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Unveiling the potential of eDNA/eRNA approaches for monitoring the critically endangered Yangtze finless porpoise

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ABSTRACT

Assessing the population distribution and dynamics of the critically endangered Yangtze finless porpoise (*Neophocaena asiaeorientalis*) is essential for informing conservation strategies. Traditional monitoring techniques, while valuable, are labor-intensive and time-consuming. Environmental nucleic acids (eNAs) offer a non-invasive alternative, yet prior research has predominantly relied on single-locus eDNA analysis, limiting resolution. Here, we developed a dual-marker eDNA/eRNA approach targeting mitochondrial COX3 (Cytochrome C Oxidase subunit 3) and CYTB (Cytochrome B) genes, coupled with droplet digital PCR (ddPCR) quantification, to enhance detection sensitivity and reliability. Serial dilution experiments established stringent detection limits (LOD: 0.076 copies/μL; LOQ: 0.35 copies/μL), while degradation trials at the Tongling *ex situ* conservation site revealed eDNA persistence for 65.24 (COX3) and 50.13 (CYTB) hours, compared to rapid eRNA degradation (~30 hours). Field surveys across 20 Yangtze River Estuary locations detected porpoise eDNA at 13 sites and eRNA at 11 sites, with 5 sites showing concurrent detection via both markers—predominantly near the Dongfeng Xisha Reservoir. Detection zones overlapped significantly with passive acoustic monitoring records, yet eNAs revealed broader spatial coverage. Our findings demonstrate that multi-locus eNA analysis provides a sensitive, scalable tool for monitoring critically endangered aquatic species, offering actionable insights to optimize conservation efforts.

1. Introduction

The Yangtze finless porpoise (*Neophocaena asiaeorientalis*), the world's only freshwater porpoise, is endemic to the middle and lower reaches of the Yangtze River, China, including its adjoining Poyang and Dongting Lakes (Turvey et al., 2007; Wang, 2009). Once abundant, its population has plummeted over recent decades due to habitat degradation, pollution, and overfishing, leading to its classification as Critically Endangered on the IUCN Red List (Mei et al., 2014; Wang, 2013). While conservation efforts, such as *ex situ* reserves and fishing bans, have stabilized populations (Huang et al., 2017; Huang, 2020), with a 2022 survey estimating 1249 individuals (Hao et al., 2023), the species remains at risk. Persistent challenges include inefficient monitoring technologies, unknown dynamics of habitat selection, and managing small population vulnerabilities (Lu et al., 2023; Wang et al., 2020; Wang, 2024).

Environmental DNA (eDNA) has emerged as a transformative tool for non-invasive biodiversity monitoring in aquatic ecosystems

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(Ruppert et al., 2019; Zhang et al., 2022). However, eDNA's limitations in quantifying species abundance and detecting recent biological activity hinder its utility for real-time conservation decisions (Beng and Corlett, 2020; Schenekar, 2023). Environmental RNA (eRNA), with its faster degradation rate, complements eDNA by reflecting metabolically active or temporally recent presence, enhancing spatiotemporal resolution (Cristescu, 2019; Littlefair et al., 2022; Yates et al., 2021). Despite these advantages, eRNA applications remain underexplored in endangered species monitoring, particularly for aquatic mammals.

Traditional monitoring methods for the Yangtze finless porpoise, such as visual surveys and passive acoustics, are labor-intensive and spatially constrained, limiting timely population assessments (Tang et al., 2019). Recent studies confirm eDNA's potential for detecting porpoise presence (Chen et al., 2024; Qu and Stewart, 2019), yet critical gaps persist. For instance, studies on environmental RNA (eRNA) for the Yangtze finless porpoise remain underexplored, and existing eDNA studies rely on single-locus markers, which may reduce detection sensitivity and quantification accuracy (Brys et al., 2023; Drummond et al., 2015). Furthermore, the small population in the ecologically sensitive Yangtze River estuary (~31 individuals; Tang et al., 2018) remains understudied despite its role as an indicator of ecosystem health (Ma et al., 2009; Shi et al., 2018).

Droplet digital PCR (ddPCR), a high-precision nucleic acid quantification method, overcomes limitations of traditional qPCR by eliminating standard curves and resisting PCR inhibitors, making it ideal for low-abundance eDNA analysis (Capo et al., 2021; Hindson et al., 2013). Here, we integrate dual mitochondrial markers, COX3 (Cytochrome C Oxidase subunit 3) and CYTB (Cytochrome B) with ddPCR to compare eDNA and eRNA efficacy in monitoring the Yangtze finless porpoise. Our objectives are threefold: (1) establish detection limits (LOD/LOQ) for eDNA/eRNA markers; (2) characterize degradation rates of eDNA/eRNA in a controlled *ex situ* environment; (3) map porpoise distribution in the Yangtze River estuary, validating results against passive acoustic monitoring. By addressing gaps in multi-locus eNAs quantification and temporal resolution, this study advances monitoring frameworks for critically endangered aquatic species, offering actionable insights to bolster conservation strategies.

2. Materials and methods

2.1. Primers/probe design and validation

The Yangtze finless porpoise is currently the only extant cetacean species in the Yangtze River basin, following the functional extinction of the baiji (*Lipotes vexillifer*) in 2006 (Mei et al., 2014; Turvey et al., 2007; Wang, 2009). Species-specific primers and probes were designed using Primer Express 3.0.1 software (Thermo Fisher Scientific) to target two mitochondrial gene regions of the Yangtze finless porpoise: a 114-bp fragment of the Cytochrome C Oxidase subunit 3 (COX3) gene and a 129-bp fragment of the Cytochrome B (CYTB) gene. Probes were labeled with 6-FAM (COX3) and VIC (CYTB) fluorophores and incorporated Minor Groove Binder (MGB) modifications to enhance binding specificity (Table 1). Primer and probe specificity were validated *in silico* using the NCBI Primer-BLAST tool (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>), which confirmed specificity for the target species within the study area, with no cross-reactivity to non-target species. The primer specificity was empirically validated against non-target DNA from swine, goat, and cow (supplementary Fig. S1). To confirm the authenticity of amplified porpoise DNA, positive PCR products obtained from water samples were verified through Sanger sequencing (supplementary Fig. S2 & S3).

2.2. Amplification efficiency assessment

The amplification efficiency of primers and probes was evaluated via quantitative PCR (qPCR). A positive control DNA sample (56.7 ng/μL), extracted from *N. asiaeorientalis* tissue and quantified using a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific), was subjected to a 10-fold serial dilution series (undiluted, 1:10, 1:100, 1:1000, 1:10,000). A standard curve was generated by plotting threshold cycle (Ct) values against template concentration. Amplification efficiencies of 99.67 % (COX3) and 97.71 % (CYTB) confirmed robust assay performance (see Supplementary Fig. S4 for standard curve, slope, and R² values).

2.3. Limit of Detection (LOD) and Limit of Quantification (LOQ)

Sensitivity thresholds were determined using droplet digital PCR (ddPCR). A total DNA sample (1 ng/μL, quantified via Thermo Scientific NanoDrop One) was diluted 1:100, followed by an seven-step 5-fold dilution series (1:100–1:7812,500). Each dilution was replicated 10 times. The LOD is defined as the lowest template concentration yielding ≥ 95 % positive replicates. The LOQ is defined as the lowest concentration with a coefficient of variation (CV) < 35 % across replicates (Brys et al., 2023; Forootan et al., 2017).

Table 1

Primers and probes for targeting a 114-bp COX3 sequence and a 129-bp CYTB region of the Yangtze finless porpoise.

Target gene	Primer/probe	Name	Sequence (5' – 3')
COX3	Forward	COX3-F	GACGGAGTCTACGGGTCTACATTT
	Reverse	COX3-R	GAAGTGAATGTCAAGTTGACGTATG
	Probe	COX3-P	(6-FAM)CCACAGGCTTCCACGG(MGB)
CYTB	Forward	CYTB -F	AACCTTATTTTCACCCGACCTTCT
	Reverse	CYTB -R	GGGATTGATCGTAGGATTGCATAT
	Probe	CYTB -P	(VIC)CAAAACCACTGAGCACT(MGB)

Curve-fitting methods were applied to calculate both thresholds.

2.4. Degradation experiment design

Degradation experiments were conducted using water samples collected from the Jiajiang Enclosure (Anhui Tongling Finless Porpoise National Nature Reserve; Fig. 1), a semi-natural aquatic habitat spanning 20 ha (1600 m length $\times$ 80–200 m width; depth: 5–8 m). The enclosure supports 11 Yangtze finless porpoises (5 males, 5 females, 1 calf) under controlled feeding conditions: four daily feedings (07:00, 10:00, 14:00, 17:00) of fresh common carp (*Cyprinus carpio*) and crucian carp (*Carassius auratus*). On October 12, 2024, at 10:30 AM (post-feeding), surface and mid-water samples (35 L total per section) were collected from three designated feeding zones (left, middle, right) using pre-sterilized white plastic buckets (Supplementary Fig. S5). Prior to sampling, buckets were cleaned with laboratory detergent, 10 % bleach, and rinsed repeatedly with hot tap water to eliminate contaminants.

At intervals of 0, 4, 8, 12, 24, 48, 72, 96, 144, and 192 hours post-collection, two 500 mL aliquots were drawn from each bucket ($n = 3 \text{ buckets} \times 2 \text{ aliquots} = 6 \text{ samples per time point}$) for parallel DNA/RNA analysis. Before aliquoting, water was homogenized by thorough mixing. Samples were filtered through 0.45 μm mixed cellulose ester membranes (47 mm diameter; Whatman, UK) using sterilized tweezers and disposable filter holders. A 500 mL Milli-Q water control was processed at each time point for control. Filter membranes were stored in PowerWater Bead Pro Tubes (Qiagen, Cat. 14700 & 14900, GER) at -80°C . DNA and RNA extractions were performed within one week of filtration.

2.5. eDNA and eRNA sample collection and passive acoustic monitoring in the Yangtze River Estuary

Twenty sampling sites were established across the Yangtze River estuary (October 24–25, 2024; Fig. 1). Sites S1–S6 and S18–S20 were spaced 5–8 km apart, while sites S6–S17, previously identified as Yangtze finless porpoise hotspots (Tang et al., 2018), were sampled at 2 km intervals to enhance spatial resolution. At each site, a vertical transect was divided into three positions (left, middle, right), with water collected from three depths: 1 m (surface), 5 m (mid-layer), and 10 m (bottom). From each depth, 1 L of water was collected, yielding 9 L per site. Water samples were homogenized in a sterile container, and six 500 mL aliquots were subsampled for eDNA/eRNA analysis. To prevent cross-contamination, disposable sterilized tweezers and filter holders were used, and buckets were

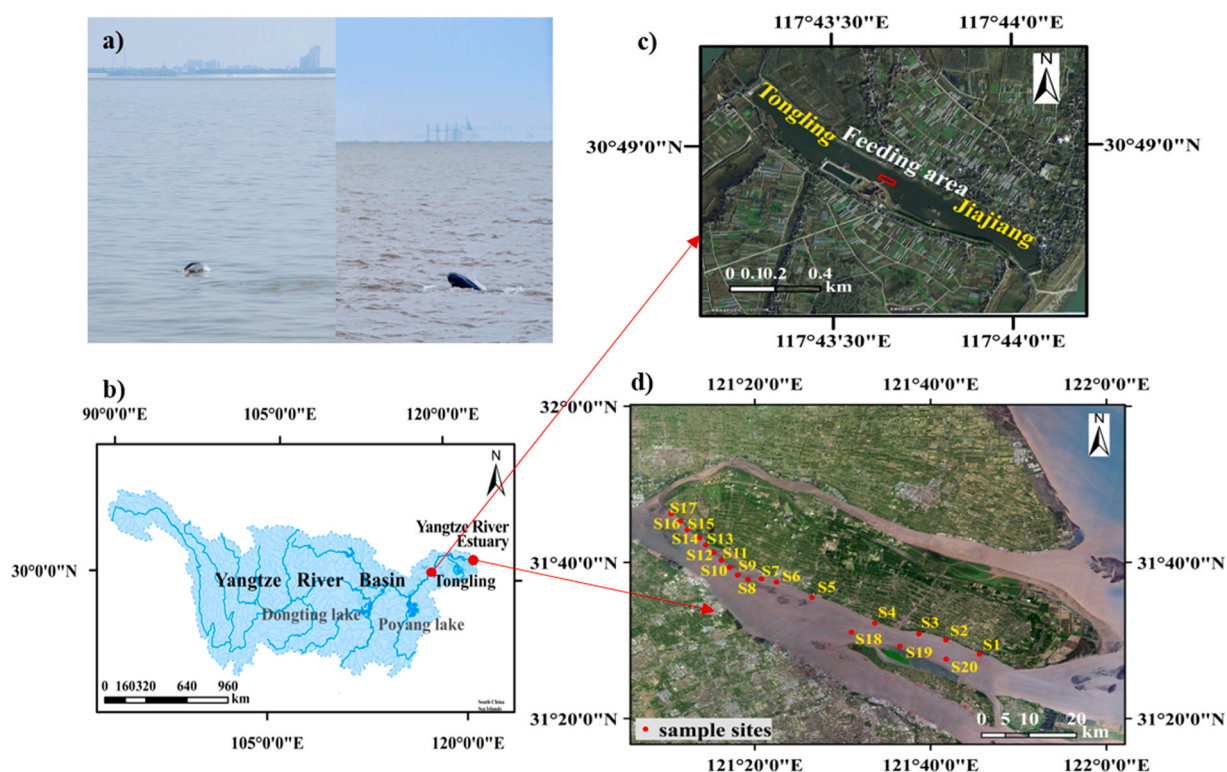


Fig. 1. Map of sampling sites: a) Photos of the Yangtze finless porpoises taken by the authors during the survey in the Yangtze River estuary, b) the Yangtze River Basin ($24^\circ30'–35^\circ45'N$, $90^\circ20'–122^\circ25'E$), c) Jiajiang Enclosure, Anhui Tongling Finless Porpoise National Nature Reserve ($30^\circ48'–30^\circ49'N$, $117^\circ43'–117^\circ44'E$), where degradation experiments were conducted, and d) Yangtze River Estuary ($31^\circ44'–31^\circ78'N$, $121^\circ16'–121^\circ82'E$), showing 20 field sampling sites (S1–S20) for eDNA/eRNA analysis. Red stars denote key sampling zones, with S6–S17 prioritized as historical porpoise hotspots. Base maps were generated using ArcGIS 10.0 with geographic data sourced from China Centre for Resources Satellite Data and Application. The Jiajiang Enclosure (inset) houses a semi-natural population of critically endangered Yangtze finless porpoises.

disinfected (10 % bleach, rinsed with Milli-Q water) between sites. A 500 mL Milli-Q water negative control was processed at each location.

On-site filtration followed protocols outlined in Section 2.4, using 0.45 μm mixed cellulose ester membranes (Whatman, UK). Membranes were stored in PowerWater Bead Pro Tubes (Qiagen, Cat. 14700 & 14900, GER) on dry ice and transferred to a -80°C freezer within 24 hours. DNA/RNA extractions were completed within one week.

A real-time porpoise click detector (RPCD-II; Institute of Hydrobiology, Chinese Academy of Sciences and Wuhan Pindu Technology Co. Ltd.) was mounted at the vessel's stern to record acoustic signals during transects. The device, validated for Yangtze finless porpoise detection (Li et al., 2022), operated continuously during outbound and return trips.

2.6. DNA/RNA extraction and cDNA synthesis

DNA from tissue samples was extracted using the DNeasy Blood & Tissue Kit (Qiagen, Cat. 69506), while eDNA from filters was extracted using the DNeasy PowerWater Kit (Qiagen, Cat. 14700). RNA was isolated from filter membranes using the RNeasy PowerWater Kit (Qiagen, Cat. 14900), followed by on-column DNase digestion. To ensure complete DNA removal, eluted RNA underwent additional DNase treatment (Thermo Fisher Scientific, Cat. AM1906).

All extractions were performed in a dedicated sterile laboratory under sequential workflows (DNA $\rightarrow$ RNA) to prevent cross-contamination. Workstations and equipment were UV-sterilized for ≥ 15 minutes before/after use and disinfected with 10 % bleach and absolute ethanol. The total eDNA was eluted in 100 μL DNase-free water and stored at -20°C until ddPCR analysis. The eRNA was eluted in 100 μL RNase-free water and 60 μL was immediately used for cDNA synthesis, while the remaining 40 μL was archived at -80°C .

cDNA was synthesized from 60 μL eRNA using SuperScript IV VILO Master Mix (Thermo Fisher Scientific, Cat. 11756050). Reactions were incubated at 25°C (5 min), 55°C (10 min), and 80°C (5 min). The cDNA was stored at -20°C and is hereafter termed "eRNA" for analytical consistency.

2.7. qPCR and ddPCR assay

qPCR amplifications were conducted on a QuantStudio™ 5 Real-Time PCR System (Thermo Fisher Scientific, USA) in 20 μL reactions containing 10 μL TaqMan™ Multiplex Master Mix (Thermo Fisher Scientific, Cat. 4461881), 1.8 μL each primer (900 nM final concentration), 0.5 μL each probe (250 nM final concentration), 3.4 μL nuclease-free water, 2 μL template DNA. Thermal cycling conditions: initial denaturation: 95°C for 2 min, 40 cycles of: 95°C for 10 s (denaturation), 60°C for 30 s (annealing/extension). Negative controls ($n \geq 2$ per run) were included for tissue-derived DNA assays.

Droplet Digital PCR (ddPCR) was performed using a QuantStudio Absolute Q Digital PCR System (Thermo Fisher Scientific, USA). Reactions (10 μL total) comprised 2 μL Absolute Q DNA Digital PCR Master Mix (5 $\times$; Thermo Fisher Scientific, Cat. A52490), 0.9 μL

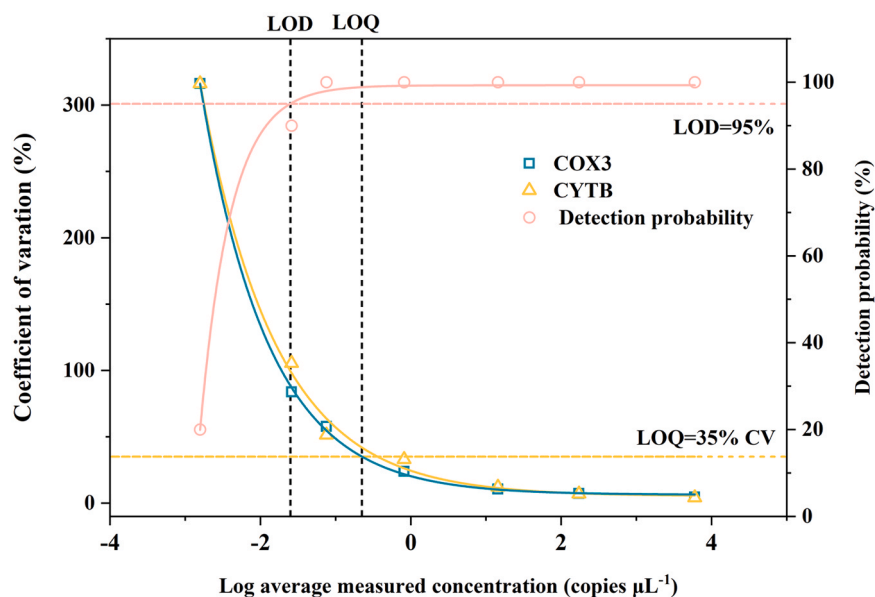


Fig. 2. Droplet digital PCR (ddPCR)-based estimation of the limit of detection (LOD) and limit of quantification (LOQ) for the Yangtze finless porpoise (*Neophocaena asiaeorientalis*) DNA. LOD ($0.076 \text{ copies } \mu\text{L}^{-1}$) and LOQ ($0.35 \text{ copies } \mu\text{L}^{-1}$) were derived from an eight-step dilution series of tissue-derived DNA, with ten replicates per concentration. The coefficient of variation and detection probability are fitted to sigmoidal curves for the estimation of LOD and LOQ (solid line). Dashed lines mark the LOD/LOQ thresholds. Analysis was performed using the QuantStudio Absolute Q system (Thermo Fisher Scientific).

each primer (900 nM final), 0.25 μL each probe (250 nM final), 5.7 μL template DNA. Reaction mixtures (9 μL) were loaded onto MAP16 plates (Thermo Fisher Scientific, Cat. A52688) with 15 μL Isolation Buffer (Thermo Fisher Scientific, Cat. A52730) per well. Compartmentalization, thermal cycling, and data acquisition were automated on the QuantStudio system. The thermal protocol included preheating: 95°C for 10 min, 45 cycles of: 95°C for 30 s (denaturation), 60°C for 1 min (annealing/extension). Threshold determination was performed using QuantStudio Absolute Q Software v6.0, with manual thresholds calculated based on no-template controls (NTCs), positive controls (tissue-derived DNA) and environmental negative controls.

3. Results

3.1. LOD and LOQ estimation

Droplet digital PCR (ddPCR) analysis of an eight-gradient dilution series of Yangtze finless porpoise tissue DNA generated an average of 20,447 droplets per well (range: 20,432–20,472 droplets), demonstrating consistent droplet generation across replicates. A linear regression model effectively described the relationship between input DNA concentration and target detection for dilution gradients 1–5 (highest concentrations), while gradients 6–8 fell below the limit of detection (LOD; Fig. 2). The LOD was estimated to be 0.076 copies μL^{-1} . Despite being below the LOD, one positive replicate was detected in gradient 7 (1/10 replicates). For quantification reliability, the limit of quantification (LOQ) was established at 0.35 copies μL^{-1} , with gradients 1–4 meeting the criteria of coefficient of variation (CV) < 35 % and 95 % detection probability. Gradients 5–8 exhibited variability exceeding the LOQ threshold, rendering them unsuitable for precise quantification (Fig. 2).

3.2. Environmental DNA/RNA decay rates

qPCR assays confirmed the absence of residual DNA in DNase-treated RNA eluents, with all samples returning negative results. Both eDNA and eRNA concentrations for *COX3* and *CYTB* genes peaked immediately post-collection and declined exponentially over time (Figs. 3, 4). Decay rate constants (mean $\pm$ SD) were $0.174 \pm 0.047 \text{ h}^{-1}$ (*COX3*) and $0.229 \pm 0.062 \text{ h}^{-1}$ (*CYTB*) for eDNA, and $0.342 \pm 0.16 \text{ h}^{-1}$ (*COX3*) and $0.313 \pm 0.09 \text{ h}^{-1}$ (*CYTB*) for eRNA, respectively.

The Wilcoxon Signed-Rank test (Paired Samples) showed no significant difference in the decay rate between the *COX3* and *CYTB* genes for eDNA ($p = 0.074$) or eRNA ($p = 0.426$). The statistical test also indicated a significant difference in the decay rate constants between eDNA and eRNA for the *COX3* ($p = 0.003$), but not *CYTB* ($p = 0.301$). The eDNA signals for the *COX3* and *CYTB* gene dropped below the ddPCR detection limit after 65.24 h and 50.13 h, respectively. The eRNA signals became undetectable after 29.80 h (*COX3*) and 32.72 h (*CYTB*).

3.3. Environmental DNA/RNA detection results in the Yangtze River Estuary

The results showed that for eDNA, the Yangtze finless porpoises was detected at 13 of the 20 sites (Fig. 5), 10 sites had both *COX3* and *CYTB* markers detected, and 3 sites had only *COX3*. The eDNA detection sites were concentrated near the Dongfeng Xisha Reservoir, Qingcaosha Reservoir, and the western side of the Yangtze River Bridge near Chongming Island. For eRNA, the Yangtze finless porpoises were detected at 11 of the 20 sites, with 6 sites had both *COX3* and *CYTB* markers detected, and 5 sites had only *COX3*. Notably, for both eDNA and eRNA, the *COX3* gene was detected in all locations where the *CYTB* gene was identified. The eRNA detection sites were predominantly located in the Dongfeng Xisha Reservoir and the Qingcaosha Reservoir. The passive acoustic device recorded Yangtze finless porpoise acoustic signals at 9 sites (Supplementary Fig. S6), fewer than eDNA/eRNA detections. The sites where the acoustic signals were recorded also coincided with those eDNA/eRNA hotspots.

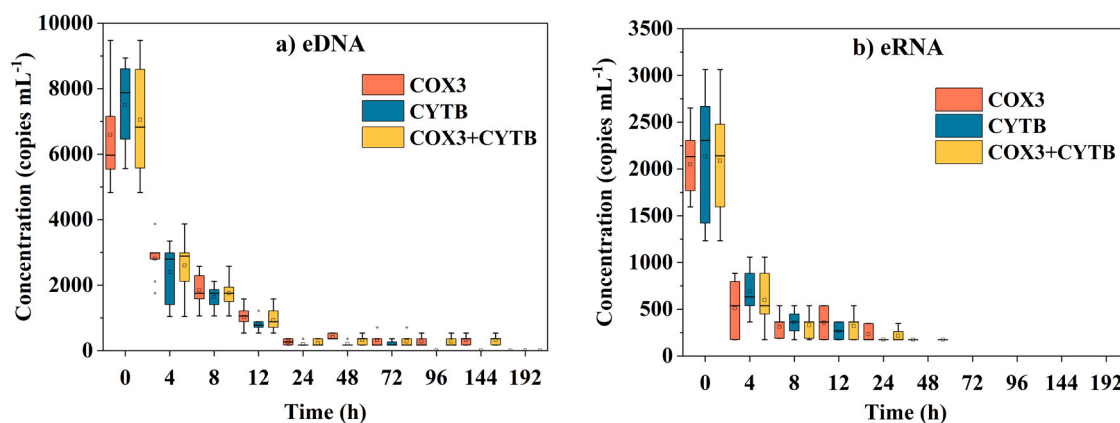


Fig. 3. a) Mean copies per mL of Cytochrome C Oxidase subunit 3 (*COX3*) and Cytochrome B (*CYTB*) in environmental DNA (eDNA). b) Mean copies per mL of *COX3* and *CYTB* in environmental RNA (eRNA). Vertical error bars represent standard errors. Note that y-axis scales differ between panels.

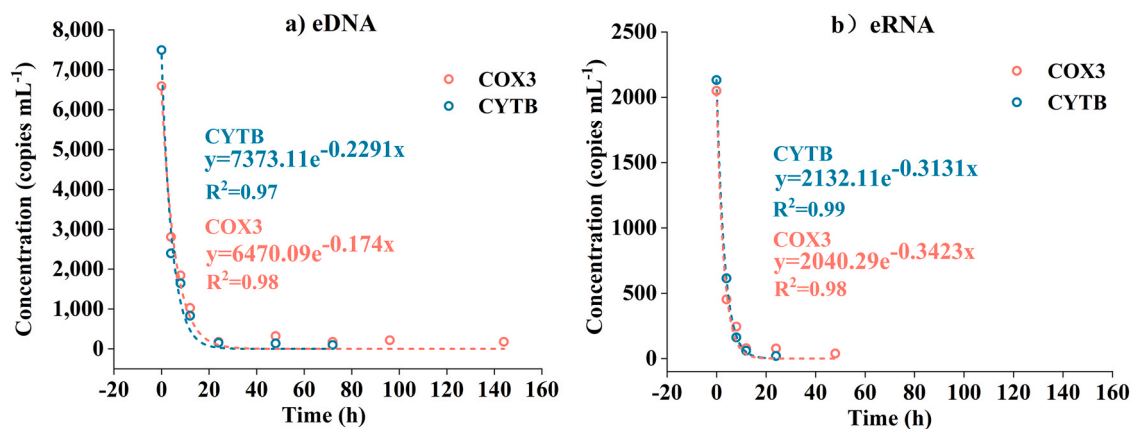


Fig. 4. Time-dependent changes in the average concentrations of environmental DNA (eDNA) and RNA (eRNA), quantified via the Cytochrome C Oxidase subunit 3 (COX3) and Cytochrome B (CYTB) genes, for: a) eDNA and b) eRNA. The exponential decay model $N(t) = N_0e^{-\lambda t}$ was applied to the raw data, with equations illustrating decay rates. R^2 values indicate the goodness of fit between raw data and the model.

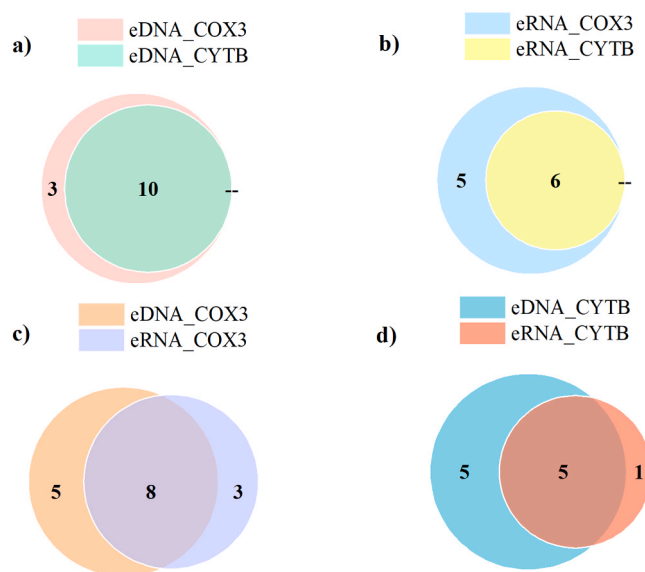


Fig. 5. Venn diagrams showing the eDNA and eRNA detection results of Yangtze finless porpoises at 20 sites in the Yangtze Estuary. a) number of sites detected by eDNA based on COX3 and CYTB; b) number of sites detected by eRNA based on COX3 and CYTB; c) number of sites detected by eDNA and eRNA based on the COX3; d) number of sites detected by eDNA and eRNA based on the CYTB.

A comparison of eDNA and eRNA detection results for the COX3 and CYTB genes across 20 sites revealed the following: for COX3, both eDNA and eRNA were detected at 8 sites, while only eDNA was detected at 5 sites and eRNA at 2 sites. For CYTB, both eDNA and eRNA were detected at 5 sites, while only eDNA was detected at 5 sites and eRNA at 1 site. Additionally, in 5 of the 20 sites, concurrent detection of both eDNA and eRNA using the dual markers COX3 and CYTB occurred, primarily concentrated in the Dongfeng Xisha Reservoir (Fig. 5).

The concentrations of eDNA and eRNA at each site, as measured by ddPCR, are shown in Fig. 6. For eDNA, concentration of the COX3 peaked at site S11, followed by S8, S19, and S15, while relatively lower concentrations were found at sites S2, S3, and S5. For the CYTB gene, the highest detection concentration was observed at site S15, followed by S3, S12, and S14, lowest at sites S1, S2, S8, S13, and S19. For eRNA, the concentration of COX3 peaked at sites S2 and S14, followed by S11, S12, and S8, while relatively lower concentrations were found at sites S7, S13, S15, and S19. Conversely, eRNA concentration of CYTB peaked at site S15, followed by sites S13 and S19, lowest at site S7. The Wilcoxon Signed-Rank test (Paired Samples) showed significant difference in detection concentrations between COX3 and CYTB gene for eDNA ($p = 0.036$), but no significant difference for eRNA ($p = 0.237$). The statistical analysis also showed no significant difference in detection concentrations between eDNA and eRNA for both COX3 ($p = 0.120$) and CYTB ($p = 0.547$).

Passive acoustic monitoring identified two large porpoise aggregations in Dongfengxisha waters (groups of 17 and 10 individuals;

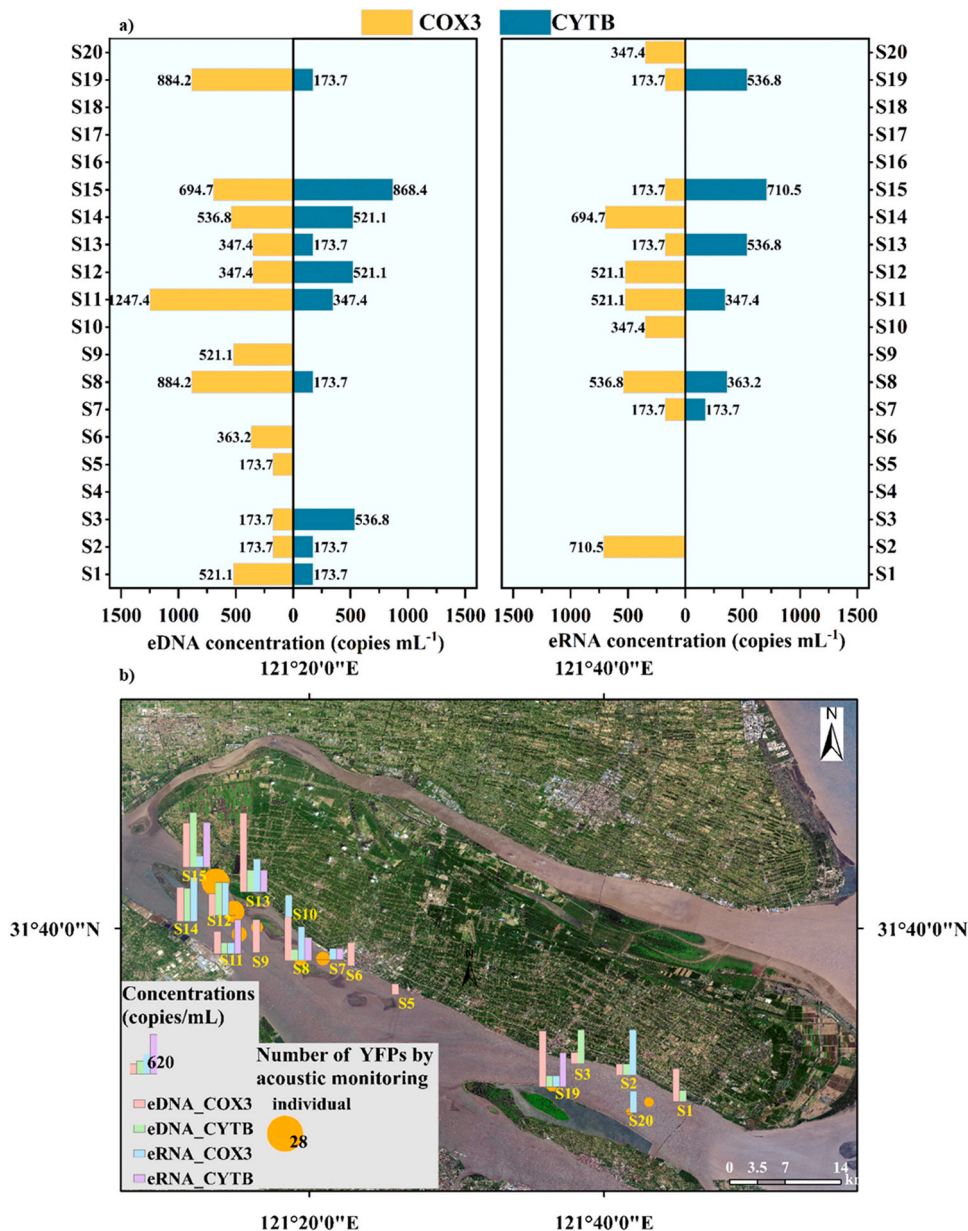


Fig. 6. Distribution of the Yangtze finless porpoises' eDNA and eRNA in the Yangtze River Estuary. a) Concentrations of the eDNA and eRNA of the Yangtze finless porpoises estimated based on *COX3* and *CYTb* at the 20 sampling sites; b) Distribution patterns of Yangtze finless porpoise (YFP) eDNA and eRNA, bars indicate concentrations (copies per mL) of eDNA and eRNA; orange dots represent acoustic signals recorded via passive acoustic monitoring, with dot size corresponding to the estimated number of YFP individuals based on acoustic signals.

Fig. 6, Supplementary Table 1), aligning spatially with high eDNA/eRNA concentrations. Spatial distribution of the dolphin revealed by Kernel Density Analysis (ArcGIS) integrating eDNA, eRNA, and acoustic data is shown in Fig. 7. While eDNA, eRNA, and passive acoustic monitoring all identified high-density hotspots within Dongfeng Xisha waters, the regions pinpointed by passive acoustic monitoring are more narrow compared to those detected by eDNA and eRNA methods.

4. Discussion

This study pioneers the integration of dual-marker eDNA and eRNA approaches to monitor the critically endangered Yangtze finless porpoise (*Neophocaena asiaeorientalis*), establishing a framework for high-resolution, non-invasive population assessment. By addressing methodological gaps in sensitivity, reliability, and ecological relevance, our findings advance environmental nucleic acid (eNA)-based monitoring for aquatic megafauna.

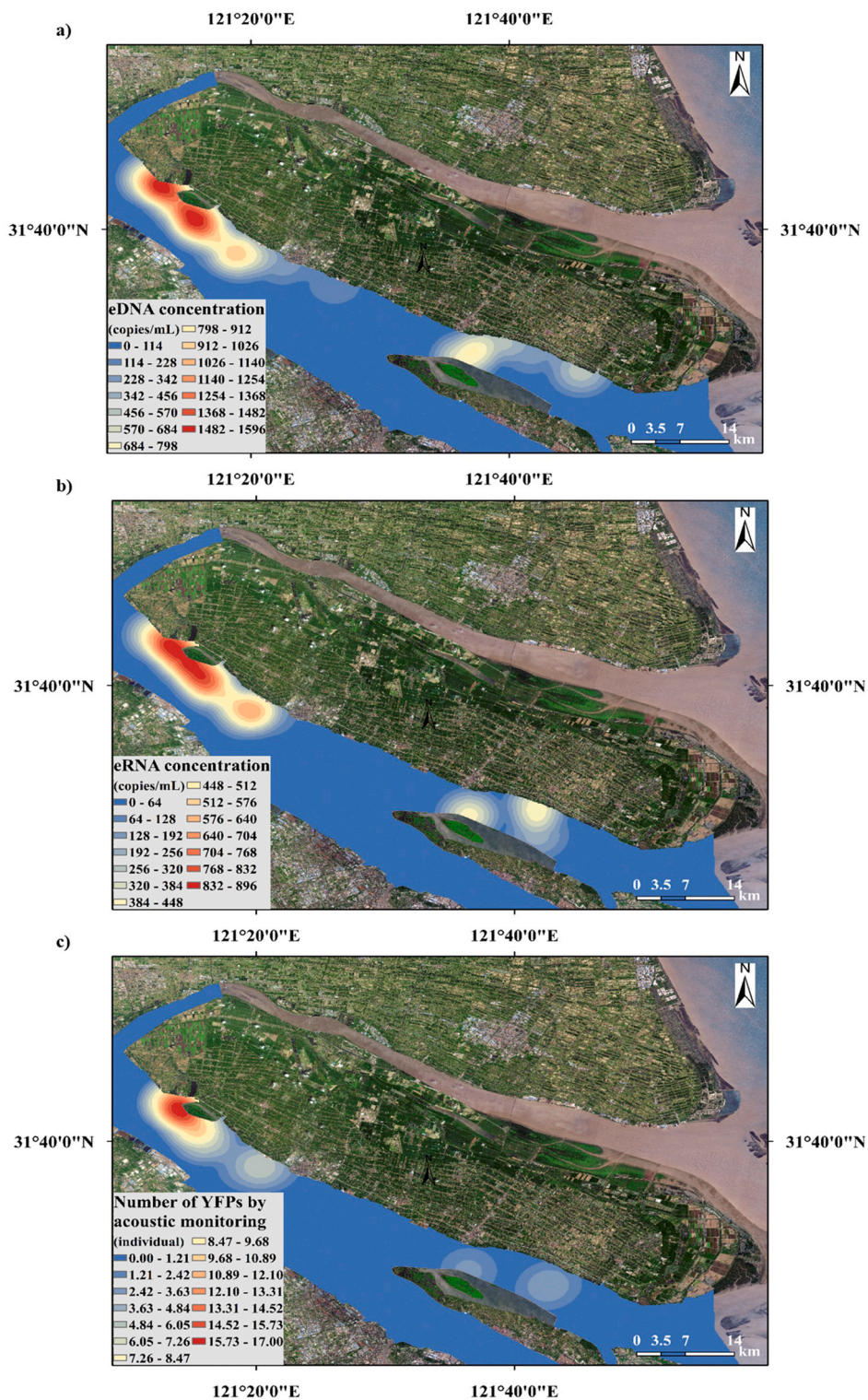


Fig. 7. Predicted distribution of the Yangtze finless porpoises by eDNA, eRNA and acoustic data using the ArcGIS Kernel Density Analysis tool. a) eDNA; b) eRNA; c) acoustic monitoring.

4.1. Defining LOD and LOQ: enhancing the precision and reliability of eDNA monitoring

Rigorous quantification of detection limits is critical for standardizing eDNA workflows (Goldberg et al., 2016; Klymus et al., 2020). Here, we established a limit of detection (LOD) of 0.076 copies μL^{-1} and limit of quantification (LOQ) of 0.35 copies μL^{-1} for Yangtze finless porpoise DNA, resolving a key limitation in prior studies that lacked such metrics (Chen et al., 2024; Qiao et al., 2024). Our LOD/LOQ criteria, 95 % detection probability and coefficient of variation (CV) < 35 %, provide actionable thresholds for field applications, ensuring reproducibility and minimizing false negatives in low-abundance scenarios.

4.2. Dual-Marker development and performance comparison

Previous studies on eDNA monitoring of the Yangtze finless porpoise have primarily relied on single-marker assays. However, research has shown that combining multiple markers significantly enhances the sensitivity and precision of eDNA-based single-species analyses (Brys et al., 2023; Farrington et al., 2015; Matsuo et al., 2024; McClenaghan et al., 2020; Weitemier et al., 2021). This study builds upon existing literature to develop duplex markers, ensuring that the performance of duplex markers surpasses that of simplex marker.

Firstly, the target loci must be sufficiently distanced from each other on the mitochondrial genome to avoid interference (Doi et al., 2021; Eastwood and Orsini, 2021; Zhu et al., 2023). The *COX3* and *CYTB* markers developed in this study are located 5784 bp apart on the mitochondrial genome. Secondly, the eDNA target sequences at the two loci exhibit similar sizes, ensuring that the concentrations of different target eDNAs in the water are comparable. However, when comparing our results to previous studies, it is important to note that although the eDNA target sequences *COX3* and *CYTB*, which are similar in size and located at different loci, occur at almost identical concentrations in tissue samples, this is not the case in natural samples. In the Yangtze River estuary, the number of detection sites for the Yangtze finless porpoise based on the *COX3* marker exceeded that based on the *CYTB* marker by three sites. Moreover, at half of the 10 sites where both markers were detected, there was a significant difference in concentrations.

A possible explanation for this difference is that the stability and integrity of DNA in tissue samples, which are less affected by external environmental factors, result in consistent target sequence concentrations. In contrast, eDNA in natural water bodies exhibits more variable concentrations due to uneven sources and distribution, susceptibility to environmental factors, and the presence of PCR inhibitors, which can hinder the amplification of specific target sequences (Lance and Guan, 2020; Rishan et al., 2023). Additionally, the low concentration of eDNA from the Yangtze finless porpoise in the Yangtze River estuary may contribute to the stochastic difference in detection concentrations between the two markers.

In this study, ddPCR assays were employed instead of qPCR to mitigate delays or limitations caused by competitive interactions during PCR reactions. In ddPCR, interaction effects are minimized because each reaction is partitioned into tens of thousands of nanoliter-sized droplets, reducing the likelihood of multiple target DNA fragments being encapsulated within a single droplet. The results of this study indicate that using multiplex markers combined with digital PCR is an effective approach for detecting low-abundance species.

4.3. Integration of eDNA and eRNA for enhanced monitoring

A major breakthrough in this study is the integration of both eDNA and eRNA methodologies to conduct degradation rate studies in natural aquatic environments, subsequently facilitating extended field surveillance efforts. Traditional studies about the degradation rates of eDNA and eRNA have largely relied on controlled laboratory microcosm experiments. Here we have gained a deeper understanding of how these molecules persist or degrade within semi-natural aquatic environments, which is more representative of real-world field conditions compared to controlled laboratory microcosm experiments. This approach enhances our ability to generalize findings from lab studies to field scenarios, thereby contributing significantly to both theoretical understanding and practical applications.

Previous research has shown that eRNA degrades more rapidly than eDNA (Qian et al., 2022). The faster degradation rate of eRNA enables real-time inference of species presence or absence, which improves the identification of living organisms, increases sensitivity, and reduces false positives. In this study, the distribution predictions for the Yangtze finless porpoise in the Yangtze River Estuary based on eRNA monitoring and acoustic monitoring exhibit greater consistency, further confirming this conclusion. This also allows for more accurate estimates of species abundance (Bowers et al., 2021; Farrell et al., 2021; Miyata et al., 2021). In an aquarium study on the degradation of eDNA and eRNA in marine invertebrates, eDNA was detectable up to 94 hours after the organism's removal, while eRNA persisted for a maximum of only 13 hours (Wood et al., 2020). Our findings support this conclusion, demonstrating that eDNA signals persist nearly twice as long as eRNA signals.

In flowing aquatic systems, hydrological dynamics (e.g., flow velocity, turbulence), eDNA particle characteristics (size, density, shape), and biodegradation rates collectively determine eDNA transport distance and detection reliability (Barnes et al., 2014; Pont et al., 2024; Shogren et al., 2018). eDNA degradation is modulated by multiple factors, including molecular state (extracellular or intracellular), fragment sizes, genomic origin (mitochondrial or nuclear), and environmental conditions (both abiotic and biotic) (Barnes et al., 2014; Scriver et al., 2023). This inherent complexity prevents the derivation of universal degradation rate constants across spatiotemporal gradients (Bowers et al., 2021; Farrell et al., 2021; Jo, 2023), fundamentally constraining the precision of hydraulic models that fail to account for degradation heterogeneity and water-sediment dynamics (Fremier et al., 2019; Jerde et al., 2016; Wilcox et al., 2016). In the Yangtze Estuary, hydrodynamic complexity arising from river-tide coupling and three-dimensional turbulent processes (Wan et al., 2014) further intensifies these methodological challenges.

To overcome these limitations, this study developed a dual-marker eDNA/eRNA assay using digital PCR (dPCR) with concurrent acoustic monitoring. The detection rate of eDNA was significantly higher than that of eRNA. Notably, in locations where eRNA signals were detected, passive acoustic devices also recorded vocal behaviors of the Yangtze finless porpoise, confirming species presence. Spatial congruence between eDNA hotspots and acoustic clusters demonstrate that the proposed multi-locus eNA methodology effectively captures localized biota signals in complex estuarine environments, consistent with multiple studies demonstrating that fluvial eDNA predominantly mirrors proximal biological communities (Civade et al., 2016; Spear et al., 2015; Tillotson et al., 2018). This study represents the first attempt to combine eDNA and eRNA for monitoring the endangered Yangtze finless porpoise, highlighting the potential of eRNA analysis to enhance the positive predictability in biological monitoring.

4.4. Conservation implications

Our integrated analysis of environmental nucleic acids (eNAs) and passive acoustic monitoring identified two critical activity hotspots for the critically endangered Yangtze finless porpoise (*Neophocaena asiaeorientalis*) in the Yangtze River estuary. The first hotspot, located near the Dongfeng Xisha Reservoir on Chongming Island, exhibited consistently high detection rates across both eDNA/eRNA and acoustic methods, suggesting sustained porpoise presence and potential breeding or foraging activity. This area aligns with prior reports of high-density porpoise aggregations in regions with stable hydrological conditions and abundant prey resources (Tang et al., 2018). The second hotspot, near the Yangtze River Bridge on Chongming Island, showed comparatively lower detection signals, possibly reflecting transient use or suboptimal habitat quality due to anthropogenic disturbances such as vessel traffic and underwater noise pollution (Wang et al., 2020).

The stark contrast in activity levels between these regions underscores the importance of habitat heterogeneity in shaping porpoise distribution. The Dongfeng Xisha Reservoir's quieter, resource-rich environment likely provides refuge from anthropogenic stressors, whereas the bridge-adjacent zone may serve as a migration corridor with limited long-term residency. These findings are consistent with those from other distribution areas, where differences in conservation measures and habitat conditions, particularly between core habitats and peripheral zones, significantly impact the population size and distribution of the Yangtze finless porpoise (Huang et al., 2020; Zhang et al., 2024).

The congruence between eNAs and acoustic data reinforces the utility of multi-method monitoring for mapping critical habitats. While eNAs provided broad spatial resolution of porpoise presence, acoustic records added temporal granularity, capturing real-time vocal behaviors indicative of live individuals (Li et al., 2022). This synergy highlights the value of integrating molecular and acoustic tools to overcome limitations inherent to single-method approaches, such as false positives from eDNA persistence or acoustic masking by ambient noise.

Both identified hotspots warrant urgent protection, but management strategies should reflect their distinct ecological roles. The Dongfeng Xisha Reservoir should be prioritized as a core protected zone, with strict regulations on fishing, dredging, and vessel traffic to mitigate habitat degradation. Conversely, the bridge-adjacent area necessitates targeted mitigation of anthropogenic noise and pollution to enhance its viability as a functional corridor. These measures align with established conservation frameworks for the Yangtze finless porpoise in recent years (Hao et al., 2023; Lu et al., 2023), and could serve as a model for balancing human activity and biodiversity preservation in heavily industrialized estuaries.

Future studies should prioritize establishing an integrated observational technology framework to investigate seasonal variations in porpoise habitat utilization, while assessing impacts of specific stressors (e.g., acoustic disturbance, chemical pollutants) on site fidelity. Such empirical insights will systematically refine spatially explicit conservation strategies for this flagship species.

5. Conclusion

This study establishes a dual-marker eDNA/eRNA framework for monitoring the Yangtze finless porpoise, addressing critical gaps in detection sensitivity, temporal resolution, and ecological relevance. Key innovations include: standardized LOD/LOQ thresholds for reproducible eDNA quantification, mitochondrial multi-locus optimization enhancing detection reliability, eRNA integration for real-time presence confirmation. While our findings highlight eNAs' potential, limitations persist. Single-season sampling restricts insights into population dynamics, and degradation rates require validation across diverse habitats. Future efforts should prioritize longitudinal monitoring to track seasonal/behavioral shifts, environmental transcriptomics to link eRNA signals to physiological states, cross-method validation integrating acoustics, genomics, and citizen science. By advancing non-invasive monitoring tools, this work supports targeted conservation strategies for the Yangtze finless porpoise, a flagship species for freshwater ecosystem health.

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Ethics Statement

Not applicable: This manuscript does not include human or animal research. If this manuscript involves research on animals or humans, it is imperative to disclose all approval details.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.gecco.2025.e03645](https://doi.org/10.1016/j.gecco.2025.e03645).

Data availability

Data will be made available on request.

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Further reading

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