19$ overlaps in the middle of spring) (Figure 6). However, when considering individual sites, only three of the 58 total plant species recorded were identified with both methods: *Galium* sp. (bedstraw) and *Juncus* sp. (rushes) at SS2 and *Plantago* sp. (plantain) at SS3 (Figure S11). Similar to the zooplankton results, most of the eDNA detections were to the genus level, while the botanical surveys primarily resolved plants to the species level (Table S14). However, greater similarities were observed between methods when comparing at the genus level (e.g., *Azolla*, *Coprosma*, *Glyceria*, *Holcus*, *Iris*, *Juncus*, *Rubus*, and *Ulex* were identified with both methods). Interestingly, SS3 and SS4 are dominated by introduced *S. fragilis* (crack willow) and *S. cinerea* (gray willow), but neither of these species were detected at species level using eDNA. Instead, each was identified at the tribe level (*Saliceae*). Meanwhile, an unusual eDNA detection was *Fuscospora* sp. (beech tree) at SS3 and SS4.

4 | DISCUSSION

We tested different eDNA field techniques to understand how biodiversity signals vary spatially and temporally in Aotearoa New Zealand wetland environments. We found an overall pattern in which conventional survey methods showed key differences to the eDNA results, elucidated the residence time of eDNA at Opuatia Wetland, and identified significant differences between filtering methods, spatial sites, and temporal sampling points.

Understanding the persistence and transport of eDNA signals is important because it indicates the relative age of the deposited DNA (Jo, 2023) and its spatial representativeness (Civade et al., 2016), ultimately assisting the interpretation of eDNA data. Our experiments showed that foreign DNA was detectable for up to 1 week following release within a 10 m radius from the source, and for only 2 days post-release in our in situ experiment. The disparity between these two experiments highlights the impact of environmental factors, such as weather and water flow, on eDNA persistence and future work should combine these two factors (i.e., in situ and dispersal distances) into a single larger experiment to investigate this issue further. The absence of the foreign DNA at the 25 m point in our dispersal distance experiment may have been due to the presence of impeding vegetation between the 10 and 25 m transect lines and/or limited water flow. However, the vegetation did not completely obstruct the flow path, particularly during flooding. Our findings were consistent with the literature, where eDNA concentrations have been shown to decrease with increasing distance from the source point and increasing time (Goldberg et al., 2016; Harrison et al., 2019). In

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wetland environments, DNA persistence is likely to be significantly impacted by abiotic factors (e.g., pH <5, water temperature > 25°C, limited dispersion; Goldberg et al., 2018). However, different environments have varying intensities of physicochemical parameters and hydrological processes, which may be attributed to varying eDNA decay rates (Jo & Minamoto, 2021; Seymour et al., 2018). For example, Ely et al. (2021) could no longer detect in situ foreign DNA after 7.5 h in a nearshore rocky reef habitat, while a caged fish experiment in a marine environment showed detection of target DNA up to 1 km from the cage, but recovered no signal just 2 h after the cage was removed (Murakami et al., 2019). Meanwhile, in riverine environments, target eDNA could be detected at distances from 9 km (Deiner & Altermatt, 2014) to 100 km (Pont et al., 2018) downstream from the release point.

The type of filter used when collecting eDNA dictates the size of particles that can permeate through, ultimately affecting DNA detection sensitivity (Schabacker et al., 2020). For example, in a stream environment, 5 μm filters have been shown to process larger volumes of water and return higher DNA concentrations than 1.2 μm filters (Banks et al., 2021). In turbid environments—where filter clogging is more apparent—a 20 μm filter has been shown to be effective in capturing eDNA (Cooper et al., 2021, 2022; Robson et al., 2016). Consistent with this, we found that the dacron filter exhibited the highest mean species richness, followed by the smaller 5 μm and 1.2 μm filters. Thus, unsurprisingly, filtering larger volumes of water using larger filter sizes can increase the mean species richness detected in wetlands, as has previously been shown in river and marine environments (e.g., Banks et al., 2021; Jensen et al., 2022; Jeunen et al., 2022; Macher et al., 2021; Smith et al., 2023). However, from a taxon-specific perspective, opting for a smaller filter size may be sufficient in some cases while also requiring shorter filtration times. For example, for fish, the 1.2 μm filter yielded similar proportion of species richness compared to the dacron filter in our study. The number of collected sample replicates is also important in wetland sampling, with species accumulation curves in our study indicating that 5–6 replicates are required to capture the highest biodiversity, as recommended in eDNA sampling guidelines for streams, rivers, and lakes (De Brauwer et al., 2022; Smith et al., 2023). However, the replicate-based species accumulation curves in our study did not reach a plateau—indicating that further sampling would likely return higher species richness.

Understanding of spatial and temporal heterogeneity of eDNA signals is fundamental for providing new insights into shifts in biodiversity signals and defining when and where to sample to attain a comprehensive evaluation of overall biodiversity. Here, we found statistically significant spatiotemporal variation in DNA sequence composition. Despite SS2-SS4 all being classified as marsh wetlands situated within a 1 km proximity at the same wetland site, significant pairwise differences in biodiversity (i.e., DNA sequence composition) were observed among these sites. Less surprising, SS1—a swamp—exhibited significant differences in biodiversity to the marsh sites (except for SS2, which might be due to their relatively closer proximity), highlighting the value of spatial sampling especially for wetlands where the wetland type varies within the overall site. Other research has also found distinct geographic patterns in species distribution (for fishes, amphibians, and mammals) when sampling a transect from freshwater to marine in coastal wetlands (Saenz-Agudelo et al., 2021) and differences in biodiversity patterns between coastal and temporary inland wetlands (Coleman et al., 2023), while riverine and lake environments also show variation in eDNA samples from different spatial sites (Civade et al., 2016; Hänfling et al., 2016). Here, as the number of sampling sites increased, so did species richness, consistent with previous studies conducted in pond and riverine environments (Bylemans et al., 2018; Evans et al., 2017; Macher et al., 2021). This suggests that sampling at one site within a wetland is unlikely to yield a representative result, underscoring the importance of accounting for even small-scale (~1 km) spatial sensitivity. However, similar to the replicate-based species accumulation curves, the site-based curves in our study did not level off; thus, the optimum number of both replicates and sampling sites required to yield representative results for the biodiversity of wetland sites remains unknown and should be specifically tested in future.

Temporal variation was also evident in our study, with the end of spring yielding the highest mean species richness. Compared to spatial variation, relatively few studies have investigated temporal shifts in eDNA, with the research that has been undertaken typically investigating short time scales (i.e., within a year; Mathieu et al., 2020). However, Gabrielsen et al. (2022), used a combination of eDNA and remotely sensed imagery over 3 years to show that habitat variability at both spatial and temporal scales played a pivotal role in shaping the occurrence and abundance of three amphibian species. Meanwhile, hourly collections over a 32 h period in a marine environment revealed short-term temporal variation in fish and eukaryote richness, with species richness highest during dawn (Jensen et al., 2022). Temporal fluctuations in the abundance of scalloped hammerhead (*Sphyrna lewini*) and tiger sharks (*Galeocerdo cuvier*) over a 13-week sampling period during summer have also been recorded (Mariani et al., 2021).

To gain insight and validate best practice approaches, eDNA is often compared to conventional biomonitoring methods, where a key finding has been that eDNA should ideally complement rather than replace such methods (Schenekar, 2022). Our findings support this perspective, particularly in the case of the zooplankton surveys, 5-min bird counts, and botanical surveys, where certain species were only detected by one method or the other. For example, previous floral records for Opuatia Wetland (Barnes et al., 2001; Reeves, 2011) showed minimal overlap with the eDNA results observed here for many genera and most species. Meanwhile, greater overlap was observed for bird and fish species, with 8/11 bird species and 10/15 fish species detected in the eDNA versus conventional methods used here (Reeves, 2011; https://nzffdms.niwa.co.nz/). These patterns are consistent with other research, where eDNA has detected more zooplankton species than morphological-based methods in marine and lake environments (Qiu et al., 2022; Suter et al., 2021). Similarly, Mejia et al. (2021) observed low concordance between a botanist survey and eDNA results at Mojave

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Desert Springs. Conversely, Coleman et al. (2023) observed a similar resolution between conventional methods and eDNA for fish and frog species from wetland habitats. Meanwhile, eDNA has generally been shown to detect all species recorded through conventional fish monitoring (David et al., 2021; Griffiths et al., 2020; Wang et al., 2021). Consistent with this, our eDNA samples detected all the fish species that were caught in the gee-minnow traps. However, they also detected additional fish species (and insects) that were not trapped. Notably, gee-minnow traps are small and are designed to target small-bodied species, such as rare mudfish (Lake, 2013); using additional traps, such as fykes nets, would likely have caught at least some of the other species only detected in the eDNA dataset.

A common theme observed when comparing conventional and eDNA methods here was that conventional methods more often involved taxonomic identifications to the species level. This highlights the dependence of eDNA on reference databases (e.g., the National Centre for Biotechnology Information—NCBI GenBank; and the Barcode of Life Data System—BOLD) for matching unknown genetic sequences, that suffer from a significant amount of missing data (Hotaling et al., 2021). Indeed, more than a quarter of the 3000 most encountered sequences across eight metabarcoding assays could not be appointed to a phylum in a recent study (Wilkinson et al., 2024), and >50% of sequences were unassigned to any taxonomic rank here in our study. This emphasizes the importance of current plans to develop a reference database for biodiversity In Aotearoa New Zealand (https://www.landcareresearch.co.nz/events/national-dna-database-webinar-series/), as well as global sequencing initiatives (e.g., Vertebrate Genome Project; https://vertebrategenomesproject.org/ ; Bird10K; https://b10k.genomics.cn/index.html; i5K Project; https://i5k.github.io/; Earth BioGenome Project; https://www.earthbiogenome.org/) that may help to address these data gaps.

Notably absent from our eDNA data were the rotifer *P. plicatum*, matuku (Australasian bittern *Botaurus poiciloptilus*), crack willow, and gray willow. Conversely, eDNA detected *E. coregonia*—a non-native water flea potentially new to Aotearoa New Zealand—and *Fuscospora* sp. (beech tree). The absence of *P. plicatum* and matuku in the eDNA detections can be attributed to the current unavailability of their reference sequences in the eDNA reference database—it is possible that eDNA sequences for these species are present in our results but could not be identified at the species level. The absence of crack and gray willow at the species level is due to current eDNA assays being unable to distinguish *Salix* sp. to species level, and the sharing of identical barcodes between these and other *Salix* and *Populus* species. Meanwhile, the *E. coregoni* detection may actually represent *Bosmina meridionalis*—a native species for which *E. coregoni* is the closest database match, while the *Fuscospora* sp. detection may represent pollen that traveled into the wetland from a local garden. These non-detections highlight the strengths and limitations associated with eDNA, particularly its variation in effectiveness across taxa that are more or less likely to be detected in a water sample. When used in conjunction, eDNA and conventional methods thus likely offer a more comprehensive view of the overall ecosystem biodiversity.

Nevertheless, eDNA methods are continuing to improve, with the development of new assays, decreasing costs of sequencing, and regular additions to the reference database together enhancing their potential for species detection and identification. Indeed, eDNA has revolutionized the way we monitor biodiversity, and significant recent efforts dedicated to protocol development and optimization (Schenekar, 2022), have enhanced eDNA efficiency and use (Gleeson, 2021). Here, we explored the effects of filter size and spatial and temporal sampling schemes on biodiversity detection using eDNA in wetland environments. Outstanding questions include whether eDNA can detect macroorganisms in other challenging wetland environments (e.g., high-temperature geothermal wetlands and low pH bogs), and whether quantitative measures of ecosystem quality and health (e.g., the taxon-independent community index, "TICI"; Wilkinson et al., 2024) can be extended to wetlands and other environments.

AUTHOR CONTRIBUTIONS

SB, AM, and PD conceived the research project. SW advised on the study's experimental design and analyses, JS and ID performed conventional surveys. SB collected eDNA samples and analyzed the data, with AM helping with the former. SB wrote the first draft of the manuscript and all authors provided feedback, with SB leading subsequent revision of the final manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

eDNA data are available at Wilderlab (https://www.wilderlab.co.nz/explore; Accession numbers: 603518, 603589, and 605453), and raw sequence files are available on the SRA (BioProject number: PRJNA1102914). The ASV table is further provided in Table S4, and all data files required to reproduce the results in this manuscript are available at: https://github.com/invasomics/eDNA_OpuatiaWetland.

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