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Monitoring vertebrate biodiversity of a protected coastal wetland using eDNA metabarcoding

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Abstract

Monitoring plans using environmental DNA have the potential to offer a standardized and cost-efficient method to survey biodiversity in aquatic ecosystems. Among these ecosystems, coastal wetlands are key elements that serve as transition zones between marine and freshwater ecosystems and are today the target of many conservation and restoration efforts. In this sense, eDNA monitoring could provide a rapid and efficient tool for studying and generating baseline biodiversity information to guide coastal wetland management programs. Here, we test an eDNA metabarcoding assay as a tool to characterize vertebrate biodiversity in one of the largest coastal wetlands of Chile, the Rio Cruces Wetland, a Ramsar designated site since 1981. We sampled surface water from 49 sites along the entire wetland. Our eDNA approach detected 91 genera of vertebrates including amphibians, fishes, mammals, and birds, as well as identified several cryptic, exotic, and endangered species. Our results also indicated that the spatial distribution of eDNA from different species is spatially structured despite the complex hydrodynamics inherent in this wetland due to the influence of daily tidal regimes. For amphibians and fishes, the number of taxa detected with eDNA was higher in the periphery of the wetland, and increased with proximity to the ocean, a pattern consistent with small-scale spatial sensitivity for some species and eDNA accumulation downstream for others. Birds and mammals showed somewhat more idiosyncratic distributions. Taken together, our results add to the growing body of evidence showing eDNA can serve as a rapid cost-effective tool

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to characterize vertebrate communities in protected coastal wetlands, where visual surveys are difficult and animal collections are often prohibited. The use of multiple primer sets is also recommended as it facilitates the detection of ephemeral terrestrial organisms and resident aquatic organisms that make use of these wetlands.

KEYWORDS

amphibians, biological monitoring, birds, coastal wetland, community structure, DNA sequencing, fishes, mammals

1 | INTRODUCTION

Natural and anthropogenic threats to natural ecosystems are increasing worldwide (Hoegh-Guldberg et al., 2019; Malhi et al., 2020; Steffen et al., 2015). Monitoring plans are one of the tools that can help evaluate how community structure and function change over time. They also inform about how environmental, habitat, or climate changes are affecting biodiversity. Long-term monitoring programs can help reveal what species are most susceptible to environmental variations, identify new threats, and understand how different species in an ecosystem are connected (Gauthier et al., 2013; Lindenmayer et al., 2012, 2020). These programs can also provide valuable information for conservation and restoration planning efforts that target threatened ecosystems (Halme et al., 2013; Hartig et al., 2009; Sergeant et al., 2012). However, conventional monitoring programs can be costly and require a substantial workforce, often relying on specialized taxonomic expertise and sampling skills (Qu & Stewart, 2019; Valentini et al., 2016). In this sense, novel methods that provide cheaper, faster, simpler, but still standardized protocols might substantially increase the efficiency of biodiversity monitoring programs (Deiner et al., 2017).

Environmental DNA (eDNA) metabarcoding is rapidly revolutionizing the way we perform biodiversity monitoring, particularly in marine and freshwater ecosystems. Some of the significant advantages of eDNA metabarcoding include its non-invasive nature, flexibility in species targeted (from single species to entire communities; e.g., Stat et al., 2017) and the relatively rapid and cost-effective application of protocols to a broad range of ecosystems (Barnes & Turner, 2016; Bohmann et al., 2014; Rees et al., 2014; Ruppert et al., 2019; reviewed in Taberlet et al., 2012). In particular, eDNA metabarcoding has been successfully applied to evaluate a full range of situations in different aquatic ecosystems (e.g., Adrian-Kalchhauser & Burkhardt-Holm, 2016; Andruszkiewicz et al., 2017; Ardura & Planes, 2017; Deiner et al., 2016). These methods have also been used to address different objectives, including tracking of biological invasions (Ardura & Planes, 2017; Ardura et al., 2015; Ficetola et al., 2008; Hunter et al., 2019), detection of cryptic species (Bryson et al., 2020; Nester et al., 2020; Rose, 2020), delimiting species assemblages (Berry et al., 2019; DiBattista et al., 2020; Sales et al., 2020; Shackleton et al., 2019), and ecological assessment (Seymour et al., 2020). Traditional surveys to determine the abundance and distribution of macroorganisms often involve invasive (i.e., capture

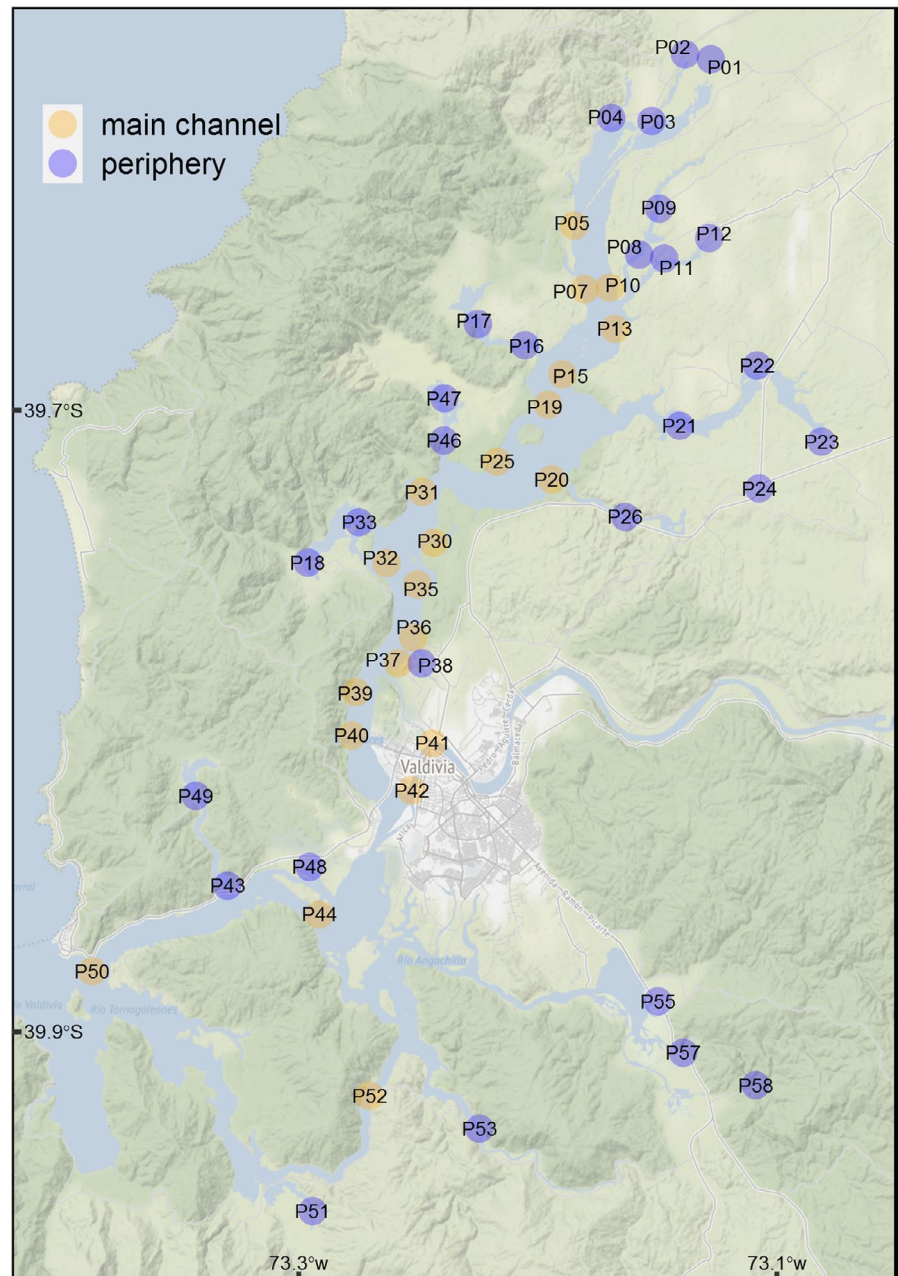
and sometimes lethal collection methods. In this sense, eDNA offers a non-invasive, cost-efficient approach for biomonitoring of aquatic systems, especially to characterize assemblages of large, highly mobile, or cryptic vertebrates.

The application of eDNA to study and monitor vertebrates is rapidly expanding. Most eDNA studies so far have targeted fishes and amphibians, and the ability of these techniques to detect other groups such as mammals or birds has received less attention. However, new studies are beginning to emerge that show that eDNA can be used to detect and monitor terrestrial vertebrates such as mammals or birds (Sales et al., 2020) at different timescales (Domaizon et al., 2017). Similarly, most eDNA metabarcoding studies have targeted marine or freshwater ecosystems such as rivers and lakes (reviewed in Beng & Corlett, 2020). The use of eDNA to characterize biodiversity in coastal wetlands and estuaries with significant tidal ebb and flow, however, remains somewhat limited.

Coastal wetlands are an essential interface between marine and land ecosystems (Semeniuk & Semeniuk, 2018). They provide unique habitat to a broad range of organisms (Figuerola et al., 2016) and supply several beneficial essential ecosystem services to human populations (Barbier, 2017; Engle, 2011; Marquet et al., 2017). However, estuaries are currently considered highly vulnerable, given that more than 10% of humans live in coastal areas that overlap with these ecosystems (McGranahan et al., 2007). The lack of biodiversity information is one of the key obstacles to proper management of wetlands as whole ecosystems. In Chile, wetlands cover approximately 4.5 million ha and 5.9% of the territory, which includes 12 Ramsar sites that spread across 359,989 ha (Donoso et al., 2018). In general, wetlands in Chile are classified as biodiversity hotspots; however, there is no complete understanding of the extent and conservation status of these environments (Figuerola et al., 2016). In this context, it is a priority to generate long-term biodiversity monitoring plans that are effective in this type of environment (Goldberg et al., 2015).

Here, we used eDNA to characterize vertebrate biodiversity at the Río Cruces Coastal Wetland in Southern Chile. This wetland, designated a Ramsar site since 1981, is today a Fauna and Flora Sanctuary and among the largest coastal wetland systems in the country (Marquet et al., 2017). The current hydrological configuration of this wetland was partly the result of tectonic subsidence during the 1960 mega earthquake in Chile and subsequent flooding (Plafker & Savage, 1970). It is dominated by a dynamic tidal regime where the extent of saltwater intrusion upstream displays daily,

FIGURE 1 Map of the study area and indicating sampling sites. Each site is represented by a colored circle. Sampling sites codes correspond to those indicated in all other figures and detailed in Table S2. Color codes correspond to the channel zone classification (periphery vs main channel) used for some of the statistical analyses



monthly, and even seasonal oscillations. It is a system with recurrent anthropogenic threats, including one well-documented environmental catastrophe associated with polluted water discharge in 2004 (Jaramillo et al., 2018; Lagos et al., 2008). To our knowledge, no comprehensive spatial community structure study exists for this wetland. Herein, we tested the efficiency of eDNA metabarcoding assays targeting targeted mammals, birds, and fishes as a monitoring tool comparing the species recovered using eDNA versus historical species records obtained through conventional surveys. We then explored the spatial structure of vertebrate communities in this coastal wetland inferred from eDNA. We hypothesized an increase in species and genera richness from tributaries to the main channel of the Río Cruces river and from upriver toward the sea. The rationale was twofold: first, marine ecosystems harbor richer vertebrate communities of taxa such as fishes and birds and second, the hydrology of

the system with net water downstream transport should result in species eDNA accumulation toward the sea (Sales et al., 2021). Thus, we expected to detect freshwater species upriver and downriver, but marine species were expected only in the lower, downstream reaches of the wetland. Following a similar rationale, we expected to find higher richness in the main channel compared to the tributaries. Possible departures from this expectation could result from dilution and degradation of eDNA of terrestrial or freshwater origin, as it is transported toward the sea (Barnes et al., 2014). Beyond species richness, we also studied spatial changes in species composition. Given our fine sampling grain and habitat heterogeneity, we hypothesized geographically structured species communities, and a major community composition gradient along the freshwater–marine hydrological network. We looked for indicator species and representative sites that best reflect these patterns. We show that eDNA

recovers species historically recorded in the area by conventional methods and that the species composition inferred from eDNA displays spatial structuring. In particular, our data indicate an increase in species detections seawards, suggesting that despite significant tidal flow upstream twice a day, eDNA from both freshwater and marine habitats accumulates near oceanic connections.

2 | METHODS

2.1 | Sample collection and laboratory processing

We collected water from the Río Cruces Coastal Wetland including its subsidiaries. Water was collected by boat (45 sites) or from the shore (4 sites) under research permit N SNRC01/2018 issued by the Corporación Nacional Forestal. A total of 49 sampling points at least 1.5 km apart from each other were distributed along most of the wetland and encompassed many of its tributaries (Figure 1). 45 sites were sampled within 4 consecutive days (12–15 February 2019) during a period of neap tide. Four sites (P18, P55, P57, and P58) that could not be accessed by boat were sampled a month later by land (14–15 March 2019). At each sampling site, three 1L water samples were taken 30 cm below the surface. Water samples were stored in glass bottles previously treated for at least 10 minutes with 10% bleach. All water samples were filtered immediately after collection in the field using manual vacuum pumps and 0.45 µm pore size (47 mm diameter) hydrophilic mixed cellulose esters sterile filters (Pall Corporation, NY, USA). All filtering and handling materials were soaked in the 10% bleach solution before water filtration. A filtration negative control ($n = 1$) was included by filtering 1L of molecular grade water (Milli-Q® filtered) in the field following the same procedure as all other water samples. Given that water quality (including the amount of particulate matter) is highly variable in estuaries, the volume of water that we filtered varied among the sites. The general rule that we followed was to filter 1L of water whenever possible or filter water until the membrane became saturated. Filters were then stored in the laboratory in 2 ml plastic tubes containing 1 ml of lysis buffer (Thermo Fisher Scientific, Waltham, MA, USA) at 4°C until extraction within two months of collection. Overall, we filtered 148 samples of water (3 from each of the 49 sites and one Milli-Q® water control). See Table S2 for more details.

2.1.1 | eDNA extractions

DNA extraction was performed by first placing each tube in a Mini-Beadbeater-16 (BioSpec products inc, Bartlesville, USA) for two minutes at $2.5 \times 1,000$ stroke/min. Tubes were then opened in the laminar flux chamber where 500 ml of supernatant was taken and transferred to a 1.5 ml microcentrifuge tube for DNA extraction using the GeneJET Genomic DNA Purification Kit (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's protocol with some modifications (50 µl of the Proteinase K solution was

added and the sample vortexed; samples were then incubated at 56°C for 3 hr). The rest of the procedure followed the manufacturer's recommendations. DNA samples were eluted in 60 µl of elution buffer. DNA extractions were processed in batches of 11 samples, and for each batch, a negative extraction control (six in total) was included (using 500 µl lysis buffer and no added sample). Purified extracted DNA samples were stored at -20°C until PCR amplification.

2.1.2 | PCR amplification

We used a two-step PCR method to amplify eDNA and to add a unique combination of dual barcodes. DNA samples, including the filter and DNA extraction negative controls, were first amplified with three primer sets targeting specific fragments of the mitochondrial 12S and 16S rRNA genes. These primers included an Illumina primer sequence, a 12 base pair (bp) barcode with a 0–4 bp spacer, and the actual target primer sequence (Table S2). Each sample ($n = 147$) and all negative controls were amplified in duplicate. In addition, we also incorporated three PCR negative controls. Details of the PCR protocol can be found in the Appendix S1.

2.1.3 | Complementary sequences to the reference database

The number of local vertebrate species for which 12S and 16S sequence data are publicly available was limited. We supplemented this resource by obtaining tissue samples from 14 mammal, 19 bird, and 6 fish species expertly identified by colleagues, as well as from the vouchered mammal collection at the Universidad Austral de Chile. We extracted DNA from these samples using the GeneJET Genomic DNA Purification Kit following the manufacturer's protocol. We performed PCR reactions using the same primer sets and PCR conditions as described above. Fragment sizes were verified with UV light on a 1% agarose gel stained with GelRed™ (Biotium, Inc., Fremont, CA, USA). The PCR products were then Sanger sequenced at the AUSTRAL-omics bioscience core facility at the Universidad Austral de Chile. Sequences were quality checked and trimmed using Geneious Prime v. 2019.2.3 and verified by the BLAST algorithm against the National Center for Biotechnology Information (NCBI) nucleotide database. A positive match was accepted if the most similar sequence was of the same genus as the query sequence. Taxonomic information for these species can be found in Table S3.

2.1.4 | Laboratory environment

Sample processing was performed at the Genética y Ecología Molecular laboratory at the Universidad Austral de Chile. Every day, before processing samples, all surfaces were cleaned using 10% bleach solution and then wiped with 70% ethanol. All eDNA sample manipulations were performed in a laminar flux chamber fitted with

a Hepa filter. Prior to use, the chamber was cleaned with DNAZap™ (Thermo Fisher Scientific, Waltham, MA, USA) and then irradiated for 10 min with UV light. All equipment (micropipettes and micro-centrifuge) were also cleaned prior to each use with DNAZap™. The laminar flux chamber and the centrifuge are of exclusive use for eDNA samples. Tissue samples for reference data building were processed using a different set of micropipettes, centrifuge, and thermocycler. All of these were disinfected every day using the same procedure as for the equipment used exclusively for eDNA.

2.1.5 | Sequence data analysis

Illumina sequences were first demultiplexed using Geneious Prime 2019.2.3, allowing for up to one mismatch in the barcode or spacer. Demultiplexed sequence files were then processed using the Anacapa tool kit (Curd et al., 2019). First, we created one CRUX reference library for each of the three primer sets using the public databases NCBI_blast_nt and EMBL std. This was done by following instructions in (https://github.com/limey-bean/CRUX_Creating-Reference-libraries-Using-eXisting-tools). Once these custom reference libraries were built, we added sequences from our reference sequence library described in the previous section. We then ran the first Anacapa pipeline "Anacapa_QC_dada2.sh" to quality filter sequence data and to generate Amplicon Sequence Variants (ASV) (Callahan et al., 2016). Briefly, for each raw demultiplexed file, adapters and primers were trimmed using cutadapt (Martin, 2011), then low-quality reads ($Q < 30$) were removed and reads were sorted by primer sequence using fast-toolkit (Gordon & Hannon, 2010). These sequences were then passed through a custom script that sorts the reads as forward, reverse, and unmerged read files, which were then input into the DADA2 program where they were merged (when possible) denoised, tested for chimeric sequences, and finally grouped into ASVs. As a conservative measure, a sequence had to occur at least four times in the dataset to be retained as an ASV. We then input the ASV.fasta files into the second Anacapa pipeline "Anacapa_classifier.sh" to assign taxonomy to each ASV. Here, all ASVs were globally aligned to the CRUX reference catalogs using bowtie2 (Langmead & Salzberg, 2012), and the best 100 hits were retained. These ASVs and hits were then input into BCLA where bootstrap confidence scores were assigned to taxonomic assignments (Gao et al., 2017). The output consisted of summary tables of ASVs and taxonomy count data for each sample and each primer set.

2.2 | Contamination denoising and data consolidation

Summary tables produced by Anacapa were converted into phyloseq class objects and imported into R v 3.5.1 (R Core Team, 2018) using the "phyloseq" package (McMurdie & Holmes, 2013). We then used the package "decontam" (Davis et al., 2018) to remove contaminant ASVs that appeared in the negative controls ($n = 10$). We used the

prevalence method to remove all contaminant ASVs with a threshold of 0.5. In other words, if an ASV was present in a given proportion of the negative controls, it was considered a contaminant if it was present in an equal or lesser proportion of the samples.

To assess taxonomic assignments and compare these to historical records of vertebrate species from this coastal wetland, we accepted an assignment at the genus and species level with a $\geq 80\%$ bootstrap confidence. We then compared these taxonomic assignments with historical records from the literature (see Table S4, for details).

2.3 | Statistical analyses

2.3.1 | Spatial variation in richness

We tested the spatial variation in richness hypothesis by fitting Poisson generalized linear models, where we modeled the number of taxa as a function of both fluvial distance (km) from the sampling location to the lowest site representing near-marine conditions (distance to site P50 or D.P50), and a categorical zone variable coded as "tributaries" or "main channel". We built one model for each of the four taxonomic classes instead of incorporating taxonomic class as a covariate in one statistical model. We did this because it was out of our scope to statistically compare richness among taxonomic groups and to avoid statistical complexity that arises from complex interactions when dealing with three explanatory variables. In each case, we began by fitting a full model with interaction and dropped terms if they were not significant. We repeated these analyses for fishes where the number of taxa as a function of distance to the ocean was modeled for marine, euryhaline, and freshwater species separately. Model comparisons were performed using likelihood ratio tests and a chi-square distribution and only the best models were presented. Given that data for different taxonomic groups were not independent, we applied a Bonferroni correction for these tests accordingly.

2.3.2 | Spatial variation in community structure

To study the drivers behind gradients of species richness and spatial structure, we conducted a series of multivariate analyses. First, indirect and direct gradient analyses were conducted to study if, and to what extent, the freshwater-marine gradient, represented by fluvial distance to the sea (D.P50), was related to the observed community structure. Second, non-hierarchical cluster analysis was used to identify groups of sites that share similar species composition, to examine whether these clusters were geographically structured, and to identify diagnostic species for each cluster, as well as representative sites. We implemented this analytical strategy separately for each of the four taxonomic classes.

The gateway for most of the analyses was the calculation of a general-purpose beta diversity, pairwise dissimilarity matrix. We computed the Sørensen dissimilarity index with the function "vegdist" using the R package "vegan" (Oksanen et al., 2019). This index

gives double weight to the number of double ones (species shared by pairs of sites). Bearing in mind that our presence–absence matrix had many zeroes (putative absences), and because it was reasonable to assume that this matrix was imperfect, especially with regard to low detectability of certain species (e.g., false negatives of rare species), this asymmetric dissimilarity measure seems an appropriate choice as it relies heavily on common species occurrences between pairs of sites (Borcard et al., 2011).

Indirect gradient analysis was carried out by unconstrained ordination of sites through principal coordinates analysis (PCoA), based on the Sørensen dissimilarity matrix. The vector of distances to the sea (D.P50) was fitted *a posteriori* onto the first two canonical dimensions of the ordination, and the significance of the association was assessed through a permutation test. This allowed a distended graphical representation of the community composition variation that can be represented in two dimensions, while passively gauging if and how this variation related to distance to the sea. Additionally, we conducted direct gradient analysis by constrained ordination through distance-based redundancy analysis (db-RDA). The input for db-RDA was the same dissimilarity matrix as outlined above, with D.P50 now used as a constraining variable. The significance of the constraining axis was assessed through a permutation test for constrained ordination. This allowed a more targeted and powerful assessment of whether D.P50 explained a significant proportion of the multivariate community composition variance. PCoA and db-RDA were fitted with “cmdscale” and “capscale,” respectively, the *a posteriori* D.P50 fitting and test of significance with “envfit,” and the test of significance of db-RDA with “anova.cca”; all functions were used in the R package “vegan” (Oksanen et al., 2019).

The clusters of sites were identified through the Partitioning Around Medoids (PAM) method, a non-hierarchical clustering algorithm with few assumptions compared to other common clustering methods. It has the added advantage of identifying typical sites (medoids) as a central feature. Other clustering methods did not materially alter the general conclusions of this study (data not shown). The number of clusters (k) must be specified *a priori*, and so to choose an optimal k , we ran PAM iteratively while varying k incrementally. These results were screened for cluster-specific and average silhouette widths, matrix correlation statistic (Pearson), and indicator values of species in clusters considered both as sum of significant indicator values and proportion of clusters with significant indicator species (Borcard et al., 2011). The optimal number of clusters for fishes was $k = 2$, since the above criteria were mostly convergent. Results from other taxa were less clear; nevertheless, $k = 2$ also offered an adequate benchmark to facilitate comparative interpretation. Species-cluster associations were investigated through Pearson's ϕ (phi) coefficient of association corrected by unequal group sizes, and its significance was assessed through a permutation test between clusters, followed by the Holm correction of p values for multiple testing (Cáceres & Legendre, 2009). Species with significant Pearson's ϕ values for a given cluster were interpreted as “good diagnostic species” for that cluster. PAM were fitted with the function “pam” of the “cluster” R package (Maechler et al., 2019),

whereas Pearson's ϕ was fitted with function “multipatt” of package “indicpecies” (Cáceres & Legendre, 2009). For all permutation tests, the number of permutations was set to 9999, and the level of significance for all tests was set to $\alpha = 0.05$.

3 | RESULTS

A total of 19,798,006 paired-end reads were successfully demultiplexed into individual samples. From these, 6,426,316 corresponded to the 12S Bird primer set, 7,447,686 to the 16S Fish primer set and 4,668,162 to the 16S Mammal primer set. The number of reads per site, per primer set, for each sampling site, and for each of the negative controls before and after quality filtering are presented in Table S1. The average number of reads that passed quality filtering per site was 49,773 for the 12S Bird primer set ($SD: 48,250$), 69,488 for the 16S Fish primer set ($SD: 45,297$), and 40,599 for the 16S Mammal primer set ($SD: 30,159$).

3.1 | Taxonomic assignments

The vast majority of ASVs were annotated at the taxonomic rank of order or lower. The proportion of ASVs that were annotated to the family level was lowest for the 16S Mammal primer set (84%), followed by the 16S Fish primer set (91.2%), and was highest for the 12S Bird primer set (96.6%) (Table S1). After contamination denoising, ASVs were agglomerated to the taxonomic level of genus or species. Only ASVs with a posterior probability $\geq 80\%$ to each of these taxonomic levels were considered.

With a confidence of 80% or higher, eDNA sequences were assigned to a total of 91 genera and 90 species of vertebrates, including amphibians (7 genera and 9 species), bony fish (23 genera and 25 species), birds (31 genera and 29 species), and mammals (24 genera and 27 species). These taxonomic assignments include between 17% and 71% of the previously reported genera and species in the region (Table 1). The lowest proportion of matches to previously reported taxa was for birds and the highest was for fishes and amphibians. Most of the taxonomic assignments to taxa that did not have previous records in the Río Cruces Coastal Wetland were those that either have an overlapping geographic distribution or sister species that occur in the area but are absent from our reference library. Finally, the number of taxa that have previous records in the wetland but were not detected with eDNA was highest for birds and lowest for amphibians (Table 1). The full list of taxonomic assignments can be found in Table S4.

3.2 | eDNA relative abundance

We found that eDNA read abundance varied substantially among classes and among genera within classes (Figure 2). Sequence reads from fishes and birds were more abundant than that of mammals and

TABLE 1 Distribution of taxonomic assignments for eDNA sequences at the genus and species level with 80% or higher posterior probability of assignment

Class	Taxonomic level achieved with eDNA at $\geq 80\%$ confidence	Number of taxa				
		HRC	eDNA	eDNA & HRC	eDNA only	HRC only
Amphibia	Genus	6	7	4 (67%)	3 (3; 0)	2 (0)
	Species	8	9	6 (75%)	3 (3; 0)	2 (0)
Actinopterygii	Genus	27	23	21 (77%)	2 (0; 2)	7 (3)
	Species	34	25	23 (66%)	2 (0; 2)	12 (5)
Aves	Genus	91	31	27 (30%)	3 (2; 1)	64 (20)
	Species	106	29	18 (17%)	10 (1; 5)	87 (52)
Mammalia	Genus	22	24	16 (73%)	8 (2; 2)	6 (6)
	Species	25	27	16 (64%)	11 (3; 2)	10 (6)

Note: HRC refers to the number of taxa reported and published for the Río Cruces Coastal Wetland. Numbers in parentheses in the eDNA & HRC column indicate the percentage of taxa historically reported for the region recovered by eDNA. In parentheses in the eDNA only column: the first number indicates the number of taxa that have a distribution which includes the sampled area, and the second number indicates the number of taxa with a known sister taxon that occurs in the geographic location but is not in our reference library. The numbers in parentheses in the HRC only column indicate taxa that are absent from the reference library.

amphibians. For amphibians, the most abundant reads were assigned to the genus *Batrachyla* (South American wood frogs), followed by *Eupsophus* (ground frogs), and *Alsodes* (alsodid frogs). For fish, the most abundant reads in decreasing order were from the genera *Galaxias* (galaxids), *Gambusia* (gambusids), *Percichthys* (temperate perches), *Brachygalaxias* (galaxids), *Oncorhynchus* (salmon and trout), *Salmo* (salmon and trout), *Odontheistes* (neotropical silversides), and *Cyprinus* (carps). For mammals, the most abundant reads were from the genera *Homo* (humans), *Myocastor* (coypu), followed by *Bos* (domestic cattle), *Canis* (domestic dogs), *Olygorizomys* (rodents), and *Otaria* (South American sea lions). Finally, for birds, the most abundant reads were from the genera *Mareca* (ducks), *Nannopterum* (neotropical cormorants), *Elaenia* (South American flycatchers), *Anas* (dabbling ducks), *Turdus* (true thrushes), and *Cygnus* (swans).

3.3 | eDNA richness and community spatial structure

3.3.1 | Spatial variation of the number of taxa detected with eDNA

For class Amphibia, the best model indicates that the number of genera detected with eDNA decreases with increasing distance from the ocean. This decay was similar for samples taken from the main channel and in the tributaries. However, samples taken from peripheral sites (i.e., tributaries) had, on average, higher detections versus those from the main channel (Figure 3, Table 2).

For fishes (including ray-finned fishes and one lamprey), the best model indicated that the number of species detected also decreased with increasing distance from the ocean. However, this model indicated marginal differences between the peripheral and main channel sites in terms of slope or intercept (Figure 3, Table 2). Furthermore,

the relationship between the number of taxa and distance to the ocean differed among fishes that use different habitats (marine, euryhaline, and freshwater). The number of freshwater fish taxa tended to increase with distance from the ocean, but this trend was not significant. On average, the number of freshwater fish taxa did not differ between samples taken from the main channel or the periphery. The number of euryhaline fish taxa decreased as distance to the ocean increased, and on average, the number of detections was higher in peripheral sites versus those in the main channel. However, these differences were only marginally significant. Finally, the number of marine fish taxa decreased significantly as the distance to the ocean increased, but there were no statistical differences between samples taken from the periphery versus those in the main channel (Figure S1, Table S5).

For mammals, the number of taxa detected did not change as a function of increasing distance from the ocean, and on average, the number of detections in peripheral sites was only marginally higher than detections in the main channel (Figure 3, Table 2). The same trend was also observed for birds (Figure 3, Table 2).

3.3.2 | Spatial variation in eDNA community structure

Fishes, amphibians, and mammals exhibited a clear geographic pattern in species distributions related to the freshwater–marine gradient. For these three classes, site scores of the first two canonical axes of unconstrained ordinations (PCoA) were significantly correlated with distance to the ocean (D.P50) (Figure 4). This suggests that major systemic features of these communities were related to the marine–freshwater gradient. For birds, the correlation of the first two canonical axes of unconstrained ordinations (PCoA) was not correlated to distance to the ocean (D.P50). With db-RDA, by

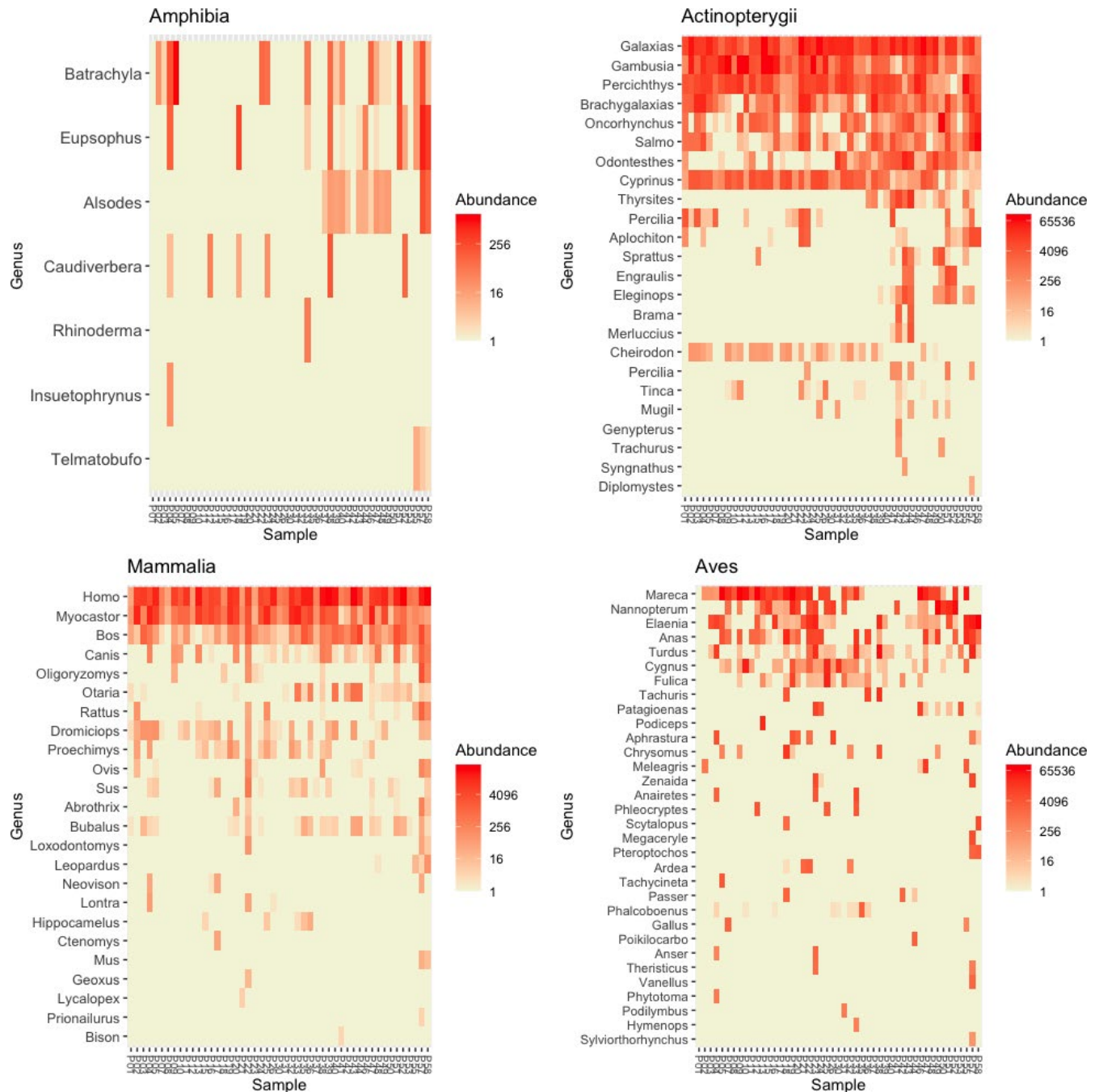


FIGURE 2 Heat map indicating the number of reads (color scale) for each of the genera (rows) and sampling sites (columns). Genera were ordered by decreasing total read abundance across sites. Sampling sites were ordered according to their numerical code. Details of their geographic location and other attributes can be found in Figure 1 and Table S1

calculating the best possible relationship between species composition and D.P50, we conclude that the proportion of community composition variance explained for all taxa by D.P50 was modest (adjusted $R^2 < 10\%$) and, again, only significant for fishes, amphibians, and mammals. This means that other processes we did not account for were driving most of the variation in eDNA community composition (Figure 5).

Non-hierarchical clustering (PAM) helped visualize eDNA community patterns in a more discrete fashion. In fishes, amphibians,

mammals, and birds, 6, 1, 5, and 3 diagnostic species associated with particular clusters were identified, respectively, and most were related to the lower reaches of the Valdivia River system. Fishes were detected in all 49 sites screened, and exhibited the clearest spatial segregation of sample clusters based on species composition. The eDNA of six freshwater and diadromous species including the natives *Galaxias maculatus*, *Brackigalaxias bullocki*, and *Percichthys trucha*, as well as the introduced *Cyprinus carpio* and *Gambusia holbrooki*, were nearly ubiquitous and hence poor clustering diagnostics. However,

FIGURE 3 Number of taxa identified with eDNA at each sampling site in relation to the fluvial distance to the ocean (D.P50 in Table S2) and the channel zone (main channel vs. tributaries). For amphibians and fishes, the best model included different intercepts but similar slopes for samples from the main channel and tributaries. Therefore, two regression lines were fitted following the color code indicated in the legend. For mammals and birds, neither effect on its own nor the interaction differed significantly from zero, and thus, no regression lines were fitted. See Table 2 for model details

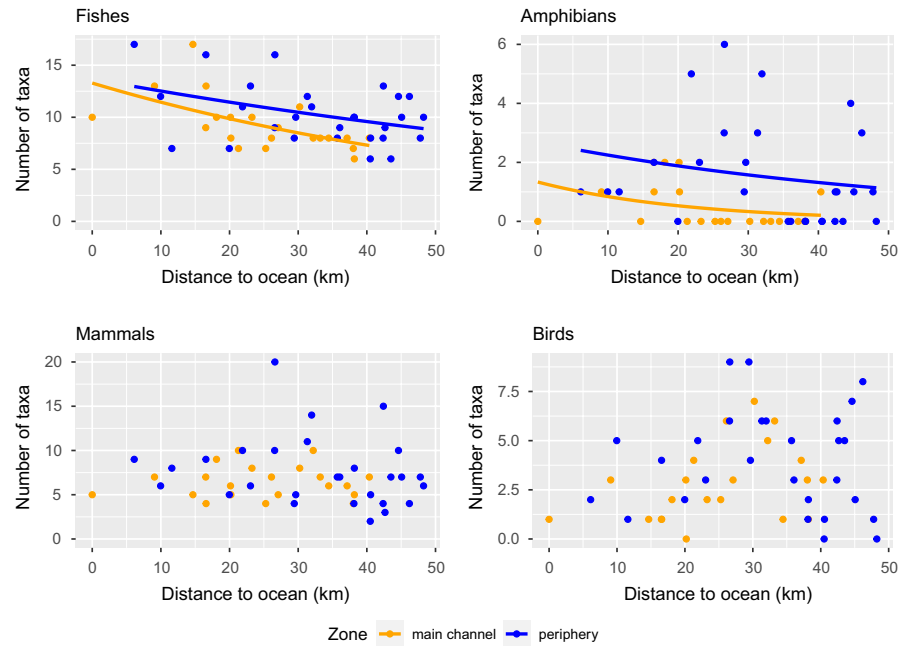


TABLE 2 Results of Poisson GLM best models describing the association between the number of taxa detected with eDNA within each of the four vertebrate classes considered here and the distance from the ocean where samples were taken as well as the zone (main channel vs. periphery)

Coefficient	Estimate	SE	z	p-value
Amphibians				
Intercept ^a	-0.222	0.403	-0.551	0.581
Zone ^b	1.334	0.359	3.711	<0.001
Distance (slope)	-0.021	0.011	-1.971	0.049
Fishes				
Intercept ^a	2.492	0.117	21.272	<0.001
Zone ^b	0.185	0.097	1.910	0.056
Distance ^c	-0.010	0.003	-2.778	0.005
Birds				
Intercept ^a	0.900	0.218	4.125	<0.001
Zone ^b	0.302	0.170	1.773	0.076
Distance ^c	0.005	0.006	0.767	0.443
Mammals				
Intercept ^a	1.988	0.142	13.949	<0.001
Zone ^b	0.208	0.115	1.804	0.071
Distance ^c	-0.005	0.004	-1.113	0.266

Note: Significant *p* values after Bonferroni correction for multiple tests are indicated in bold.

^aCorresponds to the main channel.

^bDifference between periphery and main channel.

^cSlope in relation to distance from the sea (D.P50).

the detection of other species was variable, and useful to identify community clusters. In particular, cluster 1, which dominated in the lower reaches, was related to the presence of estuarine and marine species such *Thyrsites atun* and *Eleginops maclovinus* (Figure S2).

Amphibians were the most sparsely detected (26/49 sites), with the most common eDNA corresponding to *Batrachyla taeniata* and *Alsodes sp* (62 and 50%, respectively, of the sites that revealed at least one amphibian). The only significant diagnostic species was *Caudiverbera caudiverbera* (currently accepted as *Calyptocephalella gayi*) associated with cluster 2, which was common in inland tributaries (Figure S3). Mammals were detected in all 49 sites studied, and eDNA of three species was ubiquitous (present in >95% of the samples). These reads were assigned to the species *Homo sapiens*, *Bos taurus*, and *Myocastor coypus*. The last species is a frequently observed aquatic rodent locally referred to as coipo. While both clusters were mixed in the upper reaches, only cluster 1 occupied the lower reaches of the study area. Five species including the sea lion *Otaria flavescens*, dog *Canis lupus familiaris*, and domestic pig *Sus scrofa domesticus* appeared as indicators of cluster 1, and yet were also occasionally detected in sites of cluster 2. No species was diagnostic of cluster 2 (Figure S4). Birds were detected at most sites (46/49), and eDNA of three species was diagnostic of clusters. The South American flycatchers *Elaenia albiceps* and neotropical cormorant *Nannopterum*, most likely *N. brasilianus* (currently accepted as *Phalacrocorax brasilianus*), were diagnostic of cluster 1, whereas the striated caracara *Phalcooboenus australis*, most likely the chimango caracara *Milvago chimango*, was diagnostic of cluster 2 (Figure S5).

4 | DISCUSSION

Our eDNA approach was able to recover the majority of fish, amphibians, mammals, and to a lesser extent birds, historically recorded in the Río Cruces Coastal Wetland. Our eDNA assay also identified several cryptic and exotic species that are the target of conservation efforts. Despite the dynamic hydrology of these wetlands, our results indicate that the distribution of community eDNA is not

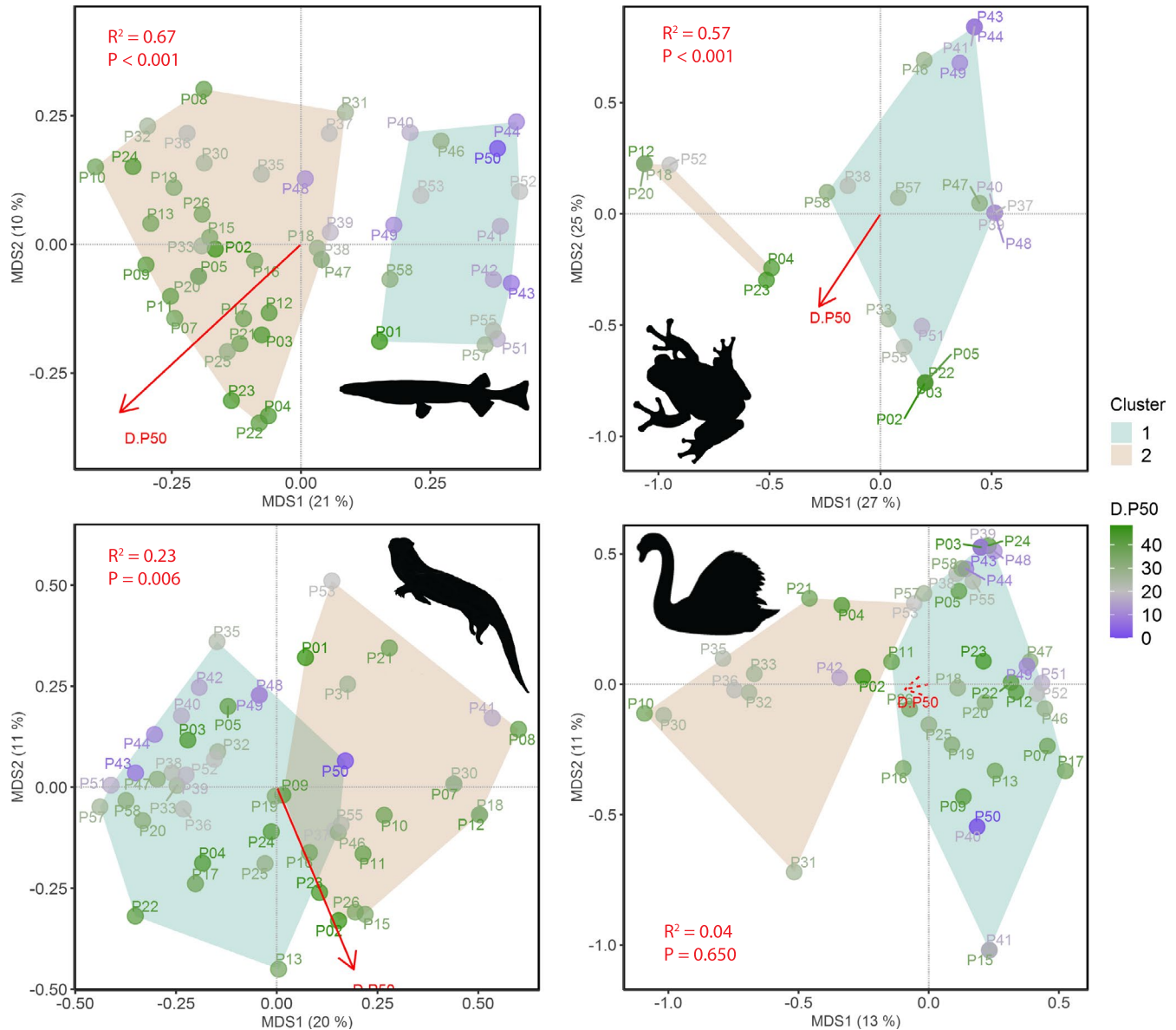


FIGURE 4 eDNA community structure for fish, amphibians, mammals, and birds by indirect gradient analysis through principal coordinate analysis (PCoA). Symbols represent sampling sites in community-composition space; color-coded hulls correspond to PAM clusters (see text, and Figures S2–S5). The color of each point represents the distance of each sampling site to the lowest site nearby the ocean (D.P50, color gradient). Red arrows represent the overall association of D.P50 and the first two dimensions of PCoA, with corresponding magnitude (R^2) and significance (p -value) of the association indicated in red text. Adjusted significance level after Bonferroni correction for multiple tests $\alpha = 0.0125$

random and reflects, to some extent, the distribution and abundance of many of the taxa that we were able to identify. Below we discuss these findings, the limitations, and perspectives of our work.

The proportion of genera and species identified with our eDNA assay varied considerably among taxonomic classes. Our results indicate that for birds, the most diverse group of vertebrates in the Río Cruces Coastal Wetland in terms of the number of genera and species (Muñoz-Pederos, 2003), the percentage of identified genera and species with eDNA was the lowest. However, of the 106 avian species reported previously for this area, 42 are not resident (either seen occasionally or migratory), and 52 are absent from the genetic sequence library that we used. As expected, most of the avian

species we identified are the ones that live in prairie fields or native forest close to or in obligate association with the wetland and therefore have higher chances of releasing DNA that can be recovered with water sampling. As for the other taxonomic classes, our results indicate that at least half of the species previously reported from the region were detected with eDNA. In terms of species that appeared in eDNA but have no previous records in the literature, we note that most of them either have distributions that do include the study area or have sister species that have been reported in the Río Cruces Coastal Wetland previously but are not in our reference library. The only exception to this trend was for mammals, where the majority of species present in the eDNA but not in historical records did not

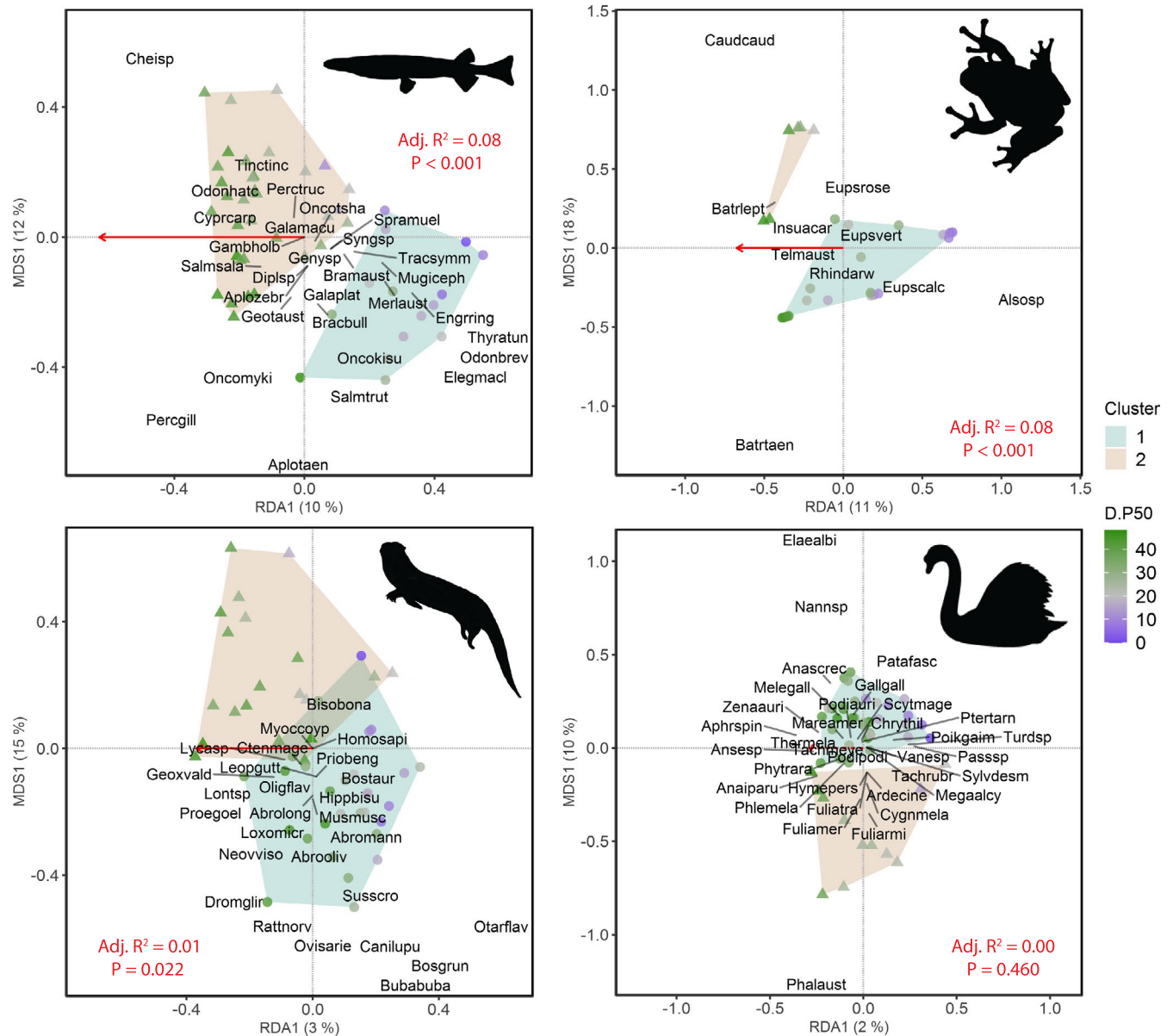


FIGURE 5 eDNA community structure for fish, amphibians, mammals, and birds by direct gradient analysis through distance-based redundancy analysis (db-RDA). Red arrows indicate the direction of the constraining axis D.P50, and its adjusted explanatory power and statistical significance are indicated in red text. Remaining conventions follow Figure 4, except that species scores are also indicated using 8-character codes: The first four characters correspond to genus and last four characters correspond to species. Adjusted significance level after Bonferroni correction for multiple tests $\alpha = 0.0125$

conform to either of these categories. Thus, we suggest that these detections may be erroneous type I errors given that it is extremely unlikely the assigned species are actually present in the study area or even in the region. This higher error rate at the genus and species level for the 16S mammal primer set is consistent with the lower taxonomic resolution linked to the shorter PCR size of this amplicon compared to the bird and fish amplicons that we used (Taberlet et al., 2018). Despite this shortcoming, our results support previous eDNA surveys that indicate fish, amphibian, and mammal detection with eDNA can be efficient and in some regards preferable to traditional approaches (Sales et al., 2020; Valentini et al., 2016), and now avian species as well. However, we note that for birds the efficiency of

eDNA from water samples is lower compared to mammals or fishes. Further work is required, in terms of improving the available DNA sequence catalog and testing alternative sources of eDNA as well as other avian barcodes to improve the taxonomic coverage.

Taxonomic assignment of eDNA sequences is also dependent on the marker length, genetic variability within the amplicon, and amplifiability of the degraded DNA fragments in the sample based on the primer set used. Although the COI gene does appear to have the most reference barcodes available online (Leray et al., 2019), this comes at a cost of amplifiability across a broad range of taxa given the difficulty of designing universal primers with more mutations in the primer binding region (Deagle et al., 2014; Yu et al., 2012). The

alternative markers used in this study (16S and 12S) are more easily amplified across the broad range of taxa but in turn are shorter, and in our case unable to resolve certain taxa to the species level. The development of approaches based on shotgun sequencing (Eisen, 2007; Stat et al., 2017), mitochondrial enrichment (Liu et al., 2016; Zhou et al., 2013), or metabarcoding by capture using a single probe (Mariac et al., 2018) to survey eDNA represent promising alternatives to classic PCR-based approaches (Hebert et al., 2003), although these methods are not without difficulty.

Estimating species relative abundance using eDNA remains a challenging endeavour (Deiner et al., 2017). Here, we found that at least for some groups, when we ranked species according to the abundance of eDNA, there was some degree of coherence with previous reports using traditional methods. For example, in amphibians, abundance data from Méndez et al., (2003) that surveyed 28 localities along the coastal mountain range south of our sampling area found that *Eupsophus*, *Batrachyla*, and *Alsodes* were the three most common genera. These were also the three most abundant genera in our eDNA survey, although not in that exact order. Capture-per-unit-effort data using traditional fishing methods (fyke nets) is also available for class Actinopterygii (Correa & Boisjoly, unpublished data). From these traditional surveys, the six most abundant species or genera reported are *Cheirodon*, *Galaxias maculatus*, *Gambusia*, *Odonthestes*, *Cyprinus carpio*, and *Percichtys trucha*. All of these taxa, with the exception of *Cheirodon*, are within the top-eight most abundant fish species in terms of number of reads and within the top most prevalent species in terms of sites with positive detections. Similar trends have been reported for deepwater fishes (Thomsen et al., 2016). Taken together, our basic comparisons suggest that eDNA abundance can be, to some extent, a rough proxy of relative species abundance. However, we note that our comparisons remain anecdotal and more complete quantitative studies, including testing with mock communities and mesocosms within the study design are warranted before these speculative ideas can be taken further (Beng & Corlett, 2020; Lacoursière-Roussel et al., 2016).

We were able to identify several cryptic and exotic species that are the target of current local and national conservation and eradication plans, respectively. For instance, we recovered eDNA from the American mink (*Neovison vison*). This mammal was introduced at the beginning of the century in the south of Chile to support the fur trade but was released into the wild after this economy collapsed (Valenzuela et al., 2013). The American mink has rapidly expanded toward the north and recently reached the Río Cruces Coastal Wetland; this species is currently a target of an intensive eradication program. Similarly, we also detected two endangered and cryptic species of amphibians (*Insuetophrynus acarpicus* and *Rhinoderma darwini*), both of which are listed as “endangered” according to the International Union for Conservation of Nature (IUCN) Red List of Threatened Species (Dulvy et al., 2004; IUCN Ssc Amphibian Specialist Group, 2018a, 2018b). We also detected two iconic but elusive mammal genera *Lontra* and *Lycalopex*. Unfortunately, we were not able to determine the species identity with high confidence in either case as the 16S segment that we amplified for these taxa is

identical between the described species available in our catalog (all four species were Sanger sequenced as part of our effort to complete the reference database), which led to a species posterior assignment probability below our threshold (0.8). Both species of *Lontra* are classified as “endangered” by the IUCN and occupy adjacent habitats that are connected by waterways: *L. felina* is a marine otter, while *L. provocax* is associated with freshwater environments. Thus, the lack of taxonomic resolution in this particular case can be an obstacle for the specific monitoring plans of each species. Whether longer segments of the 16S gene or a different marker altogether can be amplified from water eDNA samples and resolve species identity as in *Lontra* or *Lycalopex* remains to be tested. Taken together, these results add to the growing body of evidence that highlights the utility of eDNA metabarcoding to first detect exotic and elusive species (Adrian-Kalchhauser & Burkhardt-Holm, 2016; Brys et al., 2020; Hunter et al., 2019; Jerde et al., 2011; Rose, 2020) and then monitor their movement and/or efficacy of their removal. However, our result also indicates the importance of more complete reference databases to improve the taxonomic assignments.

Despite the complex hydrodynamics of this coastal wetland system, the community structure inferred from eDNA was heterogeneous and differed among the taxonomic classes. As expected, we found that eDNA-based species richness increased seawards, but only for fishes. A similar, marginally significant trend was also found for amphibians. This result is consistent with the idea that waterborne eDNA accumulates as it passes through rivers and estuaries (Barnes & Turner, 2016; Deiner et al., 2016). However, an increase in biodiversity downstream in riparian and estuarine ecosystems has also been associated with increasing habitat heterogeneity, complexity, and sea connectivity toward river deltas (Béjar et al., 2020; Rice et al., 2006), which can confound the richness accumulation hypothesis discussed here. Nevertheless, the detection of freshwater species such as creole perch (*P. trucha*) and puyecito (*B. bullocki*) in locations with great marine influence—and not viceversa—suggests downstream eDNA accumulation for fishes and perhaps amphibians. The corollary of this pattern explains the counterintuitive paucity of indicator or cluster-diagnostic species for freshwater fish assemblages: No single fish species was identified as a good freshwater community indicator. Despite the transport, mixing and likely accumulation of eDNA in our study system, we found a modest but statistically significant community structure associated with the freshwater–marine gradient in all taxa but birds. In fishes, this pattern was most easily explained by the consistent appearance of locally common estuarine and marine species in many sites close to the sea, such as anchovy (*E. ringens*) and snoek (*Thyrstites atun*). The latter species, however, is sold in Valdivia city's local fish market that spills untreated residues directly to the river, and could therefore be an additional source (or amplifier) of marine eDNA. Also, in amphibians, species richness was significantly higher in the periphery compared to the main channel, and fishes showed a similar yet marginal tendency. One possible explanation is that eDNA from species that occupy peripheral aquatic habitats or adjacent terrestrial habitats such as

the two ground frogs (*Eupsophus* spp.) we detected gets diluted from its near-shore source into the mainstream (Jeunen et al., 2019; O'Donnell et al., 2017), but many terrestrial mammalian or bird species as well as amphibians did not seem to conform to this expectation. To some extent, this result supports the prospect that assays based on waterborne eDNA can reveal local biodiversity patterns even in dynamic systems. Clearly, however, given the complexity of these systems and variability in eDNA sources, dispersion, and rates of decay and detection, further studies that combine traditional and eDNA methodologies are needed to further dissect eDNA patterns and processes.

As anthropogenic environmental stressors intensify exponentially, threatening biodiversity worldwide, the demand for high-resolution spatial and temporal biodiversity data underpins management and mitigation measures increases. Overall, our results indicate that eDNA is a promising tool for biomonitoring vertebrate diversity in Chilean wetlands. We contend that most of what is needed for specific applications of eDNA is already in working order, but considerable refinement discussed herein will be required to achieve the high standards environmental management requires. Perhaps foremost, eDNA taxonomic assignments' accuracy is contingent on still sparse DNA reference databases (Delrieu-Trottin et al., 2020; Stoeckle et al., 2020), a limitation we partially mitigated by sequencing several local vertebrate species, but much more effort in this regard is warranted. The Chilean government has recently approved a national law for urban wetlands to protect the ecosystem services, structure, and functions that contribute to environmental sustainability and well-being (Law N°21.202, 2020). Our study system is but one of hundreds of wetlands that will be protected by implementing this new legislation since it overlaps with an urban area, the city of Valdivia. Public policy and law enforcement are perhaps the most significant drivers of biomonitoring worldwide (Friberg et al., 2011), and eDNA assays are beginning to complement traditional approaches in the endeavor to supply the increasing demand for sustained, standardized, and cost-effective high-throughput biodiversity data in Chile and the world.

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AUTHOR CONTRIBUTIONS

PS-A, ED-T, MS, and CR conceived and designed the study. PS-A, DM, SM-G, FP, PR, MS, and CR participated in fieldwork, data acquisition, and sample processing. SM-G, PR, FP, and AS performed all genetic laboratory work and DNA sequencing. PS-A, ED-T, and CR analyzed the data. PS-A wrote the first draft with input from all the authors.

DATA AVAILABILITY STATEMENT

All raw sequence and data files that support the findings of this study are available in Dryad: <https://doi.org/10.5061/dryad.pg4f4qrp4>.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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