Environmental DNA

WILEY

Environmental DNA
Open Access
Dedicated to the study and use of environmental DNA for basic and applied sciences

ORIGINAL ARTICLE OPEN ACCESS

Real-Time PCR Assay and Environmental DNA Workflow for Detecting Irukandji Jellyfish, *Malo bella* (Cubozoa)

Jessica K. Strickland[1] | Kylie A. Pitt[1,2] | Michael J. Kingsford[3,4] | Scott J. Morrissey[3,4] | Dean R. Jerry[3,5,6]

[1]Centre for Marine and Coastal Research, Australian Rivers Institute, and School of Environment and Science, Griffith University, Southport, Queensland, Australia | [2]Griffith Institute for Tourism, Griffith University, Southport, Queensland, Australia | [3]Marine Biology and Aquaculture, College of Science and Engineering, James Cook University, Townsville, Queensland, Australia | [4]ARC Centre of Excellence for Coral Reef Studies, James Cook University, Townsville, Queensland, Australia | [5]ARC Research Hub for Supercharging Tropical Aquaculture Through Genetic Solutions, James Cook University, Townsville, Queensland, Australia | [6]Tropical Futures Institute, James Cook University, Singapore, Singapore

Correspondence: Jessica K. Strickland (jessica.strickland@griffithuni.edu.au)

Received: 27 June 2024 | **Revised:** 16 January 2025 | **Accepted:** 1 February 2025

Funding: This study was supported by the Minderoo Foundation with additional in-kind support from the Minderoo Foundation Exmouth Research Laboratory (MERL) and its staff. J. Strickland was supported by a PhD scholarship and funding from Griffith University.

Keywords: Cubozoa | environmental DNA | Irukandji | jellyfish | polymerase chain reaction

ABSTRACT

The rise in coastal populations and marine activities has intensified challenges posed by hazardous Irukandji jellyfishes, whose stings can cause severe symptoms and sometimes death. Despite their significant impact on health services and marine-related industries, Irukandji jellyfishes remain poorly understood due to the challenges of studying them and the limitations of traditional sampling methods. Genetic methods and environmental DNA (eDNA) offer promising solutions. This study developed and validated a sensitive and specific quantitative PCR assay to detect and monitor *Malo bella*, an Irukandji jellyfish threatening tourism in Western Australia. *M. bella*-specific primers and a TaqMan Minor Groove Binding (MGB) probe were designed. The assay demonstrated high specificity, not amplifying non-target species, and sensitivity, with 95.6% efficiency, a slope of −3.43, and an R^2 value of 0.98. The assay's 95% limit of detection (LoD) was 0.80 eDNA copies/reaction, and the modeled limit of quantification (LoQ) was 13 eDNA copies/reaction. Validation through in silico and in vitro tests confirmed successful detection of *M. bella* eDNA in all water samples from aquaria and around medusae in the ocean. Sanger sequencing verified the amplification of the target *M. bella* sequence. This assay improves the ability to study *M. bella*, addressing critical knowledge gaps on the species' ecology. These include assessing the spatial and temporal distributions of this species and potential detection of early benthic life stages to identify source populations. Such studies will improve management of envenomation risks in tourism hotspots. Future research should explore integrating passive or automated samplers and developing real-time detection assays to further enhance monitoring capabilities and mitigate risks posed by hazardous marine species.

1 | Introduction

The surge in coastal population growth and marine activities in the tropics and subtropics has exacerbated challenges associated with hazardous jellyfishes (Kingsford et al. 2017; Purcell 2012), with the 'Irukandji jellyfishes' emerging as a significant concern (Tibballs et al. 2012). These jellyfishes constitute at least 13 carybdeid species that can cause Irukandji syndrome (Gershwin 2014; Gershwin et al. 2013), which is characterized by symptoms such as back pain, muscle cramps, laboured breathing, a sense of impending doom, and in rare cases, death (Fenner and Hadok 2002; Flecker 1952). Irukandji jellyfishes and other 'marine stingers' strain emergency and health services (Crowley-Cyr 2020;

Macrokanis et al. 2004), threaten fisheries and aquaculture (Gershwin et al. 2013; Purcell et al. 2007), and pose a significant risk to marine tourism (Crowley-Cyr 2020; Gershwin et al. 2010). Although Irukandji jellyfishes are circumtropical (Gershwin et al. 2013), climate change may facilitate their poleward expansion, impacting previously unaffected communities (Madin et al. 2012).

The Ningaloo Coast World Heritage Area, in Western Australia, attracts tens of thousands of tourists annually to swim with whale sharks, humpback whales, and manta rays (Department of Biodiversity Conservation and Attractions 2023). However, Irukandji jellyfish envenomations cause dozens of hospitalisations in the region each year (Gershwin and Hannay 2014; Keesing et al. 2020; Perera 2017). Two main Irukandji species occur along the Ningaloo Coast; *Malo bella* (Gershwin 2014) and *Keesingia gigas* (Gershwin 2014). Anecdotally, the smaller *M. bella* (bell height ≈25 mm) generally appears between March and July in the open ocean, while the larger *K. gigas* (bell height > 40 cm) occurs sporadically, with individuals occasionally becoming stranded ashore (Gershwin 2014; Keesing et al. 2020). A more comprehensive understanding of these jellyfishes' spatial and temporal distributions, particularly *M. bella* which occurs semi-regularly for 4–6 months per year, is needed for government agencies and tourism operators to develop management strategies to minimize the risks they pose.

Determining the presence and distribution of Irukandji jellyfishes is challenging due to the morphological similarity between many species, their patchy distributions, small size, transparency, frangibility, and dangerous stings (Bentlage et al. 2010; Kingsford and Mooney 2014; Rowley 2021). Environmental DNA (eDNA) provides an approach to accurately identify and study elusive and patchily distributed taxa. eDNA assays can complement traditional survey methods, like nets, lights, and cameras, and enable Irukandji to be identified over multiple spatial and temporal scales, often with less effort (Lewis Ames et al. 2021; Rees et al. 2014; Thomsen and Willerslev 2015). Several studies have developed species-specific eDNA assays to detect jellyfishes (e.g., Gaynor et al. 2017; Minamoto et al. 2017; Wang et al. 2021), including some cubozoans (e.g., Azama et al. 2023; Bolte et al. 2021; Morrissey et al. 2022). These assays have been used to investigate ecological hypotheses and to address jellyfish management issues (e.g., Peng et al. 2023; Takasu et al. 2019).

TaqMan quantitative PCR (qPCR) assays, employing species-specific primers and a fluorophore probe, constitute a highly sensitive tool for detecting and quantifying the eDNA of a species (Klymus et al. 2020; Kutyavin et al. 2000). This method provides a powerful and precise genetic approach for detecting rare or sporadically distributed species (e.g., Gargan et al. 2022; Harper et al. 2018, but see McCarthy et al. 2023) and can detect just a single fragment of the target species' eDNA in a water sample (Wilcox et al. 2013). Accurate ecological analyses via eDNA sampling demand the careful development and validation of PCR assays and workflows (De Brauwer et al. 2022; Thalinger et al. 2021). qPCR assays must be specific to avoid amplifying non-target species' DNA, efficient, and sufficiently sensitive to detect very low eDNA copy numbers of a target species (De Brauwer et al. 2022; Klymus et al. 2019, 2020).

The objective of this study was to develop and validate a sensitive species-specific eDNA assay and workflow to detect the Irukandji jellyfish, *M. bella* from filtered seawater samples. This was undertaken through (1) developing and optimizing a *M. bella*-specific probe-based qPCR assay and eDNA workflow and (2) testing and validating the assay and workflow in the laboratory and the field.

2 | Materials and Methods

2.1 | Assay Design

2.1.1 | Generation of Sequence Database

The mitochondrial 16S ribosomal RNA gene (16S rRNA) was chosen for assay design because it is highly conserved within the species, varies among species, and reference sequences for closely related taxa are available. Sequences from sympatric and closely related allopatric species (non-target species) (Table S1) were downloaded from the National Centre for Biotechnology Information (NCBI) database (Sayers et al. 2022), and collated in Geneious Prime 2022.2 (www.geneious.com). No sequences were available for *M. bella* or closely related species in the Ningaloo region, so cubozoan jellyfish were collected from Ningaloo Marine Park and nearby waters, identified morphologically, and preserved in ethanol (*M. bella*, $n = 9$; *K. gigas*, $n = 2$; *Carybdea cf. xaymacana*, $n = 2$). Additional specimens were obtained from scientific collections (Reef and Ocean Laboratory Collection, James Cook University, Townsville, Australia).

Genomic DNA (gDNA) was extracted from a small segment (~200 mg) of tentacle from each specimen using a Qiagen DNeasy Blood and Tissue kit (Qiagen Pty Ltd), following manufacturer protocols, except with overnight tissue lysis. A 584 bp length of the 16S rRNA gene was amplified via end-point PCR using jellyfish primers 16SL (Ender and Schierwater 2003) and Aa_H16S_15141H (Bayha and Dawson 2010), as used by Bolte et al. (2021), with the SimpliAmp Thermal Cycler (Applied Biosystems) (initial denaturation step of 95°C for 3 min, followed by 38 cycles of 94°C for 30 s, 59°C for 1 min, and 72°C for 75 s, with a final extension at 72°C for 10 min). Each 25 μL reaction contained 1 μL gDNA template (approximately 25–225 ng), 2.5 μL 10× PCR buffer (−Mg), 0.5 μL [10 mM] dNTP mix, 1.5 μL [50 mM] $MgCl_2$, 0.1 μL [5 U/μL] Taq DNA polymerase, 0.63 μL [10 μM] forward and reverse primers, and UltraPure DNase/Rnase-free distilled water (all ThermoFisher Scientific Inc.).

After visualization by gel electrophoresis on an agarose gel, the PCR product was sent to the Australian Genome Research Facility (AGRF; Brisbane, Australia) for bidirectional Sanger sequencing. Forward and reverse sequences were imported into Geneious Prime 2022.2 (www.geneious.com), trimmed to remove low-quality bases, pairwise aligned (Geneious alignment), and searched using NCBI's Nucleotide Blast search (Altschul et al. 1990) to review taxonomical identification. New sequences were submitted to NCBI GenBank (Sayers et al. 2022) (Table S1).

All 16S rRNA sequences for target and non-target species were compiled in Geneious Prime 2022.2 (www.geneious.com). A *M. bella* consensus sequence was generated after aligning all

sequences ($n = 9$) with the multiple alignment tool (Geneious alignment) (Table S1). The 16S rRNA sequences for non-target species ($n = 273$) were reviewed, and identical or similar sequences were removed. The resulting non-target sequences ($n = 58$) (Table S1) were compiled for primer and probe design. Finally, the target and non-target sequences (using the consensus sequences where applicable) were multiple-aligned (MUSCLE alignment with 10 iterations) to create an all-sequence alignment. A phylogenetic tree was also generated based on 16S rRNA jellyfish sequences obtained from NCBI or generated as part of this study (Figure S1). The ModelFinder analysis (Kalyaanamoorthy et al. 2017) and ultrafast bootstrapping (1000 replicates) (Hoang et al. 2017) were used in IQ Tree 1.6.12 (Minh et al. 2020) (1). The tree was maximum likelihood generated using the GTR+F substitution models with sequence regions of 255–710 bp.

2.1.2 | Primer and Probe Design

Candidate *M. bella* primers and TaqMan Minor Groove Binding (MGB) probes were designed using Geneious Prime 2022.2 (www.geneious.com) and AlleleID 7.0 (Premier Biosoft, USA). Inspection of the all-sequence alignment identified potential primer and probe regions, ensuring interspecific variation while avoiding intraspecific SNPs. Primers and probes were generated from the *M. bella* consensus sequence using the design new primers tool in Geneious Prime 2022.2 (www.geneious.com), with specificity enhanced by using the option to test against an off-target database (i.e., the non-target sequences). Selected primers maximized 3′ base pair mismatches with non-target sequences. Additionally, potential MGB probes were generated from *M. bella* sequences using AlleleID 7.0 (Premier Biosoft, USA).

In silico specificity was tested using NCBI's Primer-Blast (Ye et al. 2012) and Nucleotide Blast (searched against the nr database) tools (Altschul et al. 1990), per Klymus et al. (2020). The test with saved primers tool in Geneious Prime 2022.2 (www.geneious.com) then tested the likelihood of the candidate primers and probe binding to non-target sequences. Evaluation of primer and probe combinations was based on parameters specified by Klymus et al. (2020), determined using Geneious Prime 2022.2 (www.geneious.com), IDT OligoAnalyzer (Owczarzy et al. 2008), and the Sequence Manipulation Suite (Stothard 2000) (Table S2). The best combinations were selected for in vitro testing and subsequently, the primers and probe were synthesized (ThermoFisher Scientific Inc.).

2.1.3 | Specificity

The selected candidate assays were tested for specificity against gDNA from *M. bella* and 10 exclusion species (Table 1). Initially, end-point PCR was used to confirm the assay would amplify *M. bella* but not the exclusion species. Each PCR reaction, conducted in triplicate, utilized ~80 ng of *M. bella* or exclusion species gDNA template and occurred on the SimpliAmp Thermal Cycler (Applied Biosystems) as above (except using 0.7 μL [10 μM] of the candidate primers). One reaction containing jellyfish primers 16SL (Ender and Schierwater 2003) and Aa_H16S_15141H (Bayha and Dawson 2010) in place of *M. bella* primers was used for each species as a control. Gel electrophoresis confirmed the correct amplicon size.

Subsequent specificity testing via qPCR incorporated the TaqMan MGB probe, using a QuantStudio 3 Real-Time PCR system (ThermoFisher Scientific Pty Ltd., Australia) (initial denaturation step of 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min). Each 10 μL reaction, run in triplicate contained ~80 ng *M. bella* or exclusion species gDNA template, 5 μL TaqMan Environmental Master Mix 2.0, 0.7 μL [10 μM] forward and reverse primers, 0.25 μL [10 μM] TaqMan probe, and UltraPure DNase/RNase-free distilled water (all ThermoFisher Scientific Inc.). Threshold cycle (C_t) values were

TABLE 1 | Exclusion species sequences utilized for assay design, with NCBI accession numbers, collection locations and sources (ROEL—Reef and Ocean Ecology Laboratory, SLSQ—Surf Life Saving Queensland).

Accession number	Species	Collection location	Source
OP877035	*Alatina alata*	Waikiki, Hawaii	Sample provided by Lisa Gershwin to ROEL
OP877033	*Carukia barnesi*	Palm Cove, Queensland, Australia	Sample provided by Jamie Seymour to ROEL
PP889593	*Carybdea cf. xaymacana*	Bussleton, WA, Australia	Collected by the authors
OP877034	*Carybdea cf. xaymacana*	Townsville, Queensland, Australia	ROEL
	Cassiopea sp.	Ningaloo Coast, WA, Australia	Collected by the authors
OP877024	*Chironex fleckeri*	Townsville, Queensland, Australia	Sample provided by SLSQ to ROEL
OP877032	*Copula sivickisi*	Townsville, Queensland, Australia	ROEL
PP626683	*Keesingia gigas*	Ningaloo Coast, WA, Australia	Collected by the authors
	Morbakka sp.	Queensland, Australia	ROEL
	Pelagia noctiluca	Ningaloo Coast, WA, Australia	Collected by the authors
	Tamoya sp.	Queensland, Australia	ROEL

analyzed using QuantStudio Design and Analysis Software (v2.6.0, ThermoFisher Scientific Pty Ltd., Australia), and any amplicons were bidirectionally Sanger sequenced for taxonomic verification (AGRF, Brisbane, Australia).

2.1.4 | Efficiency and Sensitivity

Assays were optimized via a series of end-point and quantitative PCR reactions with varying annealing temperatures (Ta) and primer/probe concentrations. The optimized *M. bella* assay's efficiency and sensitivity were assessed using standard curves of known DNA copy numbers to calculate the 95% limit of detection (LoD) and limit of quantification (LoQ). For qPCR, the LoD is the lowest concentration of target DNA detectable with a defined level of confidence, usually 95%, while the LoQ is the minimum concentration that can be quantified with specified precision under defined conditions (Klymus et al. 2019).

Double-stranded synthetic DNA fragments (sDNA; gBlocks; Integrated DNA Technologies Pty Ltd. (IDT), Australia), based on the *M. bella* 16S rRNA consensus sequence, were synthesized for the standard curve. These 198 bp sDNA fragments included primer and probe binding regions and a mid-sequence 7 bp modification to distinguish between sDNA and gDNA/eDNA amplicons via Sanger sequencing as a cross-contamination check (Budd et al. 2021; Klymus et al. 2020).

Ten-point standard curves were generated from a 10:1 serial dilution of *M. bella* sDNA (ranging from a theoretical 10^8 to 0.01 copies/μL). These curves, run on a QuantStudio 3 Real-Time PCR system (ThermoFisher Scientific Pty Ltd., Australia) using the optimized assay, were analyzed using the QuantStudio Design and Analysis Software (v2.6.0, ThermoFisher Scientific Pty Ltd., Australia). C_t values from multiple standard curves ($n = 10$–24 qPCR replicates per standard) were collated and analyzed in RStudio v2023.9.0.463 (RStudio Team 2023) running R v4.2.1 (R Core Team 2022) using the Generic qPCR LoD/LoQ Calculator R Script (Klymus et al. 2019; Merkes et al. 2019). This provided slope, R^2, 95% LoD and LoQ values. qPCR efficiency (E) was calculated as $E = 10^{-1/\text{slope}}$.

2.1.5 | Endogenous Control Assay

To incorporate an additional quality assessment into the eDNA workflow, the *M. bella* assay was multiplexed with the 16S rRNA generic fish assay developed by Furlan and Gleeson (2017) as an endogenous control. This fish assay used a different fluorophore (VIC) to the *M. bella* assay (FAM), allowing distinction during qPCR. *In silico* testing against 16S rRNA and complete genome sequences for fish species in the Ningaloo Marine Park confirmed the assay's suitability for this region (Table S3). The endogenous control assay had no potential hetero-dimer formation with the *M. bella* assay. Additionally, the test with saved primers tool in Geneious Prime 2022.2 (www.geneious.com) verified its non-amplification of *M. bella* DNA.

The specificity of the multiplexed assays was verified through in vitro trials to ensure previously tested exclusion species were not amplified when run together. A QuantStudio 3 Real-Time PCR (ThermoFisher Scientific Pty Ltd., Australia) was used (initial denaturation step of 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 58°C for 1 min). Each 10 μL reaction, run in triplicate, contained ~80 ng *M. bella* or exclusion species gDNA template, 5 μL TaqMan Environmental Master Mix 2.0, 0.75 μL [10 μM] forward and reverse *M. bella* primers, 0.5 μL [10 μM] forward and reverse fish primers, 0.25 μL [10 μM] *M. bella* TaqMan probe and fish probe, and UltraPure DNase/RNase-free distilled water (all ThermoFisher Scientific Inc.).

2.2 | General eDNA Sampling and Quantification Methods

2.2.1 | Collection and Preservation of eDNA Samples

To minimize eDNA contamination risk, different sterile gloves and equipment (e.g., jars, forceps) were used for each water sample. All equipment was sterilized with 10% bleach, rinsed thoroughly, and exposed to UV light. For field sampling, water samples were collected in 2.5 L plastic jars at least 1 m from the vessel. For aquaria sampling, hand-held 250 mL plastic jars were used. Water was either filtered immediately or kept on ice and filtered within 4 h.

Samples were filtered through 5 μm nylon net filters (47 mm; Merck Millipore Ltd., Cork, Ireland). Filters were cut in half and preserved in DNA-free Longmire's solution (Renshaw et al. 2015; Williams et al. 2016) for short-term storage at 4°C or long-term storage at −20°C. To control for potential eDNA contamination, 250 mL reverse osmosis (RO) water samples were filtered and preserved as procedural controls. One procedural control was processed before processing each set of replicate eDNA samples.

2.2.2 | Extraction of eDNA from Preserved Samples

eDNA was extracted from filters using the 'preserve, precipitate, lyse, precipitate, purify' (PPLPP) workflow (Edmunds and Burrows 2020), as adapted by Cooper et al. (2021) for filter papers in 2 mL tubes. For the final purification stage, a OneStep PCR Inhibitor Removal Kit (Zymo Research Corp, California, USA) was used following manufacturer protocols. The PPLPP method yields significantly more DNA than other methods (Cooper et al. 2021; Misutka et al. 2023; Renshaw et al. 2015). Extracted eDNA samples were stored at −20°C.

2.2.3 | Detection of *M. bella* eDNA by Quantitative PCR

The TaqMan qPCR assay detected *M. bella* eDNA from eluate extracted from preserved eDNA samples. Six 10 μL technical replicate reactions were run per eDNA sample (using 18% of the eluate). Each reaction contained 2 μL eluate (DNA template), 0.95 μL [10 μM] forward and reverse primers, 0.3 μL [10 μM] TaqMan probe, 5 μL of TaqMan Environmental Master Mix 2.0, and UltraPure DNase/RNase-free distilled water (all ThermoFisher Scientific Inc.). Reactions were run on the QuantStudio 5 Real-Time PCR system (ThermoFisher Scientific

Pty Ltd., Australia) with a protocol of 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 58°C for 1 min. ROX served as the passive reference.

Replicate reactions were placed in adjacent wells of 384-well plates, which were prepared in a laminar flow hood, typically with 24 spatially separated eDNA samples run per plate. The extraction blanks (from eDNA extraction) and procedural controls (processed in parallel with eDNA samples) were also run in replicates of six. Standards ranging from 10^5 to 1 copies/μL, were used in triplicate on each plate, to generate standard curves for consistency and eDNA quantification.

The multiplexed endogenous control assay served as a quality control for eDNA collection, extraction, storage, and amplification, and as an inhibition indicator. Field samples failing to amplify fish DNA were tested for inhibition by running qPCR reactions on serial dilutions of the sample (Cao et al. 2012). Samples failing the dilution qPCR were omitted to avoid false negatives for *M. bella* DNA.

All PCR plates were analyzed using QuantStudio Design and Analysis Software (v2.6.0, ThermoFisher Scientific Pty Ltd., Australia). All positive detections of *M. bella* were confirmed through clean-up and bidirectional Sanger sequencing of PCR products (AGRF, Brisbane, Australia). True positives required: amplification crossing the fluorescence threshold within 40 cycles in at least one technical replicate; sequence matching the target species with ≥99% pairwise identity; positive amplification of the endogenous control (for field samples); and absence of amplification in negative controls or procedural controls (adapted from Trujillo-González et al. 2019). These standards were adapted from Trujillo-González et al. (2019) and are comparable to those used in similar studies on small, sporadically distributed, rare or elusive species in open marine systems (e.g., Cooper et al. 2021; Morrissey et al. 2024a, 2024b; Budd et al. 2021).

2.3 | Aquaria and Field Validation

To validate the assay's efficacy in detecting *M. bella* eDNA, two live *M. bella* medusae (-¬x wet weight = 0.93 g) were placed in four aerated tanks, each containing 30 L of artificial seawater (salinity = 35 ppt; 30°C ± 0°C). A fifth tank without jellyfish served as a control. After 1 h, 500 mL of water was collected from each tank, filtered, and preserved. One procedural control containing RO water was processed in parallel with the eDNA samples taken from the four tanks containing *M. bella*. For field validation, five 2.5 L seawater samples were collected equidistantly (approximately 3–5 m) around each of eight wild medusae located within 1.5 m of the surface. One procedural control containing RO water was processed for each medusa sampled. Negative controls of ocean water were not able to be collected as it was not possible to ensure the absence of *M. bella* eDNA, given the species is sporadically but widely distributed in the area, is small and difficult to detect visually, and has a microscopic benthic polyp stage that could be nearby.

3 | Results

3.1 | Assay Quality

The M. bella-specific assay was designed with forward and reverse primers and a 5' FAM labeled TaqMan™ probe with a 3′ MGB nonfluorescent quencher (NFQ) between base pairs 88 and 245 on the *M. bella* 16S rRNA consensus sequence (Table 2 and Figure 1). The primers and probe were selected based on their characteristics falling within acceptable ranges (Table S2), and their specificity to the target species. In silico testing showed that primer and probe binding regions contained a minimum of 17 cumulative mismatches to sympatric species tested (Figure 1). Two allopatric species, *Gerongia rifkinae*, which occurs along the north coast of Australia, and *Morbakka virulenta* from Japan, contained the fewest cumulative mismatches (respectively 3 and 4; Figure 1). These species were not included in exclusion testing, as no tissue or genomic DNA was available; but they do not overlap geographically with *M. bella*. Another *Morbakka* species (*M. fenneri*) was included in in vitro exclusion testing and did not amplify using the *M. bella* assay. The in vitro specificity qPCR did not amplify exclusion species gDNA and confirmed the assay was species-specific.

The *M. bella* qPCR assay was efficient and sensitive. Multiple 10-point standard curves (n = 10–24 qPCR replicates per standard) using 10:1 serial dilutions of sDNA, from 10^8 to 1 copies/μL, showed 95.6% efficiency (E), a slope of −3.43, and an R^2 of 0.98. The effective 95% LoD, using six technical replicates per sample, was 0.80 copies/reaction, and the modeled LoQ was 13 copies/reaction (Figure 2).

3.2 | In Situ Assay Validation

Malo bella eDNA was detected in all four water samples retrieved from aquaria hosting medusae for 1 h and in all samples surrounding all eight medusae in the ocean (Table 3). For five of the medusae in the field, eDNA was detected in all samples collected equidistantly around the animal. For two animals, four out of five samples showed positive detections, and for only one animal, three out of five samples returned positive results. Sanger sequencing of these samples confirmed the successful amplification of the target *M. bella* sequence.

4 | Discussion

eDNA methods offer a promising approach for monitoring hazardous jellyfish; however, developing and validating specific, sensitive, and efficient assays is crucial (De Brauwer et al. 2022; Klymus et al. 2020; Thalinger et al. 2021; Wilcox et al. 2013). This study presents a novel qPCR assay for the Irukandji jellyfish, *M. bella*, which complements existing jellyfish sampling techniques (Beng and Corlett 2020; Kingsford et al. 2017; Kingsford and Mooney 2014; Rees et al. 2014). Testing and validating this assay have demonstrated its effectiveness in detecting *M. bella* eDNA, including in an open ocean environment, when the species is present.

TABLE 2 | The primers selected were Mb16S_F88 (forward) and Mb18S_R245 (reverse) and the MGB probe selected was Mb16S_MGB126.

Name	Oligo	Sequence (5′–3′)	[Final] (μM)	Ta (°C)	Target length (bp)	Efficiency (%)	R^2	95% LoD (6 reps) (copies/reaction)	LoQ (copies/reaction)
Mb16S_F88	F	TCCCTGTCTCAACTACAGAA	0.95	58	157	95.6	0.98	0.80	13
Mb18S_R245	R	AACCAAACTATCCCCTCTCT	0.95						
Mb16S_MGB126	P	TTGTCAAGACTCAA	0.3						

Note: *M. bella* species-specific assay (16S rRNA).

The foundation of a quality qPCR assay lies in developing highly specific primers (Wilcox et al. 2013). Here, *M. bella*-specific primers and TaqMan™ Minor Groove Binding (MGB) probe were developed through the systematic approach set out by Klymus et al. (2020). Including an MGB probe in the assay increases specificity compared to using conventional probes or non-probe-based assays (Kutyavin et al. 2000). MGB probes create stable duplexes with DNA targets, leading to higher melting temperatures and allowing for shorter probe lengths and reduced background fluorescence (Kumar et al. 1998; Kutyavin et al. 2000). The probe was designed to maximize mismatches in the last 2–5 bases to further enhance specificity (Kumar et al. 1998), with at least two mismatches in these bases for most non-target species (Figure 1). Subsequent *in silico* and qPCR testing confirmed specificity to *M. bella*.

The optimized assay demonstrated high efficiency and sensitivity, while the assay's low LoD and LoQ values demonstrated it can detect and quantify the target DNA when present at low levels. TaqMan Environmental Master Mix 2.0 was chosen for qPCR analysis because it is designed specifically for environmental samples, overcomes inhibition issues, and provides improved detection rates in comparison to other master mixes (Cao et al. 2012; Doi et al. 2015). Additionally, multiplexing with an endogenous positive control assay enabled additional quality assessment and helped distinguish potential false negatives caused by workflow issues or environmental inhibitors such as humic and fulvic acids (Eichmiller et al. 2016; Furlan and Gleeson 2017; Gibson et al. 2012; Jane et al. 2015).

Validating a qPCR assay and eDNA workflow is crucial to ensure its practical applicability and instil confidence in its efficacy (De Brauwer et al. 2022; Klymus et al. 2020). The *M. bella* assay was validated initially using eDNA samples taken from aquaria housing live animals, then using samples from the open ocean taken around a *M. bella* medusa. *Malo bella* eDNA was detected in all four water samples collected from aquaria. For each water sample, 6/6 technical replicates amplified, except in one instance, where only 5/6 replicates amplified. Given the high concentration of eDNA in the aquaria, it is considered that this could have been a false negative result. This was the only false negative eDNA detection observed (1/24 technical replicates, Table 3).

Malo bella eDNA was detected for all eight medusae in the ocean. However, detections were not consistent in all five replicate water samples taken around each medusa, with 12.5% of replicates failing to detect *M. bella* eDNA. *Malo bella* are active swimmers and were observed to travel at moderate speeds along the surface in a set direction. It was hypothesized that water samples with no or low amplification could have been collected 'upstream' from the jellyfish (i.e., ahead of its direction of travel), and therefore not within the plume of eDNA emanating from the medusa. The variability among these water samples may also be influenced by the clumping nature of eDNA (Furlan et al. 2016) and its sporadic distribution in marine systems (Barnes and Turner 2016). Additionally, variability in eDNA shedding rates among individuals, resulting from differences in biomass, metabolic activity, behavior, and environmental conditions, may explain the differences in detections between individual medusae (Harrison et al. 2019).

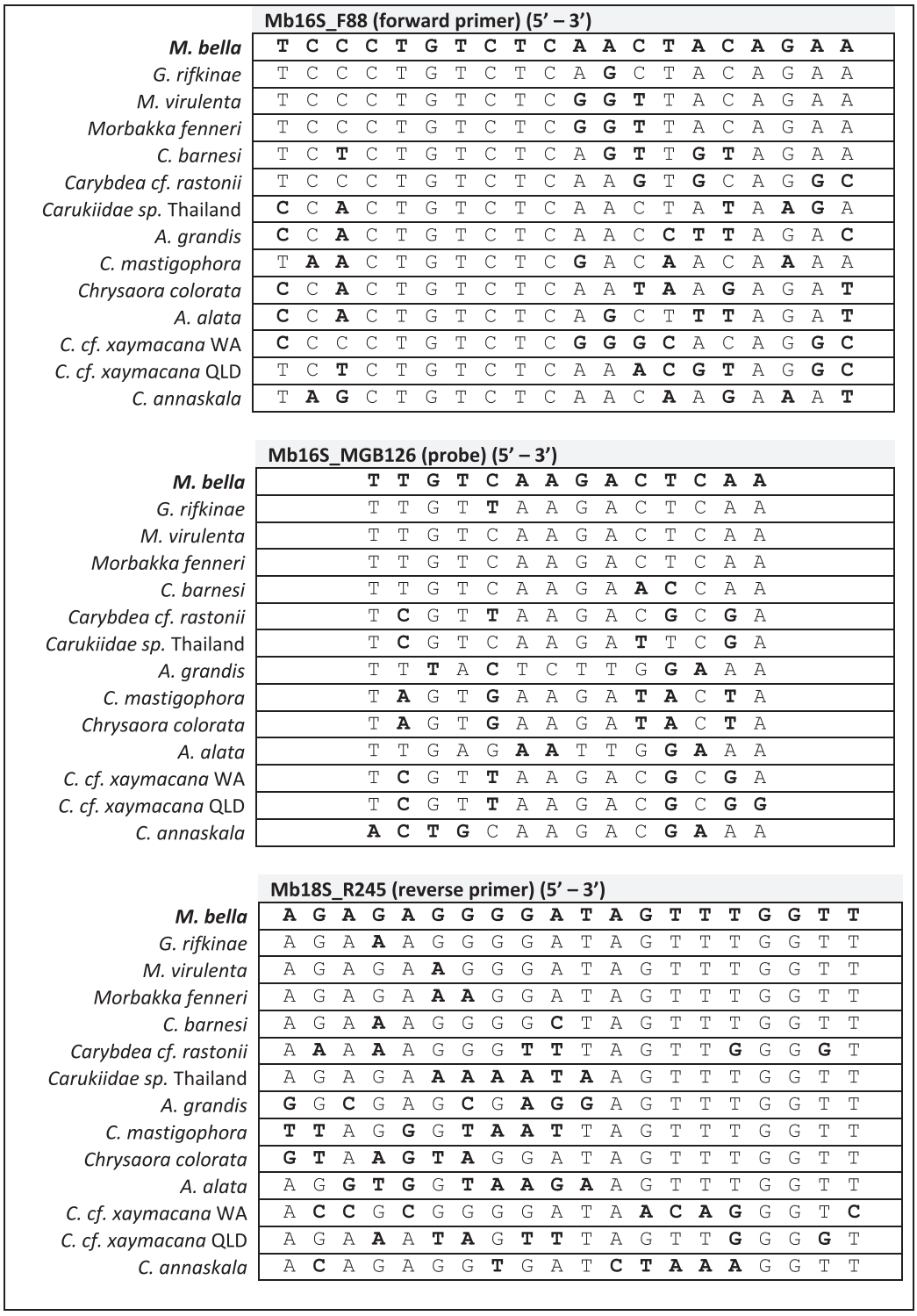

FIGURE 1 | Non-target species sequences where the assay binds in silico with fewer than seven base pair mismatches per oligo. Mismatches between the Malo bella assay and the non-target species sequences at the oligo binding sites are highlighted in bold. Sequences are ordered from fewest to most cumulative mismatches. Accession numbers: *Malo bella* (PP626684), *Gerongia rifkinae* (GQ849119), *Morbakka virulenta* (GQ849121), *Morbakka fenneri*, *Carukia barnesi* (GQ849097), *Carybdea cf. rastonii* (GQ849117), *Carukiidae* sp. Thailand (KT982721), Alatina grandis (KU707411), *Crambione mastigophora* (KY610602), *Chrysaora colorata* (MF141682), Alatina alata (KU707326), *Carybdea cf. xaymacana* Western Australia (WA) (PP889593), *Carybdea cf. xaymacana* Queensland (QLD) (OP877034), *Cyanea annaskala* (KY610738).

For studies of small, sporadically distributed species, where the target organism's eDNA may not be widespread, there is increased emphasis on replication in field sampling (Mauvisseau et al. 2019).

During field validation, while eDNA was detected around all eight medusae, some water samples had detections in just 1–2 technical replicates out of 6. Variation in technical replicates is not uncommon when eDNA concentrations are low

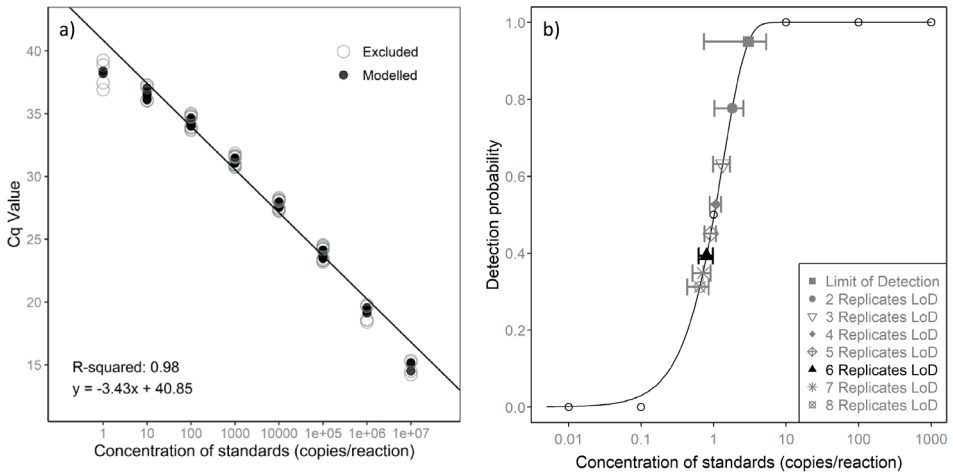

FIGURE 2 | *Malo bella* assay sensitivity: (a) An 8-point standard curve for a 10:1 serial dilution of sDNA, from a theoretical 1 to 10^7 copies/μL ($n = 10–24$ qPCR replicates per standard). Black dots represent modelled replicates while gray open circles indicate replicates falling outside the interquartile range, which were excluded from linear regression calculations. (b) Effective limit of detection (LOD) for each quantity of technical replicates, determined via a Weibull type II two-parameter function with an upper limit of one. Effective LODs ($n = 10–24$ qPCR replicates per standard) are plotted with 95% confidence intervals and open black circles represent the detection rates for each standard.

TABLE 3 | qPCR detections for assay validation samples (number of positive detections out of 6 qPCR replicates per sample, followed by the average number of 16S eDNA copies/μL ± SE).

		Controls			Replicate samples (positive qPCR detections out of 6 technical replicates) (mean 16S eDNA copies/μL ± SE)				
		Proc	**EB**	**Endo (C_t)**	**Sample A**	**Sample B**	**Sample C**	**Sample D**	**Sample E**
LAB	Aquaria	0/6	0/6	NA	5/6 (27,460 ± 4114)	6/6 (52,120 ± 3117)	6/6 (19,100 ± 766)	6/6 (27,340 ± 1508)	NA
FIELD	*Mb* 1	0/6	0/6	35.3 (± 3.3)	1/6 (23.6)	4/6 (19.2 ± 3.4)	3/6 (22.3 + 2.1)	2/6 (13.2 ± 1.4)	0/6
	Mb 2	0/6	0/6	35.6 (± 2.4)	0/6	3/6 (< 13)	2/6 (13.0 ± 0.7)	2/6 (27.8 + 1.2)	0/6
	Mb 3	0/6	0/6	21.6 (± 1.1)	0/6	5/6 (20.9)	2/6 (16.5)	1/6 (< 13)	1/6 (20.3)
	Mb 4	0/6	0/6	34.2 (± 0.6)	4/6 (22.4 ± 1.8)	2/6 (17.3 ± 1.9)	3/6 (17.7 ± 2.3)	0/6	1/6 (< 13)
	Mb 5	0/6	0/6	32.1 (± 1.3)	5/6 (< 13)	3/6 (13.1)	5/6 (< 13)	3/6 (< 13)	5/6 (< 13)
	Mb 6	0/6	0/6	28.2 (± 0.9)	5/6 (< 13)	4/6 (< 13)	3/6 (< 13)	4/6 (< 13)	3/6 (< 13)
	Mb 7	0/6	0/6	30.1 (± 2.3)	1/6 (< 13)	6/6 (31.6 ± 1.4)	3/6 (18.8 ± 2.7)	5/6 (< 13)	6/6 (< 13)
	Mb 8	0/6	0/6	29.7 (± 1.6)	3/6 (< 13)	6/6 (< 13)	4/6 (16.7 ± 2.3)	6/6 (18.2 ± 0.9)	4/6 (< 13)

Note: Results of the procedural and endogenous controls are also shown.
Abbreviations: C_t, Cycle threshold; EB, extraction blank; Endo, endogenous control; Mb, Malo bella; Proc, procedural.

(Ficetola et al. 2008), which is often the case for small and sporadically distributed species in open aquatic environments (Jerde et al. 2011). In contrast to larger or more abundant species, eDNA from species like *M. bella* likely does not saturate the water column when the species is present. Rather, trails of relatively low concentrations of eDNA may occur where medusae have traveled through a body of water. The dispersal and rapid degradation of this eDNA (Ely et al. 2021; Minamoto et al. 2017; Murakami et al. 2019) likely result in variable and sometimes very low eDNA concentrations, even when an animal is nearby, as observed in this study. When eDNA concentrations are low, stochastic effects can cause variability in amplification among technical replicates (Weusten and Herbergs 2012). In such studies, it is important to include measures to confirm the validity of detections, such as sufficient controls, a reversed sequence region for the standards,

best practice laboratory protocols, and Sanger sequencing to confirm amplification of the target species (De Brauwer et al. 2022).

Interpretation of eDNA results requires care, and approaches vary substantially across studies (Mathieu et al. 2020). Factors such as the threshold for a positive detection often depend on the specific context of the study, including the target species and environmental conditions (De Brauwer et al. 2022). Goldberg et al. (2016) suggest it is appropriate to weigh the strength of the evidence when it comes to setting detection thresholds and interpreting results, rather than using a universal approach. In this instance, sampling for a small, sporadically distributed, but highly venomous species that is difficult to detect visually, it is prudent to treat the lowest levels of detection (e.g., amplification in 1/6 technical replicates) as indicative of the possible presence of the species. However, such detections should be supported by sufficient biological replication, controls, and Sanger sequencing confirmation. This approach aligns with similar studies within the literature (e.g., Cooper et al. 2021; Morrissey et al. 2022).

Sampling eDNA in remote and hot environments poses challenges, particularly for rare or sporadically distributed species, where eDNA may be scarce. Selecting appropriate methods for collecting, preserving and extracting eDNA is vital to prevent degradation and maximize yield (Hinlo et al. 2017). The sampling methods employed in this study facilitate rapid filtration and preservation, minimizing degradation risk, and are both accessible and adaptable. Longmire's solution, which does not use ethanol and effectively preserves eDNA at ambient temperatures, including at over 40°C for up to 6 weeks, enables stable, long-term storage, and easy transportation of samples at room temperature (Edmunds and Burrows 2020; Renshaw et al. 2015; Spens et al. 2016; Wegleitner et al. 2015). This was ideal for studying *M. bella*, which inhabits the remote, hot central coast of Western Australia, where logistical challenges and high temperatures make DNA preservation challenging. Additionally, using the PPLPP precipitation-based method to extract eDNA from samples ensures higher DNA yield compared to other methods, a critical consideration for rare species (Cooper et al. 2021; Misutka et al. 2023; Renshaw et al. 2015).

The qPCR assay developed here enhances our capacity to investigate the ecology of *M. bella*. By enabling detection over multiple spatial and temporal scales and eliminating the need to locate or capture individuals, this tool reduces researcher risk and can be a cost-effective and efficient alternative to some traditional approaches (Evans et al. 2017; Jerde et al. 2011; Minamoto et al. 2017; Sigsgaard et al. 2015; Smart et al. 2016). While the results presented here demonstrate the assay's effectiveness in detecting *M. bella* in the immediate vicinity, broader ecological studies or risk assessments must consider the potential source and range of eDNA detections from *M. bella*. eDNA interacts with marine environments in complex ways, with variable shedding, degradation and dispersal rates (Barnes and Turner 2016), that are influenced by various environmental factors and hydrodynamic forces (Baetscher et al. 2024; Ely et al. 2021). This results in uneven eDNA distributions (Harrison et al. 2019), complicating the interpretation of eDNA results. However, eDNA degrades rapidly in the first 24 h (e.g., Ely et al. 2021; though see Collins et al. 2018), and is generally detected within 100 m of its source (O'Donnell et al. 2017; though see Baetscher et al. 2024). Based on these findings and on jellyfish-specific persistence rates (Bolte et al. 2021; Morrissey et al. 2022; Minamoto et al. 2017), it is considered that detections of *M. bella* utilizing this assay will likely reflect species presence nearby within the preceding 24 h.

This assay can be applied to address critical knowledge gaps, particularly the spatial and temporal distributions of this small, elusive, venomous species (Gershwin 2014). This information is critical for reducing envenomation risks in a prime tourism region of Australia, where encounters with dangerous jellyfish lead to frequent hospitalisations. This information would also enable the implementation of public safety measures, akin to those implemented during 'stinger season' in Queensland and the Northern Territory when encounters with dangerous stinging jellyfish peak (Fenner and Harrison 2000; Gershwin et al. 2010; Harrison et al. 2004; Kingsford et al. 2012).

This assay also holds promise for detecting the elusive benthic polyp stage of the jellyfish, a crucial step in understanding population dynamics and mitigating jellyfish sting risks. Cubozoans have a polymorphic life history (Kingsford and Mooney 2014), and locating the tiny, benthic, polypoid stage in situ has been challenging (Bolte et al. 2021; Van Walraven et al. 2016); cubozoan polyps have only been found twice (Cutress and Studebaker 1973; Hartwick 1991). Locating the habitat of *C. fleckeri* polyps involved extensive laboratory experiments on polyps, followed by 9 years of manual in situ searches (Hartwick 1991). In contrast, Morrissey et al. (2024a, 2024b) demonstrated the efficacy of eDNA methods in identifying *C. fleckeri* polyp habitat with comparatively little effort by detecting its eDNA in samples taken when medusae were absent. Finding polyps of *M. bella* is essential for determining the source of medusae and for understanding the population dynamics of the species, and the potential environmental conditions that trigger the metamorphosis of polyps into stinging adult medusae.

Future research should explore integrating passive or automated samplers or field-friendly methods and developing real-time detection assays to enhance monitoring capabilities for *M. bella*. Passive samplers offer continuous eDNA monitoring, providing a low-maintenance, high-frequency sampling approach (Bessey et al. 2021). Automated and robotic samplers are rapidly evolving technologies (Hendricks et al. 2022; Magalhães et al. 2020) that could be paired with this assay for efficient eDNA detection of *M. bella*. Adopting field-friendly eDNA methods (e.g., Lewis Ames et al. 2021) or developing real-time detection tools such as lateral flow tests (e.g., Doyle and Uthicke 2021) would enable instantaneous testing for *M. bella* presence, benefiting on-ground managers and tourism operators who require swift information about potential jellyfish threats. Moreover, by swabbing the sting site on victims the qPCR assay could determine whether *M. bella* is the causative species in instances of Irukandji envenomations (e.g., Sathirapongsasuti et al. 2020).

In summary, the novel qPCR assay developed for *M. bella* represents a significant advancement in the monitoring capabilities for this Irukandji jellyfish. By enabling precise detection and monitoring of this elusive and dangerous species, the assay offers a complementary alternative to traditional sampling methods and will aid in addressing critical knowledge gaps and may

help protect swimmers in an iconic Australian tourism destination. Additionally, this assay paves the way for broader applications, including real-time monitoring, which will further enhance our ability to manage and mitigate the risks posed by hazardous jellyfish.

Author Contributions

Conceptualisation and study design: J.K.S., K.A.P., D.R.J., M.J.K.; data – acquisition: J.K.S., K.A.P., M.J.K., S.J.M.; data – analysis: J.K.S.; writing – drafting: J.K.S.; writing – review and editing: J.K.S., K.A.P., M.J.K., D.R.J., and S.J.M.

Acknowledgments

This project was funded by the Minderoo Foundation with additional in-kind support from the Minderoo Foundation Exmouth Research Laboratory (MERL) and its staff. Jessica K. Strickland was supported by a PhD scholarship and funding from Griffith University. We thank James Cook University and Julie Goldsbury for laboratory support and volunteers for assistance with collecting specimens and field samples. Open access publishing facilitated by Griffith University, as part of the Wiley - Griffith University agreement via the Council of Australian University Librarians.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are archived on Griffith University servers and available from the corresponding author upon reasonable request. New sequences are published on NCBI GenBank, under accession numbers provided in Table S1.

References

Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman. 1990. "Basic Local Alignment Search Tool." *Journal of Molecular Biology* 215, no. 3: 403–410. https://doi.org/10.1016/S0022-2836(05)80360-2.

Azama, Y., M. Teruya, Y. Kuba, et al. 2023. "Real-Time PCR Assay for Detection of Environmental DNA From *Chironex yamaguchii*." *Medical Entomology and Zoology* 74, no. 1: 13–20.

Baetscher, D. S., M. R. Pochardt, P. D. Barry, and W. A. Larson. 2024. "Tide Impacts the Dispersion of eDNA From Nearshore Net Pens in a Dynamic High-Latitude Marine Environment." *Environmental DNA* 6, no. 2: e533. https://doi.org/10.1002/edn3.533.

Barnes, M. A., and C. R. Turner. 2016. "The Ecology of Environmental DNA and Implications for Conservation Genetics." *Conservation Genetics* 17, no. 1: 1–17.

Bayha, K. M., and M. N. Dawson. 2010. "New Family of Allomorphic Jellyfishes, Drymonematidae (Scyphozoa, Discomedusae), Emphasizes Evolution in the Functional Morphology and Trophic Ecology of Gelatinous Zooplankton." *Biological Bulletin* 219, no. 3: 249–267. https://doi.org/10.1086/BBLv219n3p249.

Beng, K. C., and R. T. Corlett. 2020. "Applications of Environmental DNA (eDNA) in Ecology and Conservation: Opportunities, Challenges and Prospects." *Biodiversity and Conservation* 29: 2089–2121. https://doi.org/10.1007/s10531-020-01980-0.

Bentlage, B., P. Cartwright, A. A. Yanagihara, C. Lewis, G. S. Richards, and A. G. Collins. 2010. "Evolution of Box Jellyfish (Cnidaria: Cubozoa), a Group of Highly Toxic Invertebrates." *Proceedings of the Royal Society B: Biological Sciences* 277, no. 1680: 493–501. https://doi.org/10.1098/rspb.2009.1707.

Bessey, C., S. N. Jarman, T. J. Simpson, et al. 2021. "Passive eDNA Collection Enhances Aquatic Biodiversity Analysis." *Communications Biology* 4, no. 1: 236. https://doi.org/10.1038/s42003-021-01760-8.

Bolte, B., J. Goldsbury, R. Huerlimann, D. Jerry, and M. J. Kingsford. 2021. "Validation of eDNA as a Viable Method of Detection for Dangerous Cubozoan Jellyfish." *Environmental DNA* 3, no. 4: 769–779. https://doi.org/10.1002/edn3.181.

Budd, A., M. Cooper, A. Le Port, et al. 2021. "First Detection of Critically Endangered Scalloped Hammerhead Sharks (*Sphyrna lewini*) in Guam, Micronesia, in Five Decades Using Environmental DNA." *Ecological Indicators* 127: 107649. https://doi.org/10.1016/j.ecolind.2021.107649.

Cao, Y., J. F. Griffith, S. Dorevitch, and S. B. Weisberg. 2012. "Effectiveness of qPCR Permutations, Internal Controls and Dilution as Means for Minimizing the Impact of Inhibition While Measuring Enterococcus in Environmental Waters." *Journal of Applied Microbiology* 113, no. 1: 66–75. https://doi.org/10.1111/j.1365-2672.2012.05305.x.

Collins, R. A., O. S. Wangensteen, E. J. O'Gorman, S. Mariani, D. W. Sims, and M. J. Genner. 2018. "Persistence of Environmental DNA in Marine Systems." *Communications Biology* 1, no. 1: 185. https://doi.org/10.1038/s42003-018-0192-6.

Cooper, M. K., R. Huerlimann, R. C. Edmunds, et al. 2021. "Improved Detection Sensitivity Using an Optimal eDNA Preservation and Extraction Workflow and Its Application to Threatened Sawfishes." *Aquatic Conservation: Marine and Freshwater Ecosystems* 31, no. 8: 2131–2148. https://doi.org/10.1002/aqc.3591.

Crowley-Cyr, L. 2020. "Workplace Safety, Deadly Jellyfish and Tourists: A Novel Approach to an Emergent Problem." *University of Queensland Law Journal* 39, no. 2: 549–567. https://doi.org/10.3316/informit.626999501464882.

Cutress, C., and J. P. Studebaker. 1973. "Development of the Cubomedusae *Carybdea marsupialis*." *Proceedings of the Association of Island Marine Laboratories of the Caribbean* 9, no. 1: 25.

De Brauwer, M., A. Chariton, L. J. Clark, et al. 2022. *Environmental DNA Protocol Development Guide for Biomonitoring*. National eDNA Reference Centre 10.25607/OBP-1886.

Department of Biodiversity Conservation and Attractions. 2023. "Ningaloo Marine Park Visitor Statistics (Unpublished Data)."

Doi, H., K. Uchii, T. Takahara, S. Matsuhashi, H. Yamanaka, and T. Minamoto. 2015. "Use of Droplet Digital PCR for Estimation of Fish Abundance and Biomass in Environmental DNA Surveys." *PLoS One* 10, no. 3: e0122763. https://doi.org/10.1371/journal.pone.0122763.

Doyle, J., and S. Uthicke. 2021. "Sensitive Environmental DNA Detection via Lateral Flow Assay (Dipstick)—A Case Study on Corallivorous Crown-Of-Thorns Sea Star (*Acanthaster cf. solaris*) Detection." *Environmental DNA* 3, no. 2: 323–342. https://doi.org/10.1002/edn3.123.

Edmunds, R. C., and D. Burrows. 2020. "Got Glycogen?: Development and Multispecies Validation of the Novel Preserve, Precipitate, Lyse, Precipitate, Purify (PPLPP) Workflow for Environmental DNA Extraction From Longmire's Preserved Water Samples." *Journal of Biomolecular Techniques* 31, no. 4: 125–150. https://doi.org/10.7171/jbt.20-3104-003.

Eichmiller, J. J., L. M. Miller, and P. W. Sorensen. 2016. "Optimizing Techniques to Capture and Extract Environmental DNA for Detection and Quantification of Fish." *Molecular Ecology Resources* 16, no. 1: 56–68. https://doi.org/10.1111/1755-0998.12421.

Ely, T., P. H. Barber, L. Man, and Z. Gold. 2021. "Short-Lived Detection of an Introduced Vertebrate eDNA Signal in a Nearshore Rocky Reef Environment." *PLoS One* 16, no. 6: e0245314. https://doi.org/10.1371/journal.pone.0245314.

Ender, A., and B. Schierwater. 2003. "Placozoa Are Not Derived Cnidarians: Evidence From Molecular Morphology." *Molecular Biology and Evolution* 20, no. 1: 130–134. https://doi.org/10.1093/molbev/msg018.

Evans, N. T., P. D. Shirey, J. G. Wieringa, A. R. Mahon, and G. A. Lamberti. 2017. "Comparative Cost and Effort of Fish Distribution Detection via Environmental DNA Analysis and Electrofishing." *Fisheries* 42, no. 2: 90–99. https://doi.org/10.1080/03632415.2017.1276329.

Fenner, P. J., and J. C. Hadok. 2002. "Fatal Envenomation by Jellyfish Causing Irukandji Syndrome." *Medical Journal of Australia* 177, no. 7: 362–363. https://doi.org/10.5694/j.1326-5377.2002.tb04838.x.

Fenner, P. J., and S. L. Harrison. 2000. "Irukandji and Chironex Fleckeri Jellyfish Envenomation in Tropical Australia." *Wilderness & Environmental Medicine* 11, no. 4: 233–240. https://doi.org/10.1580/1080-6032(2000)011[0233:IACFJE]2.3.CO;2.

Ficetola, G. F., C. Miaud, F. o. Pompanon, and P. Taberlet. 2008. "Species Detection Using Environmental DNA From Water Samples." *Biology Letters* 4, no. 4: 423–425. https://doi.org/10.1098/rsbl.2008.0118.

Flecker, H. 1952. "Irukandji Sting to North Queensland Bathers Without Production of Weals but With Severe General Symptoms." *Medical Journal of Australia* 2, no. 3: 89–91. https://doi.org/10.5694/j.1326-5377.1952.tb100081.x.

Furlan, E. M., and D. Gleeson. 2017. "Improving Reliability in Environmental DNA Detection Surveys Through Enhanced Quality Control." *Marine and Freshwater Research* 68, no. 2: 388–395. https://doi.org/10.1071/MF15349.

Furlan, E. M., D. Gleeson, C. M. Hardy, and R. P. Duncan. 2016. "A Framework for Estimating the Sensitivity of eDNA Surveys." *Molecular Ecology Resources* 16, no. 3: 641–654. https://doi.org/10.1111/1755-0998.12483.

Gargan, L. M., P. R. Brooks, S. R. Vye, et al. 2022. "The Use of Environmental DNA Metabarcoding and Quantitative PCR for Molecular Detection of Marine Invasive Non-Native Species Associated With Artificial Structures." *Biological Invasions* 24, no. 3: 635–648. https://doi.org/10.1007/s10530-021-02672-8.

Gaynor, J. J., P. A. Bologna, D. J. Restaino, and C. L. Barry. 2017. "qPCR Detection of Early Life History Stage *Chrysaora quinquecirrha* (Sea Nettles) in Barnegat Bay, New Jersey." *Journal of Coastal Research* 78, no. 10,078: 184–192. https://doi.org/10.2112/SI78-014.1.

Gershwin, L.-A. 2014. "Two New Species of Box Jellies (Cnidaria: Cubozoa: Carybdeida) From the Central Coast of Western Australia, Both Presumed to Cause Irukandji Syndrome." *Records of the Western Australian Museum* 29, no. 1: 19. https://doi.org/10.18195/issn.0312-3162.29(1)0.2014.010-019.

Gershwin, L.-A., M. De Nardi, K. D. Winkel, and P. J. Fenner. 2010. "Marine Stingers: Review of an Under-Recognized Global Coastal Management Issue." *Coastal Management* 38, no. 1: 22–41. https://doi.org/10.1080/08920750903345031.

Gershwin, L.-A., and P. Hannay. 2014. "An Anomalous Cluster of Irukandji Jelly Stings (Cnidaria: Cubozoa: Carybdeida) at Ningaloo Reef." *Records of the Western Australian Museum* 29: 78–81. https://doi.org/10.18195/issn.0312-3162.29(1)0.2014.078-081.

Gershwin, L.-A., A. J. Richardson, K. D. Winkel, et al. 2013. "Biology and Ecology of Irukandji Jellyfish (Cnidaria: Cubozoa)." *Advances in Marine Biology* 66: 1–85. https://doi.org/10.1016/B978-0-12-408096-6.00001-8.

Gibson, K., K. Schwab, S. Spencer, and M. Borchardt. 2012. "Measuring and Mitigating Inhibition During Quantitative Real Time PCR Analysis of Viral Nucleic Acid Extracts From Large-Volume Environmental Water Samples." *Water Research* 46, no. 13: 4281–4291. https://doi.org/10.1016/j.watres.2012.04.030.

Goldberg, C. S., C. R. Turner, K. Deiner, et al. 2016. "Critical Considerations for the Application of Environmental DNA Methods to Detect Aquatic Species." *Methods in Ecology and Evolution* 7, no. 11: 1299–1307.

Harper, L. R., L. Lawson Handley, C. Hahn, et al. 2018. "Needle in a Haystack? A Comparison of eDNA Metabarcoding and Targeted qPCR for Detection of the Great Crested Newt (*Triturus cristatus*)." *Ecology and Evolution* 8, no. 12: 6330–6341. https://doi.org/10.1002/ece3.4013.

Harrison, J. B., J. M. Sunday, and S. M. Rogers. 2019. "Predicting the Fate of eDNA in the Environment and Implications for Studying Biodiversity." *Proceedings of the Royal Society B* 286, no. 1915: 191409.

Harrison, S. L., P. A. Leggat, P. J. Fenner, D. N. Durrheim, and A. L. Swinbourne. 2004. "Reported Knowledge, Perceptions, and Behavior of Tourists and North Queensland Residents at Risk of Contact With Jellyfish That Cause the "Irukandji Syndrome"." *Wilderness & Environmental Medicine* 15, no. 1: 4–10. https://doi.org/10.1580/1080-6032(2004)015[0004:RKPABO]2.0.CO;2.

Hartwick, R. F. 1991. "Distributional Ecology and Behaviour of the Early Life Stages of the Box-Jellyfish Chironex Fleckeri." *Hydrobiologia* 216, no. 1: 181–188. https://doi.org/10.1007/bf00026460.

Hendricks, A., C. Mackie, E. Luy, et al. 2022. "A Miniaturized and Automated eDNA Sampler: Application to a Marine Environment." OCEANS 2022, Hampton Roads, Virginia, USA. https://doi.org/10.1109/OCEANS47191.2022.9977218.

Hinlo, R., D. Gleeson, M. Lintermans, and E. Furlan. 2017. "Methods to Maximise Recovery of Environmental DNA From Water Samples." *PLoS One* 12, no. 6: e0179251. https://doi.org/10.1371/journal.pone.0179251.

Hoang, D. T., O. Chernomor, A. von Haeseler, B. Q. Minh, and L. S. Vinh. 2017. "UFBoot2: Improving the Ultrafast Bootstrap Approximation." *Molecular Biology and Evolution* 35, no. 2: 518–522. https://doi.org/10.1093/molbev/msx281.

Jane, S. F., T. M. Wilcox, K. S. McKelvey, et al. 2015. "Distance, Flow and PCR Inhibition: eDNA Dynamics in Two Headwater Streams." *Molecular Ecology Resources* 15, no. 1: 216–227. https://doi.org/10.1111/1755-0998.12285.

Jerde, C. L., A. R. Mahon, W. L. Chadderton, and D. M. Lodge. 2011. "Sight-Unseen Detection of Rare Aquatic Species Using Environmental DNA." *Conservation Letters* 4, no. 2: 150–157. https://doi.org/10.1111/j.1755-263X.2010.00158.x.

Kalyaanamoorthy, S., B. Q. Minh, T. K. F. Wong, A. von Haeseler, and L. S. Jermin. 2017. "ModelFinder: Fast Model Selection for Accurate Phylogenetic Estimates." *Nature Methods* 14: 587–589. https://doi.org/10.1038/nmeth.4285.

Keesing, J. K., P. Barnes, B. Ingram, L.-A. Gershwin, D. Liu, and D. Slawinski. 2020. "Sightings, Strandings and Irukandji Syndrome Caused by Envenomations of the Large, Rarely Observed Jellyfish; *Keesingia gigas* Gershwin, 2014 (Cnidaria: Cubozoa: Carybdeida: Alatinidae) in North-Western Australia." *Plankton and Benthos Research* 15, no. 2: 156–167. https://doi.org/10.3800/pbr.15.156.

Kingsford, M. J., S. Becken, C. S. Bordehore, V. N. Fuentes, K. A. Pitt, and A. A. Yangihara. 2017. "Empowering Stakeholders to Manage Stinging Jellyfish: A Perspective." *Coastal Management* 46, no. 1: 1–18. https://doi.org/10.1080/08920753.2018.1405326.

Kingsford, M. J., and C. J. Mooney. 2014. "The Ecology of Box Jellyfishes (Cubozoa)." In *Jellyfish Blooms*, 267–302. Springer Netherlands. https://doi.org/10.1007/978-94-007-7015-7_12.

Kingsford, M. J., J. E. Seymour, and M. D. O'Callaghan. 2012. "Abundance Patterns of Cubozoans on and Near the Great Barrier Reef." *Hydrobiologia* 690, no. 1: 257–268. https://doi.org/10.1007/s10750-012-1041-0.

Klymus, K. E., C. M. Merkes, M. J. Allison, et al. 2019. "Reporting the Limits of Detection and Quantification for Environmental DNA Assays." *Environmental DNA* 2, no. 3: 271–282. https://doi.org/10.1002/edn3.29.

Klymus, K. E., D. V. R. Ramos, N. L. Thompson, and C. A. Richter. 2020. "Development and Testing of Species-Specific Quantitative PCR Assays for Environmental DNA Applications." *Journal of Visualized Experiments* 165: e61825. https://doi.org/10.3791/61825.

Kumar, S., M. Foti, J. West, et al. 1998. "Solution Structure of a Highly Stable DNA Duplex Conjugated to a Minor Groove Binder." *Nucleic Acids Research* 26, no. 3: 831–838. https://doi.org/10.1093/nar/26.3.831.

Kutyavin, I. V., I. A. Afonina, A. Mills, et al. 2000. "3′-Minor Groove Binder-DNA Probes Increase Sequence Specificity at PCR Extension Temperatures." *Nucleic Acids Research* 28, no. 2: 655–661. https://doi.org/10.1093/nar/28.2.655.

Lewis Ames, C., A. H. Ohdera, S. M. Colston, et al. 2021. "Fieldable Environmental DNA Sequencing to Assess Jellyfish Biodiversity in Nearshore Waters of the Florida Keys, United States." *Frontiers in Marine Science* 8: 640527. https://doi.org/10.3389/fmars.2021.640527.

Macrokanis, C. J., N. L. Hall, and J. K. Mein. 2004. "Irukandji Syndrome in Northern Western Australia: An Emerging Health Problem." *Medical Journal of Australia* 181, no. 11–12: 699–702. https://doi.org/10.5694/j.1326-5377.2004.tb06527.x.

Madin, E. M., N. C. Ban, Z. A. Doubleday, T. H. Holmes, G. T. Pecl, and F. Smith. 2012. "Socio-Economic and Management Implications of Range-Shifting Species in Marine Systems." *Global Environmental Change* 22, no. 1: 137–146. https://doi.org/10.1016/j.gloenvcha.2011.10.008.

Magalhães, C., A. Martins, and A. Dos Santos. 2020. "New Approaches to Study Jellyfish." In *Zooplankton Ecology*, 227–251. CRC Press.

Mathieu, C., S. M. Hermans, G. Lear, T. R. Buckley, K. C. Lee, and H. L. Buckley. 2020. "A Systematic Review of Sources of Variability and Uncertainty in eDNA Data for Environmental Monitoring." *Frontiers in Ecology and Evolution* 8: 135. https://doi.org/10.3389/fevo.2020.00135.

Mauvisseau, Q., A. Burian, C. Gibson, R. Brys, A. Ramsey, and M. Sweet. 2019. "Influence of Accuracy, Repeatability and Detection Probability in the Reliability of Species-Specific eDNA Based Approaches." *Scientific Reports* 9, no. 1: 580. https://doi.org/10.1038/s41598-018-37001-y.

McCarthy, A., H. Rajabi, B. McClenaghan, et al. 2023. "Comparative Analysis of Fish Environmental DNA Reveals Higher Sensitivity Achieved Through Targeted Sequence-Based Metabarcoding." *Molecular Ecology Resources* 23, no. 3: 581–591. https://doi.org/10.1111/1755-0998.13732.

Merkes, C., K. Klymus, M. Allison, et al. 2019. "Reporting the Limits of Detection (LOD) and Quantification (LOQ) for Environmental DNA Assays: Data." U.S. Geological Survey Data Release. https://doi.org/10.5066/P9AKHU1R.

Minamoto, T., M. Fukuda, K. R. Katsuhara, et al. 2017. "Environmental DNA Reflects Spatial and Temporal Jellyfish Distribution." *PLoS One* 12, no. 2: e0173073. https://doi.org/10.1371/journal.pone.0173073.

Minh, B. Q., H. A. Schmidt, O. Chernomor, et al. 2020. "IQ-TREE 2: New Models and Efficient Methods for Phylogenetic Inference in the Genomic Era." *Molecular Biology and Evolution* 37, no. 5: 1530–1534. https://doi.org/10.1093/molbev/msaa015.

Misutka, M. D., C. N. Glover, M. Brush, G. G. Goss, and H. D. Veilleux. 2023. "A Validated and Optimized Environmental DNA and RNA Assay to Detect Arctic Grayling (*Thymallus arcticus*)." *Environmental DNA* 5, no. 6: 1378–1392. https://doi.org/10.1002/edn3.460.

Morrissey, S. J., D. R. Jerry, and M. J. Kingsford. 2022. "Genetic Detection and a Method to Study the Ecology of Deadly Cubozoan Jellyfish." *Diversity* 14, no. 12: 1139. https://doi.org/10.3390/d14121139.

Morrissey, S. J., D. R. Jerry, and M. J. Kingsford. 2024a. "Use of eDNA to Determine Source Locations of Deadly Jellyfish (Cubozoa) in an Open Coastal System." *Coasts* 4, no. 1: 198–212. https://doi.org/10.3390/coasts4010011.

Morrissey, S. J., D. R. Jerry, and M. J. Kingsford. 2024b. "Use of eDNA to Test Hypotheses on the Ecology of *Chironex fleckeri* (Cubozoa)." *Marine Ecology Progress Series* 728: 25–41. https://doi.org/10.3354/meps14507.

Murakami, H., S. Yoon, A. Kasai, et al. 2019. "Dispersion and Degradation of Environmental DNA From Caged Fish in a Marine Environment." *Fisheries Science* 85, no. 2: 327–337. https://doi.org/10.1007/s12562-018-1282-6.

O'Donnell, J. L., R. P. Kelly, A. O. Shelton, J. F. Samhouri, N. C. Lowell, and G. D. Williams. 2017. "Spatial Distribution of Environmental DNA in a Nearshore Marine Habitat." *PeerJ* 5: e3044.

Owczarzy, R., A. V. Tataurov, Y. Wu, et al. 2008. "IDT SciTools: A Suite for Analysis and Design of Nucleic Acid Oligomers." *Nucleic Acids Research* 36, no. suppl_2: W163–W169. https://doi.org/10.1093/nar/gkn198.

Peng, S., L. Wang, Y. Ma, et al. 2023. "Application of Environmental DNA Metabarcoding and Quantitative PCR to Detect Blooming Jellyfish in a Temperate Bay of Northern China." *Ecology and Evolution* 13, no. 11: e10669. https://doi.org/10.1002/ece3.10669.

Perera, A. 2017. "Stinger Influx Sparks DPaW Investigation." Pilbara News. https://www.pilbaranews.com.au/news/pilbara-news/stinger-influx-sparks-dpaw-investigation-ng-b88442929z.

Purcell, J. E. 2012. "Jellyfish and Ctenophore Blooms Coincide With Human Proliferations and Environmental Perturbations." *Annual Review of Marine Science* 4, no. 1: 209–235. https://doi.org/10.1146/annurev-marine-120709-142751.

Purcell, J. E., S.-I. Uye, and W.-T. Lo. 2007. "Anthropogenic Causes of Jellyfish Blooms and Their Direct Consequences for Humans: A Review." *Marine Ecology Progress Series* 350: 153–174. https://doi.org/10.3354/meps07093.

R Core Team. 2022. "R: A Language and Environment for Statistical Computing." R Foundation for Statistical Computing. https://www.R-project.org/.

Rees, H. C., B. C. Maddison, D. J. Middleditch, J. R. Patmore, K. C. Gough, and E. Crispo. 2014. "The Detection of Aquatic Animal Species Using Environmental DNA—A Review of eDNA as a Survey Tool in Ecology." *Journal of Applied Ecology* 51, no. 5: 1450–1459. https://doi.org/10.1111/1365-2664.12306.

Renshaw, M. A., B. P. Olds, C. L. Jerde, M. M. McVeigh, and D. M. Lodge. 2015. "The Room Temperature Preservation of Filtered Environmental DNA Samples and Assimilation Into a Phenol-Chloroform-Isoamyl Alcohol DNA Extraction." *Molecular Ecology Resources* 15, no. 1: 168–176. https://doi.org/10.1111/1755-0998.12281.

Rowley, O. C. 2021. "Rethinking Biological Tools for Logistically Difficult Species: A Case Study—The Irukandji (*Carukia barnesi*) and Box Jellyfish (*Chironex fleckeri*)." PhD diss., James Cook University. https://researchonline.jcu.edu.au/79679/.

RStudio Team. 2023. "RStudio: Integrated Development for R." RStudio, PBC. http://www.rstudio.com/.

Sathirapongsasuti, N., K. Khonchom, T. Poonsawat, et al. 2020. "Rapid and Accurate Species-Specific PCR for the Identification of Lethal Chironex Box Jellyfish in Thailand." *International Journal of Environmental Research and Public Health* 18, no. 1: 219. https://doi.org/10.3390/ijerph18010219.

Sayers, E. W., E. E. Bolton, J. R. Brister, et al. 2022. "Database Resources of the National Center for Biotechnology Information." *Nucleic Acids Research* 50, no. D1: d20–d26. https://doi.org/10.1093/nar/gkab1112.

Sigsgaard, E. E., H. Carl, P. R. Møller, and P. F. Thomsen. 2015. "Monitoring the Near-Extinct European Weather Loach in Denmark Based on Environmental DNA From Water Samples." *Biological Conservation* 183: 46–52. https://doi.org/10.1016/j.biocon.2014.11.023.

Smart, A. S., A. R. Weeks, A. R. van Rooyen, A. Moore, M. A. McCarthy, and R. Tingley. 2016. "Assessing the Cost-Efficiency of Environmental DNA Sampling." *Methods in Ecology and Evolution* 7, no. 11: 1291–1298. https://doi.org/10.1111/2041-210X.12598.

Spens, J., A. R. Evans, D. Halfmaerten, et al. 2016. "Comparison of Capture and Storage Methods for Aqueous Macrobial eDNA Using an Optimized Extraction Protocol: Advantage of Enclosed Filter." *Methods in Ecology and Evolution* 8, no. 5: 635–645. https://doi.org/10.1111/2041-210x.12683.

Stothard, P. 2000. "The Sequence Manipulation Suite: JavaScript Programs for Analyzing and Formatting Protein and DNA Sequences." *BioTechniques* 28, no. 6: 1102–1104. https://doi.org/10.2144/00286ir01.

Takasu, H., H. Inomata, K. Uchino, et al. 2019. "Spatio-Temporal Distribution of Environmental DNA Derived From Japanese Sea Nettle Jellyfish Chrysaora Pacifica in Omura Bay, Kyushu, Japan." *Plankton and Benthos Research* 14, no. 4: 320–323. https://doi.org/10.3800/pbr.14.320.

Thalinger, B., K. Deiner, L. R. Harper, et al. 2021. "A Validation Scale to Determine the Readiness of Environmental DNA Assays for Routine Species Monitoring." *Environmental DNA* 3, no. 4: 823–836. https://doi.org/10.1002/edn3.189.

Thomsen, P. F., and E. Willerslev. 2015. "Environmental DNA—An Emerging Tool in Conservation for Monitoring Past and Present Biodiversity." *Biological Conservation* 183: 4–18. https://doi.org/10.1016/j.biocon.2014.11.019.

Tibballs, J., R. Li, H. A. Tibballs, L.-A. Gershwin, and K. D. Winkel. 2012. "Australian Carybdeid Jellyfish Causing Irukandji Syndrome." *Toxicon* 59, no. 6: 617–625. https://doi.org/10.1016/j.toxicon.2012.01.006.

Trujillo-González, A., R. C. Edmunds, J. A. Becker, and K. S. Hutson. 2019. "Parasite Detection in the Ornamental Fish Trade Using Environmental DNA." *Scientific Reports* 9, no. 1: 5173. https://doi.org/10.1038/s41598-019-41517-2.

Van Walraven, L., F. Driessen, J. Van Bleijswijk, et al. 2016. "Where Are the Polyps? Molecular Identification, Distribution and Population Differentiation of Aurelia Aurita Jellyfish Polyps in the Southern North Sea Area." *Marine Biology* 163, no. 8: 4. https://doi.org/10.1007/s00227-016-2945-4.

Wang, J., T. Mi, Z. Yu, et al. 2021. "Species-Specific Detection and Quantification of Scyphomedusae in Jiaozhou Bay, China, Using a Quantitative Real-Time PCR Assay." *Journal of Oceanology and Limnology* 39: 1360–1372. https://doi.org/10.1007/s00343-020-0160-0.

Wegleitner, B. J., C. L. Jerde, A. Tucker, W. L. Chadderton, and A. R. Mahon. 2015. "Long Duration, Room Temperature Preservation of Filtered eDNA Samples." *Conservation Genetics Resources* 7: 789–791.

Weusten, J., and J. Herbergs. 2012. "A Stochastic Model of the Processes in PCR Based Amplification of STR DNA in Forensic Applications." *Forensic Science International: Genetics* 6, no. 1: 17–25. https://doi.org/10.1016/j.fsigen.2011.01.003.

Wilcox, T. M., K. S. McKelvey, M. K. Young, et al. 2013. "Robust Detection of Rare Species Using Environmental DNA: The Importance of Primer Specificity." *PLoS One* 8, no. 3: e59520. https://doi.org/10.1371/journal.pone.0059520.

Williams, K. E., K. P. Huyvaert, and A. J. Piaggio. 2016. "No Filters, No Fridges: A Method for Preservation of Water Samples for eDNA Analysis." *BMC Research Notes* 9: 298. https://doi.org/10.1186/s13104-016-2104-5.

Ye, J., G. Coulouris, I. Zaretskaya, I. Cutcutache, S. Rozen, and T. L. Madden. 2012. "Primer-BLAST: A Tool to Design Target-Specific Primers for Polymerase Chain Reaction." *BMC Bioinformatics* 13, no. 1: 134. https://doi.org/10.1186/1471-2105-13-134.

Supporting Information

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