Use of eDNA to Detect and Study Deadly Box Jellyfish

Thesis submitted by
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Generative AI technology was not used in the preparation of any part of this thesis.

General Abstract

Cubozoans, commonly referred to as box jellyfish, are a class of marine taxa which contain members described as the most venomous organisms on the planet. They are found in warm tropical waters around the globe and have multiple unique attributes which have garnered them significant research interest. These attributes include their visual capabilities, life histories, swimming abilities, and, most notably, their potent venom. Stings from dangerous members can result in severe reactions, hospitalisation, and potentially death. The ability to manage this risk of envenomation is a challenge faced by stakeholders and decision makers globally. Despite this, cubozoan jellyfish are largely understudied and ecological data on the majority of species are limited. To overcome the threats and challenges which these taxa pose, an increased understanding of their ecology is needed. Significant knowledge gaps exist surrounding cubozoan jellyfish life histories and population structures. These gaps stem from the challenges associated with their detection, a result of their elusive nature, transparency, and spatial and temporal variability in abundance. Moreover, these gaps persist due to limitations of current detection and sampling techniques in addressing the logistical difficulties of studying these aspects of cubozoan ecology.

The broad objective of this thesis was to explore the use of eDNA as an innovative technique for detecting and studying deadly cubozoan jellyfish. The development of a robust assay (Chapter 2) was a prerequisite for the application of the technique for studying the ecology of medusae and polyps in open and closed marine environments (Chapters 3 & 4). Finally, estimates of spatiotemporal detection limits were determined by modelling the dispersal of eDNA (Chapter 5).

The development and optimisation of an eDNA assay for *Chironex fleckeri* revealed the technique's high utility for the detection of the potentially deadly jellyfish (Chapter 2). The assay was designed to target a region of the mitochondrial 16S rRNA gene which contained both interspecific and intraspecific variability. This allowed for the assay to be highly specific, ensuring it could distinguish *C. fleckeri* from closely related species, while ensuring its ability to detect the species across its biogeographic distribution. Field trials demonstrated this, with *C. fleckeri* medusae being detected at locations across the species range, where they were known and confirmed to be present. Multiplexing the assay with an endogenous control allowed for enhanced quality control, ensuring reliability in all stages of the technique's workflow. This, additionally, enabled reliability and confidence in field detections, limiting the potential for false positive or false negative detections. Additionally, the assay was found to be highly sensitive, with an

effective limit of detection of 0.45 copies/reaction when utilising six technical replicates. This sensitivity provided assurance of a powerful and reliable detection tool, especially due to the high spatial and temporal variability in *C. fleckeri* abundance. Mesocosm experimentation revealed a rapid decay rate of *C. fleckeri* eDNA (99% within 27 hours), with no detectable variation in this rate across minor temperature changes. This provided essential insights into the temporal resolution of the technique, and with knowledge on the movements and behaviours of *C. fleckeri*, suggested that detections likely reflected the species' close proximity. These findings demonstrate that eDNA offers a highly effective and precise tool for detecting *C. fleckeri*, hence addressing a key challenge in determining the species' presence.

The application of eDNA in Port Musgrave provided critical evidence that *C. fleckeri* have spatially small stock populations. The relatively enclosed estuarine system served as a habitat for both medusae and polyps (Chapter 3). eDNA samples were collected across various habitats, including sandy beaches and mangroves, during the jellyfish season, and outside of the season when only polyps would be present. Accordingly, eDNA proved to be an effective tool to locate the elusive benthic polyp stage of *C. fleckeri*, which are the source of the free-swimming medusae stage. Polyps were detected exclusively within the Port, specifically around rocky substrata along sandy beaches, with no detections in mangrove habitat. This suggested that polyps exhibit distinct habitat preferences, similar to those observed in other cnidarian species, particularly scyphozoan jellyfish. Medusae were detected at a high frequency inside, and more sporadically outside, of the estuarine system, suggesting that the species moves between these areas, aligning with known patterns of the jellyfish's movement. The distinct spatial distributions of medusae and polyps enabled an evaluation of their population boundaries, suggesting that the Port likely contains a population stock of the species. This finding supports growing evidence of spatially restricted population stocks of some cubozoan jellyfish. Finally, while eDNA proved highly effective for *C. fleckeri* detection, its use as a proxy for abundance was limited, likely due to the high variability in eDNA shed by the low abundance and spatially dispersed species.

In Chapter 4, the eDNA data collected in the open coastal environment of Magnetic Island built on the findings from Port Musgrave, providing further insights into the spatial distribution and source locations of *C. fleckeri*. The study area, encompassing Horseshoe and Maud Bays, featured oceanographic and geomorphic conditions expected to facilitate greater dispersal of the jellyfish, when compared to the semi-enclosed estuarine system of Chapter 3. Environmental DNA samples were collected across nearshore, mid-shore and offshore locations both during the jellyfish season and

non-seasonal months. Medusae were detected exclusively in nearshore areas, consistent with their known distribution, confirming the ability of eDNA to accurately capture the spatial presence of the species. Outside of the jellyfish season, polyps were detected near freshwater inflows, with the highest frequency of detections occurring in Horseshoe Bay. This supported the hypothesis, based upon empirical medusae size-distribution data, that Horseshoe Bay serves as a source location for *C. fleckeri* on the island. Polyps were detected in habitats with rocky substrata, consistent with previous findings. A comparison of the distributions of both life history stages, where medusae were exclusively detected nearshore and polyps consistently detected in Horseshoe Bay, suggested that the northern side of Magnetic Island likely represents a population stock of the jellyfish. Sampling in both semi-enclosed and open environments added further evidence to a developing paradigm of spatially restricted *C. fleckeri* stocks.

Finally, in Chapter 5, focus shifted to understanding the spatiotemporal dispersion and detectability of *C. fleckeri* eDNA through the utilisation of biophysical models, to better understand the relationship between eDNA detections and the physical presence of the jellyfish. The spatiotemporal detectability of eDNA was found to be spatially restricted, across 100s of meters to kilometres, despite potential for particles to travel distances of up to 10s of kilometres. This was, as expected, primarily influenced by both particle dilution and decay. Local hydrodynamics, including tidal flows and wind-driven surface currents, played a key role in shaping dispersal patterns, leading to heterogeneous movements and retention of eDNA across small spatial scales. Estimated spatiotemporal detection limits aligned with the results of empirical detections (Chapter 4).

In summary, this thesis has demonstrated the effectiveness of eDNA as a powerful ecological sampling tool for detecting and studying cubozoan jellyfish across their key life history stages. The development of a highly specific and sensitive eDNA assay enabled accurate detection of both medusae and polyp life history stages, and provided critical insights into their spatial distribution, habitat preferences, and population structures. Specifically, the efficient detection of polyps is a crucial advancement, offering a new approach to detect and study this life history stage. This knowledge on polyps is vital for effective management strategies as they are the source of stinging medusae. The findings of this thesis additionally add to growing evidence of some cubozoan species, including *C. fleckeri*, maintaining spatially small population stocks, to the scale of bays and estuaries, with these findings being ecologically robust across differing environmental settings. The ubiquitous nature of DNA makes this genetic detection tool applicable for all ~50 cubozoan species, highlighting its potential to further

ecological understanding on other problematic cubozoans, such as the notorious 'Irukandji' jellyfish, *Carukia barnesi*. Additionally, eDNA shows considerable promise as a potential management tool, offering an efficient method for early detection and potential mitigation of the risks posed by these taxa. While eDNA proved highly effective in detecting presence, limitations as a proxy for abundance, its inability to determine size/age distributions, and behavioural patterns were acknowledged, emphasising the need for a multifaceted approach for the comprehensive study of cubozoan ecology. As molecular genetic techniques advance, future research efforts may refine cubozoan detections by offering finer temporal and spatial resolution, potentially enabling targeted collection and *in-situ* study of these jellyfish. Collectively, the findings of this thesis contribute significantly to the field of cubozoan jellyfish ecology and showcase the high applicability of eDNA as an ecological sampling tool to detect and study these taxa.

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Thesis Outputs

PUBLICATIONS

Chapter 2

Morrissey, S.J., Jerry, D.R. and Kingsford, M.J., 2022. Genetic Detection and a Method to Study the Ecology of Deadly Cubozoan Jellyfish. *Diversity*, *14*(12), p.1139. https://doi.org/10.3390/d14121139

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Appendix V

Kingsford, M.J., Schlaefer, J.A. and **Morrissey, S.J.**, 2021. Population structures and levels of connectivity for Scyphozoan and Cubozoan jellyfish. *Diversity*, *13*(4), p.174.

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Morrissey, S.J., Jerry, D.R. and Kingsford, M.J., 2023. A Forensic Approach to Detecting Deadly Box Jellyfish. 1st Australian & New Zealand Environmental DNA (eDNA) Conference. Hobart, Tasmania, Australia.

Morrissey, S.J., Jerry, D.R. and Kingsford, M.J., 2023. Use of eDNA to Test Hypotheses on the ecology of *Chironex fleckeri*. 7th Jellyfish Blooms Conference. Thiruvananthapuram, Kerala, India.

Chapter 1.

General Introduction

Studying marine organisms and their ecology presents numerous distinct challenges in a three-dimensional environment. This complexity is further heightened by dynamic conditions, specifically within coastal regions, where factors such as visibility, organism behaviour, and habitat can vary significantly. Murky nearshore waters, in particular, are challenging to sample, necessitating innovative approaches to obtain accurate data on organism distributions and abundances. Determining the ecology of a species and its interactions with other organisms leads to a broader comprehension of ecosystems and services. Understanding these relationships is essential for management of the natural world, and by integrating ecological principles into decision making processes, informed and appropriate management strategies and solutions can be applied. The complexity of studying the ecology of marine organisms is particularly amplified when dealing with elusive organisms, whereby their cryptic nature introduces additional challenges (Thompson, 2013, Ackerman and Bellwood, 2000, Jones et al., 2002, Gaston, 1994, Kunin and Gaston, 1993, Roberts et al., 2016).

Among the diverse array of elusive organisms, jellyfish stand out as a particularly challenging group to study and manage (Fuentes et al., 2018, Gibbons and Richardson, 2013, Purcell, 2018, Kingsford et al., 2018, Kingsford and Mooney, 2014). They are often vilified as pests due to their occasional disruptive presence to infrastructure, native fauna, and industry, including tourism, aquaculture, and fisheries, yet they play integral roles in marine ecosystems (Pauly et al., 2009, Condon et al., 2011, Decker et al., 2014, Graham et al., 2003, Boero, 2013, Rothe, 2020, Bayha and Graham, 2014, Bosch-Belmar et al., 2020, Palmieri et al., 2014, Uye, 2014). Their unique characteristics and behaviours, however, further complicate challenges faced by ecologists. This is resultant of their elusive nature which stems not only from their translucent bodies, which render them difficult to observe, but also from their complex life histories (Hartwick, 1991a, Kingsford and Mooney, 2014, Goldstein and Steiner, 2020). This elusiveness perpetuates significant gaps in the understanding of their ecology (Kingsford and Mooney, 2014).

The population structures and dynamics of jellyfishes are influenced by a complex interplay of environmental forcings (Fernández-Alías et al., 2020, Loveridge et al., 2021, Schnedler-Meyer et al., 2018, Lynam et al., 2004, Benedetti-Cecchi et al.,

2015), significant temporal variation in the production of medusae (Pitt and Kingsford, 2003, Kitajima et al., 2020), life history characteristics (Schnedler-Meyer et al., 2018, Lucas, 2001, Lucas et al., 2012), prey availability (Lynam et al., 2005, Goldstein and Steiner, 2020), and anthropogenic impacts (Purcell et al., 2007). This leads to great variation in their presence both spatially and temporally. Understanding their population structures and dynamics hence poses a substantial challenge for researchers aiming to manage this group of organisms (Kingsford et al., 2021).

Developing effective management strategies for jellyfish involves deciphering their complex biology, behaviours, and ecological functions (Kingsford et al., 2018, Kingsford and Mooney, 2014, Kingsford et al., 2021, Richardson et al., 2009, Lucas et al., 2014). New innovative approaches, such as advanced monitoring techniques (Martin-Abadal et al., 2020, Houghton et al., 2006, Aleksa et al., 2018, Hidaka-Umetsu and Lindsay, 2018, Rowley et al., 2020) and predictive models (Schlaefer et al., 2018, Schlaefer et al., 2021, Purcell, 2009, Decker et al., 2007), can help to mitigate the impacts of jellyfish on both human health and commercial enterprise. However, understanding the ecology of jellyfishes while addressing the multifaceted challenges they present, still contains many challenges and knowledge gaps (Kingsford et al., 2018, Kingsford and Mooney, 2014, Kingsford et al., 2021, Richardson et al., 2009, Lucas et al., 2014). This is especially true for cubozoan jellyfishes which pose a ‘wicked’ problem globally (Kingsford et al., 2018, Gershwin et al., 2010, Crowley-Cyr and Gershwin, 2021).

1.1 Cubozoan Jellyfish and their Threat to Human Health and Enterprise

Cubozoan jellyfish are generally found in warm tropical waters around the globe (Kingsford and Mooney, 2014, Carrette et al., 2012). They possess several distinctive traits that have garnered significant research interest, including their visual capabilities (Nilsson et al., 2005, Coates and Theobald, 2003), life histories (Kingsford and Mooney, 2014, Straehler-Pohl and Jarms, 2011), swimming abilities (Schlaefer et al., 2020, Schlaefer et al., 2018, Bordehore et al., 2023, Colin et al., 2013), and their potent venom (Chung et al., 2001, Kintner et al., 2005). Members of this jellyfish class are regarded as some of the most venomous organisms on the planet (Kingsford and Mooney, 2014). Their sting can induce severe reactions and, in some cases, be fatal (Fenner et al., 1996, Fenner and Harrison, 2000, Gershwin et al., 2013, Chung et al., 2001, Kintner et al., 2005, Bentlage et al., 2010, Kingsford and Mooney, 2014). Among them, *Chironex fleckeri*, the Australian box jellyfish, is considered the most dangerous species (Brinkman

et al., 2015, Kingsford and Mooney, 2014). It reportedly has been responsible for the death of 80 plus individuals in Australian waters (Fenner and Harrison, 2000). Additionally, 'Irukandji' jellyfish, comprising 16 known species (Gershwin, 2014, Gershwin et al., 2013), can inflict harm, leading to symptoms like considerable pain, cramps, grievous bodily harm, hypertension, and breathing difficulty (Gershwin et al., 2013, Fenner and Carney, 1999). In rare instances, 'Irukandji' stings have resulted in death, with two reported cases in Australian waters (Lippmann et al., 2011, Fenner and Hadok, 2002). Consequently, members of the class Cubozoa pose a direct threat to human health.

The presence of stinging jellyfish can have significant socio-economic impacts on coastal industries and tourism-dependent communities (Kingsford et al., 2018, Gershwin et al., 2010). Local and national economies reliant on tourism, such as small island developing states where tourism accounts for nearly half of the country's exports, face threats due to the negative impact of these jellyfish on tourism (UNWTO, 2014). Managing this risk of envenomation poses a 'wicked problem', a challenge subsequently faced by stakeholders and decision makers globally (Kingsford et al., 2018, Gershwin et al., 2010). Furthermore, there are major concerns regarding potential changes in jellyfish abundances and potential range expansions, driven by climate change (Richardson et al., 2009, Orellana and Collins, 2011, Madin et al., 2012, Kingsford and Mooney, 2014, Klein et al., 2014), which would further impact upon these at-risk industries. Despite these threats posed by cubozoans, significant and critical knowledge gaps exist surrounding their ecology (Kingsford and Mooney, 2014). Filling these knowledge gaps is imperative for the effective management of these problematic jellyfish (Kingsford et al., 2018).

1.2 Knowledge Gaps in Cubozoan Ecology

The ability to effectively manage threats posed by cubozoan jellyfish to human health and commercial enterprise is largely limited by the current understanding of their ecology (Kingsford and Mooney, 2014). As a result of this, in-depth understandings on key aspects of cubozoan ecology are imperative (Kingsford and Mooney, 2014). Specifically, understandings of their life histories, the presence and distributions of their various life history stages, and their population structures (Kingsford and Mooney, 2014). These critical knowledge gaps are a consequence of the challenges associated with detecting and subsequently studying cubozoan jellyfish in their natural environment (Kingsford et al., 2018, Kingsford and Mooney, 2014).

1.2.1 Cubozoan Life Histories

Cubozoan jellyfish have a metagenetic life history consisting of two major stages; the medusae and polyp stages (Figure 1.1). The medusae stage is free swimming within the water column and is responsible for the threats and impacts to human health and commercial enterprise (Kingsford et al., 2018). Conversely, the polyp stage is benthic and rarely observed in its natural environment (Hartwick, 1991a, Cutress and Studebaker, 1973). These two stages employ distinct modes of reproduction. Medusae undergo sexual reproduction, utilising both internal and/or external fertilisation (Werner, 1973, Stewart, 1996, Hartwick, 1991b, Okada, 1927, Lewis and Long, 2005, Morandini et al., 2016). In contrast, polyps undertake asexual reproduction through budding, leading to the formation of clusters known as ‘polyp beds’ (Straehler-Pohl and Jarms, 2011, Fischer and Hofmann, 2004). Medusae produce planula larvae that settle and metamorphose into polyps and under suitable conditions, polyps detach from the substrate and metamorphose into medusae, with the remaining base giving rise to polyps again (Straehler-Pohl and Jarms, 2011). Despite there being two distinct life history stages, research has predominately focused on the medusae stage (Kingsford and Mooney, 2014). This is due to the direct threats posed by this stage, as well as the accessibility of studying it in comparison to polyps (Kingsford et al., 2018, Gershwin and Kingsford, 2019). However, there is a crucial need to better understand the polyp stage of cubozoan jellyfish.

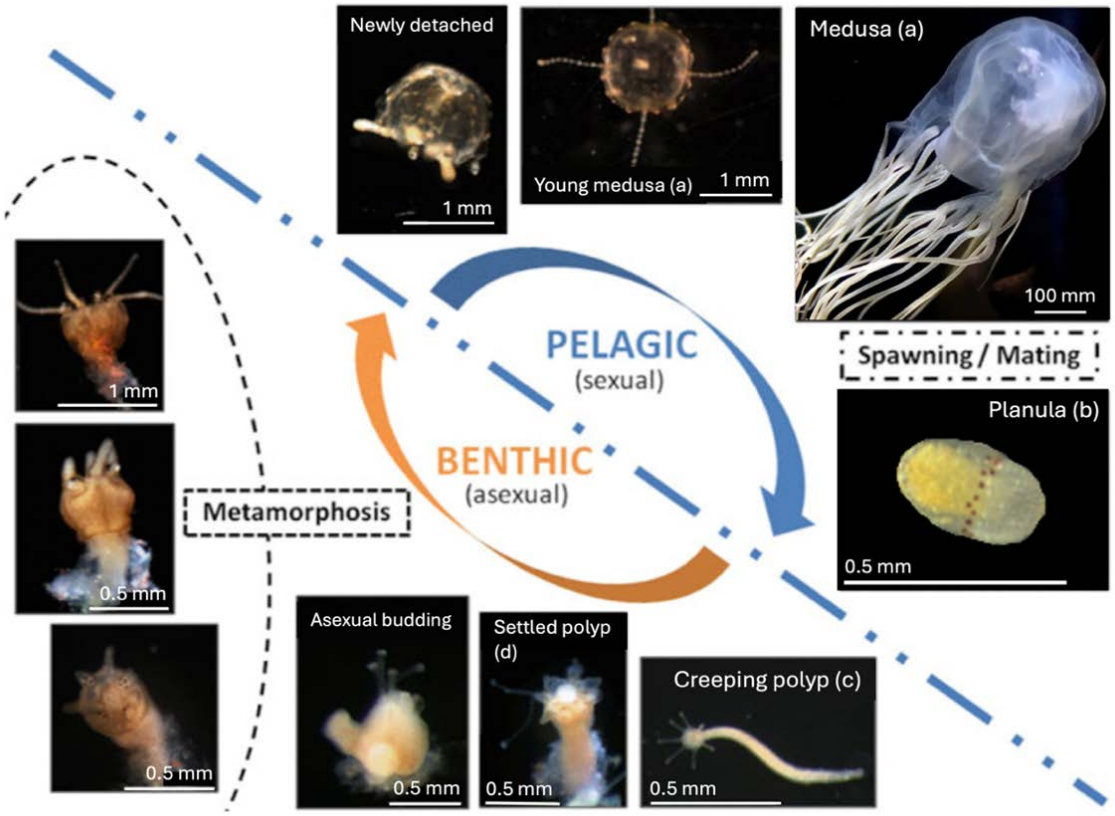

Figure 1.1. Depiction of the cubozoan life cycle showing pelagic and benthic stages. Images are from differing species, (a) *Chironex fleckeri* (Image: M. Freeman), (b) *Copula sivickisi* (Image: D. Nilsson), (c) *Carybdea* sp. (Image: I. Straehler-Pohl), (d and unlabeled) *Tripedalia cystophora* (Images: J. Bielecki). Adapted from Kingsford and Mooney (2014).

The polyp stage of cubozoan jellyfish, measuring only 1 to 2 millimetres, is the more persistent life history stage. As the benthic source of the stinging medusae (Straehler-Pohl and Jarms, 2011), it likely influences their distributional limits and potential for range expansions (Kingsford and Mooney, 2014, Kingsford et al., 2021, Courtney and Seymour, 2013, Colin and Kremer, 2002, Toyokawa et al., 2011, Shahrestani and Bi, 2018). Most investigations on cubozoan polyps have been taxonomically driven or centred on organismal development, predominately conducted in laboratory settings (Cutress and Studebaker, 1973, Stewart, 1996, Werner et al., 1971, Arneson and Cutress, 1976, Laska-Mehnert, 1985, Stangl et al., 2002, Straehler-Pohl and Jarms, 2005, Straehler-Pohl and Jarms, 2011). Beyond taxonomic investigations, a limited number of studies have aimed to locate cubozoan polyps in their natural environment (Hartwick, 1991a, Cutress and Studebaker, 1973). These studies utilised detection and sampling techniques such as netting and visual observations to infer the location of polyps via examining size-frequency distributions of medusae (i.e. assuming where medusae are present polyps must also be near), or conducted extensive and labour-intense *in-situ* searches, with instances where researchers spent years searching specific environments before finding specimens (Hartwick, 1991a, Cutress and Studebaker, 1973). Presently, only two occurrences of cubozoan polyps in their natural environment have been documented. Cutress and Studebaker (1973) discovered *Carybdea xaymacana* polyps in Puerto Rico within mangrove channels, and Hartwick (1991a) reported finding *Chironex fleckeri* polyps in Australia within an estuarine river. These findings established an anecdotal paradigm suggesting that dangerous cubozoan polyps reside within estuaries, with newly produced medusae moving into adjacent coastal waters. However, recent evidence from a study examining statolith elemental chemistry has challenged this paradigm, indicating that suitable polyp habitat may extend to the open coast (Mooney and Kingsford, 2012). Consequently, the precise locations of polyps and, hence, the source locations of the threatening medusae, remain largely unknown. Understanding on the distribution of cubozoan polyps is crucial to deepen our comprehension of cubozoan ecology, particularly regarding the role of polyps in medusae abundances and distributions (Kingsford et al., 2021). Knowledge of their locations could also aid in early detection of stinging medusae, offering warnings to water users and stakeholders.

1.2.2 Cubozoan Population Structures

Marine organism populations typically exhibit a hierarchical structure of spatially distinct units (Hastings and Harrison, 1994) (Figure 1.2). At the broadest level are metapopulations, which often align with the biogeographic range of a species. If this range is extensive, multiple metapopulations may exist (Sinclair, 1988, Kingsford and Battershill, 1998). These metapopulations can be further broken down into mesopopulations or, in fisheries terms, stocks (Gibson and Barnes, 2000, Sinclair, 1988). Mesopopulations are commonly the focus of studies investigating population structure and dynamics due to their self-contained and relatively self-sufficient nature (Sinclair, 1988). These mesopopulations can, in turn, fragment into local populations characterised by high connectivity.

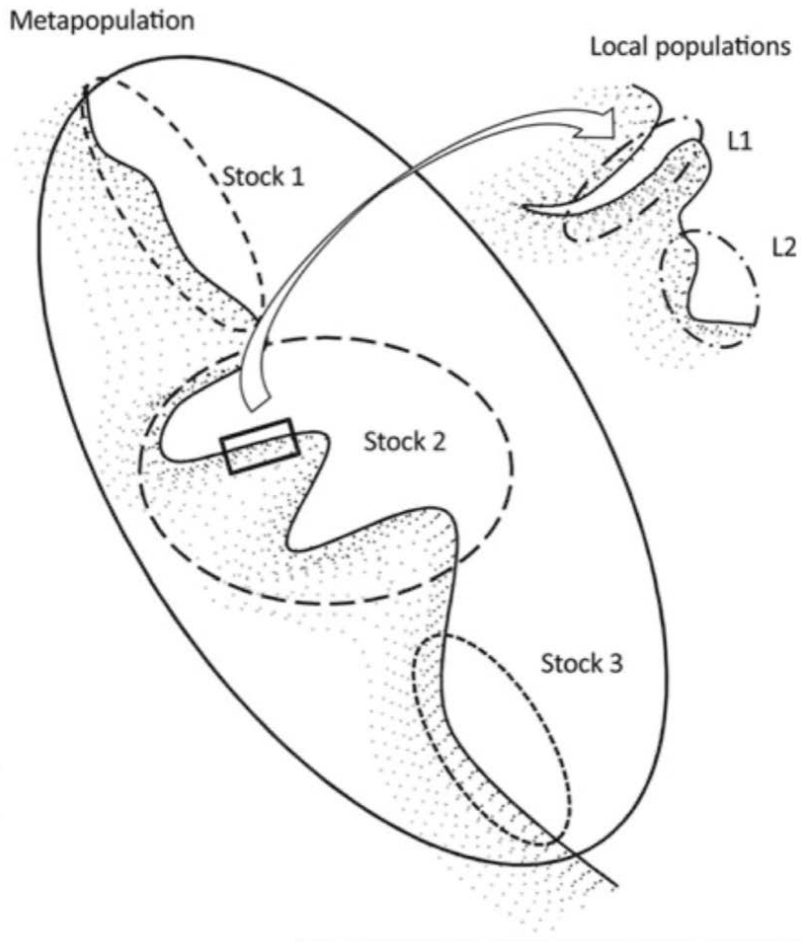

Figure 1.2. Depiction of the nested hierarchical nature of idealised population units for marine coastal organisms. The local populations (L1 and L2, dashed and dotted lines) are nested within the stocks/mesopopulations (Stock 1, 2 and 3, dashed lines), which are further nested within the metapopulation (solid line). Adapted from Kingsford and Mooney (2014).

For cubozoan jellyfish, understanding of the spatial scales and connectivity between these units is limited (Schlaefer et al., 2020, Schlaefer et al., 2018, Mooney and Kingsford, 2017, Kingsford et al., 2021, Mooney and Kingsford, 2016a, Bordehore et al., 2023). Although data on the occurrence of cubozoan species enables inferences about metapopulations, (e.g. *Carybdea rastoni* exhibiting a widespread distribution across multiple continents, each reflecting a metapopulation of the species (Kingsford and Mooney, 2014)), knowledge concerning the substructure of these metapopulations remains limited (Kingsford and Mooney, 2014, Kingsford et al., 2021, Mooney and Kingsford, 2016a, Mooney and Kingsford, 2017).

Only a limited number of studies have delved into investigating the spatial scales of cubozoan populations and their connectivity (Schlaefer et al., 2020, Schlaefer et al., 2018, Kingsford et al., 2021, Mooney and Kingsford, 2017, Mooney and Kingsford, 2016a, Bordehore et al., 2023) (Table 1.1). Mooney and Kingsford (2016a) utilised elemental chemistry analysis of *Chironex fleckeri* statoliths as a means for investigating population structure and connectivity across regions. Examining geochemical signatures within statoliths from individuals collected across regions separated by hundreds of kilometres, they found distinct signatures not only between these distant regions but also between sites only a few kilometres apart. These findings suggest substructures within metapopulations, potentially corresponding to stocks and local populations. Similarly, Mooney and Kingsford (2017) reported comparable conclusions by examining statolith morphometrics, a common technique in fisheries for stock identification (Campana and Casselman, 1993, Cardinale et al., 2004, Galley et al., 2006).

Table 1.1. The spatial scales and methods used to determine differences among cubozoan species populations. The spatial scale refers to the level at which variances were identified using the associated method and subsequently reflect reported discrete populations. Range represents the farthest linear span between detections. Tables adapted from Kingsford et al. (2021).

Species	Methods	Spatial Scale	Range (Km)	Source
Alatina alata	Genetics	1000s of km	>1000	Lawley et al. (2016)
Copula sivickisi	Statolith morphology	10s to 100s of km	>1000	Mooney and Kingsford (2017)
Chironex fleckeri	Statolith morphology	10s to 100s of km	>1000	Mooney and Kingsford (2017)
Chironex fleckeri	Elemental chemistry	Kms to 10s of km	>1000	Mooney and Kingsford (2016a)
Chironex fleckeri	Biophysical modelling	Hundreds of metres to km	>1000	Schlaefer et al. (2018)

Moreover, studies investigating the influence of cubozoan swimming behaviours on population structures and connectivity have been conducted (Schlaefer et al., 2020, Schlaefer et al., 2018, Schlaefer and Kingsford, 2024, Bordehore et al., 2023). Schlaefer et al. (2018) used biophysical modelling to explore the spatial scales of connectivity between *C. fleckeri* populations separated across medium (10's of kilometres) and small (100's of metres) spatial scales in a semi-enclosed estuarine bay. They found that jellyfish, both modelled as passive and swimming, were largely retained within the bay (0% lost for passive, <2.5% lost for swimming) due to favourable currents and medusae swimming behaviour. As a result, Schlaefer et al. (2018) concluded that cubozoan stocks may be at the scale of estuaries and bays due to the retention of jellyfish and hence unlikely connectivity to other populations. They also reported that due to the jellyfish's swimming behaviour, medusae maintained themselves at small spatial scales (100s of metres) within the bay, which resembled local populations. Interestingly, they found there to be minimal connectivity between these potential local populations. Similarly, the investigators of this study further examined how the swimming behaviours of *C. sivickisi* medusae influenced the species distribution and connectivity among populations (Schlaefer et al., 2020). They, again, found local populations which had minimal connectivity and that were maintained at small spatial scales as a result of *C. sivickisi*'s swimming behaviours and diurnal activity. These few studies, in addition to observations on the occurrence, movements and behaviours of cubozoans (Brown, 1973, Hartwick,

1991a, Gordon and Seymour, 2009, Kingsford et al., 2012, Rowley et al., 2022), provide evidence to suggest that some cubozoans may have populations (stocks) comprising small spatial scales with low levels of connectivity (Kingsford et al., 2021).

A greater resolution of these patterns of structure and connectivity are needed, especially in areas of differing geomorphic and hydrodynamic conditions. Understanding the spatial scales and connectivity of population units is of utmost importance for the effective management of cubozoans and for understanding their population dynamics. Further, this knowledge becomes particularly crucial with climate change potentially facilitating population expansions and extending seasons where dangerous jellyfish are present (Kingsford and Mooney, 2014, Orellana and Collins, 2011, Madin et al., 2012, Richardson et al., 2009).

1.3 Challenges Associated with Cubozoan Detection and Limitations of Current Detection Techniques

Detecting cubozoan jellyfish is challenging due to their unique characteristics and the limitations of existing detection techniques (Kingsford and Mooney, 2014). Their transparency, spatial and temporal dispersion, residence in turbid waters (for some species), and elusive nature make them difficult to detect (Kingsford and Mooney, 2014). Moreover, the current tools and techniques available for detecting cubozoans have some limitations (Table 1.2), which restrict the scope of ecological questions that can be explored in cubozoan ecology (Kingsford et al., 2018). The existing techniques often have restricted applicability, operational constraints, or limitations in environmental suitability. Methods like beach seines are hindered by certain environments, such as rocky substrates which limit their use. Light attraction techniques are limited in spatial coverage and species applicability. More modern tools, such as the utilisation of drones (Rowley et al., 2020, Rowe and Ahyong, 2024), are again limited in species applicability and reliant on favourable weather conditions. Moreover, these techniques are more likely to result in false negative detections which further complicates the effective detection and study of cubozoans. The likelihood of detection, however, is also dependent on sampling design/effort which in part will relate to the area of the targeted location. Given the limitations of current detection techniques, and their restricted ability to investigate critical aspects of cubozoan ecology, development and implementation of new detection techniques is imperative for advancing our understanding on these organisms. Molecular genetic techniques may be the solution to overcoming these challenges (Bolte et al., 2021, Gaynor et al., 2017, Minamoto et al., 2017).

Table 1.2. Strengths and limitations of techniques utilised to detect cubozoan jellyfish.

Technique	Strengths	Limitations	Source
Visual Observations	Real-time identification. Behavioural insight. Cost-effective. Non-invasive.	Observer expertise. Temporal constraints. Limited application to certain species/life history stages. Habitat limitations (i.e. water clarity). High false negative detection (dependent on sampling effort).	Brown (1973)
Netting – Beach Seines, Hand Netting	Collection efficiency. Selective sampling. Visual identification. Standardisation by volume. Quantitative analysis. Adaptability - various types and applications.	Specimen damage. Habitat limitations. Depth limitations. High false negative detection (dependent on sampling effort). Labour-intensive and time-consuming.	Bordehore et al. (2011) Gordon and Seymour (2012)
Netting – Trawls, Plankton Tows	Collection efficiency. Depth versatility. Standardisation by volume. Quantitative analysis. Adaptability - various types and applications.	Specimen damage. Gear selectivity. High false negative detection (dependent on sampling effort). Labour-intensive and time-consuming.	Kingsford et al. (2012) Lai (2010) Lewis and Long (2005) Kraeuter and Setzler (1975) Hartwick (1991a)

Table 1.2. Continued.

Light Attraction	Efficient for detecting cubozoans in low visibility. Quantitative analysis. Non-invasive. Above and below surface detection.	Limited application to certain species/ life history stages. Limited spatial coverage. Behavioural distortion.	Llewellyn et al. (2016) Kingsford et al. (2012) Schlaefer et al. (2021) Schlaefer et al. (2020)
Remote Sensing - Drones	Aerial perspective. Time-efficient. Non-invasive	Weather and environmental constraints (i.e. light, surface chop & water clarity). Limited application to certain species/ life history stages. Limited to surface waters. Technology constraints. Regulatory and ethical considerations.	Rowley et al. (2020)

1.4 Environmental DNA

Environmental DNA, or eDNA for short, is a technique based on the detection of minute traces of DNA released by organisms into their surrounding environments (Rees et al., 2014, Darling, 2015, Shaw et al., 2017, Huerlimann et al., 2020). These traces of DNA are extractable from diverse environmental samples, including soil, water, snow, or air (Rees et al., 2014, Leempoel et al., 2020, Clare et al., 2021, Franklin et al., 2019). Organisms release eDNA into their environment as a result of various physiological, behavioural, reproductive, and defensive behaviours and responses (Rees et al., 2014, Shaw et al., 2017), essentially creating a genetic fingerprint of the associated organism.

The premise of eDNA is that a sample collected from an environment, such as a water sample taken from an estuary, can reveal the species present or recently present at the time of sampling upon examination (Rees et al., 2014, Taberlet et al., 2012). As DNA is ubiquitous, and as all living organisms release DNA, this method can theoretically be applied to detect any organism (Huerlimann et al., 2020, Rees et al., 2014). Additionally, as eDNA is subject to degradation by microbial activity, it enables the determination of the recent presence of organisms (e.g. hours to days) (Rees et al., 2014, Thomsen et al., 2012). Consequently, eDNA facilitates the detection and identification of organisms, to species level, without the need of their direct observation, making it a valuable and complimentary tool for ecological studies (Taberlet et al., 2012, Rees et al., 2014, Shaw et al., 2017).

Initially, eDNA was utilised to detect the presence of American Bullfrogs (*Lithobates catesbeianus*) in wetlands (Ficetola et al., 2008) and has since been employed to detect and study a broad range of organisms from bacteria (Taberlet et al., 2012) to whale sharks (Sigsgaard et al., 2017), as well as utilised in various aquatic environments, such as lakes (Takahara et al., 2012, Doi et al., 2015, Uchii et al., 2016), rivers (Minamoto et al., 2012, Fukumoto et al., 2015, Ikeda et al., 2016, Yamanaka and Minamoto, 2016, Robson et al., 2016, Eva et al., 2016) and marine habitats (Thomsen et al., 2012, Yamamoto et al., 2016, Doyle et al., 2017, Minamoto et al., 2017). Comparative studies with traditional methods have predominately highlighted the equal or higher sensitivity of eDNA in species detection (Valentini et al., 2016, Ishige et al., 2017, Smart et al., 2016, Rees et al., 2014, Simpfendorfer et al., 2016, Sigsgaard et al., 2015, Clarke et al., 2017, Hänfling et al., 2016, Olds et al., 2016, Jerde et al., 2011, Wilcox et al., 2016). For example, Simpfendorfer et al. (2016) demonstrated the higher sensitivity of eDNA by successfully detecting the endangered sawfish, *Pristis pristis*, in waterholes where traditional gillnet sampling had both confirmed its presence or failed

to do so. Similarly, Sigsgaard et al. (2015) reported eDNA to be of higher sensitivity than fishing surveys in detecting the presence of the European weather loach, *Misgurnus fossilis*. Additionally, due to the cost-effectiveness, rapidity, and lower resource utilisation of eDNA compared to labour-intensive traditional methods, it has found diverse applications (Barnes and Turner, 2016, Sigsgaard et al., 2015, Smart et al., 2016, Clarke et al., 2017, Jerde et al., 2011). It has been used for biodiversity assessments, trophic interaction elucidation, population genetics, estimating organism abundances, monitoring species for conservation and ecosystem management, as well as obtaining data on species distributions (Simpfendorfer et al., 2016, Uthicke et al., 2018, Villacorta-Rath et al., 2020, Bohan et al., 2017, Srivathsan et al., 2015, Adams et al., 2019, Doi et al., 2017).

1.4.1 Application of eDNA to Monitor and Detect Problematic and Cryptic Species

Environmental DNA has been recognised and successfully employed as an efficient means to detect and monitor marine organisms, particularly those which are problematic or cryptic (Beng and Corlett, 2020, Duarte et al., 2023, Rishan et al., 2023, Larson et al., 2020, Wilcox et al., 2013, Takahara et al., 2013, Goldberg et al., 2013, Dejean et al., 2012, Jerde et al., 2011, Ficetola et al., 2008). By eliminating the need for capture and handling, it offers a less invasive detection method while also enhancing monitoring efficiency for both ecological research and management applications on these species (Mauvisseau et al., 2020, Beng and Corlett, 2020). Additionally, it can provide essential baseline data critical for assessing their current status and distributions, thereby aiding in their management (Rees et al., 2014).

eDNA has been effectively utilised to detect invasive species, such as Mozambique tilapia (*Oreochromis mossambicus*) (Robson et al., 2016), and species of environmental concern, such as the Crown-of-Thorns seastar (*Acanthaster solaris*), providing insights into their distribution and management (Uthicke et al., 2018). It has also been applied to monitor non-native genotypes which threaten biodiversity, such as invasive common carp (*Cyprinus carpio*) in Japan, which endanger native strains (Uchii et al., 2016). Moreover, eDNA, in addition to monitoring invasive and problematic species, can also be utilised as an early detection method for infestations and outbreaks (Villacorta-Rath et al., 2020, Trebitz et al., 2017, Russell et al., 2012). This versatility in eDNA applications highlights its broader potential to address challenges in managing problematic organisms.

In addition to problematic species, eDNA is invaluable for studying cryptic organisms, whose behaviour, habitat complexity, or low abundance often hinder detection using traditional methods (Beng and Corlett, 2020, Schill and Galbraith, 2019, Qu and Stewart, 2019, Reinhardt et al., 2019, Carvalho et al., 2019, Rose et al., 2020). For example, Schill and Galbraith (2019) used eDNA to detect the dwarf wedgemussel (*Alasmidonta heterodon*), demonstrating its ability to uncover cryptic populations for improved management. Similarly, Rose et al. (2020) employed eDNA to distinguish estuarine crocodiles (*Crocodylus porosus*), a significant threat to human safety, from freshwater crocodiles (*Crocodylus johnstoni*), highlighting its value as a tool for managing the threats posed by estuarine crocodiles to humans. eDNA hence serves as a valuable tool for studying these organisms, providing critical insights into their presence, distribution, and potential management strategies.

In addition to eDNA's capacity to discern presence/absence of these aquatic organisms, the genetic technique may also be utilised as a cost-effective tool for estimating species abundance and/or biomass (Doi et al., 2017, Takahara et al., 2012, Yamamoto et al., 2016, Lacoursière-Roussel et al., 2016a, Lacoursière-Roussel et al., 2016b, Minamoto et al., 2012, Pilliod et al., 2013, Moyer et al., 2014, Jo et al., 2017). The premise of this use is based on potential relationships between eDNA concentrations and species abundance/biomass (Doi et al., 2017, Deiner et al., 2017). Numerous studies have examined this potential use for a wide range of species which have shown positive correlations between the two factors (Doi et al., 2017, Takahara et al., 2012, Yamamoto et al., 2016, Lacoursière-Roussel et al., 2016a, Lacoursière-Roussel et al., 2016b, Minamoto et al., 2012, Pilliod et al., 2013, Moyer et al., 2014, Jo et al., 2017, Kutti et al., 2020, Rourke et al., 2022, Thomsen et al., 2012); however, it should be noted that this use of eDNA was not supported by some studies (Lim et al., 2016, Capo et al., 2020, Deutschmann et al., 2019, Fraija-Fernández et al., 2020, Hinlo et al., 2018, Knudsen et al., 2019, Perez et al., 2017, Bolte et al., 2021). Consequently, the use of eDNA concentration as a proxy for abundance or biomass may only be appropriate in contexts where species densities, and thus eDNA concentrations, vary substantially. This potential relationship must be calibrated on an individual species basis, and intraspecific variability in eDNA shedding rates, influenced by factors such as individual size, life history, and metabolic activity, must be considered (Kirtane et al., 2021, Dunn et al., 2017, Klymus et al., 2015, Maruyama et al., 2014). Despite the successful and diverse applications of eDNA, a number of essential considerations and understandings are needed for the accurate use and interpretation of the technique.

1.4.2 Essential Considerations and Understandings

For precise utilisation of eDNA as a detection approach, and accurate result interpretation, several critical considerations and understandings are needed. The primary consideration revolves around the accurate detection of target species (Goldberg et al., 2016, Cristescu and Hebert, 2018, Jerde, 2019, Evans et al., 2017, Xiong et al., 2016). False negative detections, indicating the failure to detect species despite their presence, are a major concern, particularly in studies utilising eDNA to detect low abundance organisms (Darling and Mahon, 2011, Rees et al., 2014, Xiong et al., 2016). This failure may arise from assay inefficiency in detecting a species' eDNA, poor quality eDNA due to mishandling or inappropriate storage, or insufficient sampling (i.e. replicate sampling or volume of water sampled) (Evans et al., 2017, Guillera-Arroita et al., 2017, Goldberg et al., 2016, Pinfield et al., 2019, Cristescu and Hebert, 2018, Rees et al., 2015, Green and Young, 1993). Equally worrisome are false positive detections, where a species is reported present despite its absence from the sampled area (Darling and Mahon, 2011, Rees et al., 2014, Roussel et al., 2015, Xiong et al., 2016). False positives may arise at two key stages, during assay development if specificity testing is insufficient, leading to cross-amplification of non-target species, or during result interpretation if decision-making frameworks are not stringent. In the latter case, weak amplification signals may reflect legacy DNA, laboratory artifacts, low-level contamination, or environmental transport processes, requiring detections to be critically assessed before acceptance (Evans et al., 2017, Cristescu and Hebert, 2018, Wilcox et al., 2013, Thomsen and Willerslev, 2015). Rigorous assay validation, strict contamination control, and conservative decision-making frameworks are therefore critical to mitigate these uncertainties (Rees et al., 2014, Cristescu and Hebert, 2018, Goldberg et al., 2016, Ficetola et al., 2015).

To ensure accurate interpretation of results, a comprehensive understanding of the "ecology" of eDNA is needed (Sassoubre et al., 2016, Sansom and Sassoubre, 2017), including the decay rate of the target species' eDNA and its physical transport and dispersion (Barnes et al., 2014, Harrison et al., 2019, Lance et al., 2017, Rees et al., 2014, Blackman et al., 2024). eDNA decay refers to the persistence time of eDNA within the environment (Harrison et al., 2019). Understanding of this temporal factor is central to interpreting and reporting organism presence (Rees et al., 2014). This needs to be explored on a per species basis (Harrison et al., 2019, Andruszkiewicz Allan et al., 2021). Multiple studies have demonstrated that the rate of decay for marine organisms falls within a 10-50 hour half-life range (Collins et al., 2018). Additionally, understanding

on the physical transport and dispersion of eDNA within the environment is crucial for linking eDNA-based detections to the physical presence of target organisms (Harrison et al., 2019). eDNA, being a passive particle (Turner et al., 2014), has the potential to travel significant distances (Thomsen et al., 2012) resultant of environmental and oceanographic influences (Andruszkiewicz et al., 2019, Murakami et al., 2019, Harrison et al., 2019). This understanding is essential for accurately interpretating species detections and distinguishing between allochthonous signals and those representative of present taxa (Blackman et al., 2024). A combined temporal and spatial understanding of eDNA's "ecology" is therefore key for its effective use in ecological applications.

1.5 Potential Application of eDNA to Cubozoans

Environmental DNA offers a promising avenue to advance our understanding and address knowledge gaps concerning cubozoan ecology. Existing gaps, as highlighted above, persist due to the limitations of current detection and sampling techniques for sampling complex coastal environments. Techniques used to detect and sample these potentially deadly jellyfish need to be accurate and reliable (Kingsford et al., 2018). Not only do we need these techniques for the management of the threat posed by these jellyfish, but also for gaining a complete understanding of their ecology which is necessary for their effective management (Kingsford et al., 2018, Kingsford and Mooney, 2014). As eDNA enables the detection of organisms without their direct observation, and due to its successful application in identifying and studying elusive and cryptic species, it shows considerable promise to fill knowledge gaps and enhance the comprehension and management of these potentially hazardous jellyfish (Minamoto et al., 2017, Bolte et al., 2021).

Notably, eDNA has shown promise in studying scyphozoan jellyfish, close relatives of cubozoans (Gaynor et al., 2017, Minamoto et al., 2017). Minamoto et al. (2017) conducted the first study to do so utilising *Chrysaora pacifica* (Japanese sea nettle) as the study species. Their mesocosm studies revealed significantly higher eDNA production rates compared to fish (Klymus et al., 2015, Maruyama et al., 2014), with eDNA persisting for approximately 4 days. Field surveys compared eDNA distribution and concentrations with jellyfish sightings, suggesting potential for estimation of abundances with eDNA. Minamoto et al. (2017)'s findings support the utility of eDNA as a method to study jellyfish, and to provide crucial information for their monitoring and management. Gaynor et al. (2017) similarly demonstrated success in mapping free-swimming larval life history stages of *Chrysaora quinquecirrha* using eDNA in estuarine

waters. These studies underscore the potential of eDNA in detecting and studying scyphozoan jellyfish, further implying its applicability to study cubozoan jellyfish.

The application of eDNA to study cubozoan jellyfish would allow for investigation into the critical knowledge gaps discussed above (section 1.3). Regarding the polyp stage of cubozoans, eDNA shows promise in facilitating their detection, bypassing the challenge of their small size and elusive nature, and aiding in understanding their location and distribution (when medusae are absent during winter months for most species; Kingsford and Mooney, 2014). Pilot studies on the relatively harmless cubozoan, *Copula sivickisi*, have showcased the potential to detect both medusae and, putatively, polyps (Bolte et al., 2021). The success of this technique to detect the polyps of potentially deadly species would allow for understanding of source locations of medusae and for investigations into numerous aspects of polyp ecology. This understanding would also significantly aid decision makers in managing the risk posed by these jellyfish.

Regarding cubozoan population structures, eDNA again presents a potential means to investigate this. To date, this understanding has been difficult to investigate, due to the challenges faced with detecting and sampling cubozoan jellyfish. Hence, quantitative data on their distributions is limited (Kingsford and Mooney, 2014). However, eDNA has been effectively utilised to monitor and examine the distributions of both problematic and cryptic species in prior studies and hence displays considerable promise to investigate this aspect of cubozoan ecology (Simpfendorfer et al., 2016, Eva et al., 2016, Ishige et al., 2017, Bálint et al., 2018, Uthicke et al., 2018, Doyle et al., 2017). Specifically, Minamoto et al. (2017) observed a correlation between the distribution of *C. pacifica* and its eDNA signal, highlighting the potential use of eDNA to study cubozoan population structures. Accordingly, eDNA provides a potential means to not only better understand cubozoan ecology, but also to enable greater and more effective management and monitoring of the risk which these jellyfish pose to both human health and commercial enterprises.

1.6 Thesis Aims and Objectives

The overarching aim of this thesis was to explore the use of environmental DNA as an innovative technique for detecting and studying deadly cubozoan jellyfish. The approach taken through the research in this thesis was to develop an eDNA assay for the detection of the Australian box jellyfish, *Chironex fleckeri*, and to utilise this genetic detection technique as an ecological survey tool to study its ecology and fill critical knowledge gaps surrounding the potentially deadly jellyfish's life history and population structures. The specific aims, by chapter, for this thesis were as follows.

Chapter 2. Develop and optimise a specific and sensitive TaqMan detection assay for *Chironex fleckeri*, multiplexed with an endogenous control for enhanced quality control. Furthermore, the persistence time of *C. fleckeri* eDNA was determined.

Chapter 3. Use eDNA to estimate the abundances of *C. fleckeri* medusae and to determine the distribution of medusae throughout and outside of a semi-enclosed estuarine system. This allowed for the validation of a biophysical model that had been used on the study area. Additionally, to explore the use of eDNA to detect the species' elusive polypoid stage while further identifying the habitats occupied by this stage. Finally, the distributions of both medusae and polyp stages were compared to infer population stocks of the species.

Chapter 4. Determine the localised distribution of the species' medusae stage in an open coastal system. This involved an examination of both polypoid and medusoid stages to determine their distribution and abundance.

Chapter 5. Assess the dispersal potential of *C. fleckeri* eDNA in an open coastal system through use of biophysical modelling. Determine the influence of oceanographic and environmental factors on said eDNA dispersal and how this influences the spatiotemporal detectability of eDNA. Finally, compare estimates of simulated eDNA with measurements made in the field to assess this application of biophysical models.

Chapter 2.

Genetic Detection and a Method to Study the Ecology of Deadly Cubozoan Jellyfish

2.1 Abstract

Cubozoan jellyfish pose a risk of envenomation to humans and a threat to many businesses, yet crucial gaps exist in determining threats to stakeholders and understanding their ecology. Environmental DNA (eDNA) provides a cost-effective method for detection that is less labour intensive and provides a higher probability of detection. The objective of this study was to develop, optimise and trial the use of eDNA to detect the Australian box jellyfish, *Chironex fleckeri*. This species was the focus of this study as it is known to have the strongest venom of any cubozoan, it is responsible for more than 200 recorded deaths in the Indo-Pacific region. Further, it's ecology is poorly known. Herein a specific and sensitive probe-based assay, multiplexed with an endogenous control assay, was developed, and successfully utilised to detect the deadly jellyfish species and differentiate them from closely related taxa. A rapid eDNA decay rate of greater than 99% within 27 h was found with no detectable influence from temperature. The robustness of the technique indicates that it will be of high utility for detection and to address knowledge gaps in the ecology of *C. fleckeri*; further, it has broad applicability to other types of zooplankton.

2.2 Introduction

Jellyfish of the classes Scyphozoa (true jellyfish) and Cubozoa (box jellyfish) are generally the largest types of non-colonial zooplankton (Castellani and Edwards, 2017). Additionally, they are of ecological importance as predators of fish larvae and other plankton, as well as structures in the pelagic environment that attract small fish and invertebrates (e.g. *Trachurus* spp. with *Desmonema chierchianum* (Kingsford, 1993). Furthermore, some jellyfish have been vilified as pests (Kingsford et al., 2018, Decker et al., 2014), invasives (Lynam et al., 2006, Graham et al., 2003) and for affecting industries such as aquaculture and fisheries (Brodeur et al., 2002, Uye, 2014, Bosch-Belmar et al., 2020). Cubozoans are represented by about 50 species (Collins and Jarms, 2018) and many are notorious for their ability to cause painful and in some cases potentially deadly stings. This class of zooplankton pose a threat to both human health and enterprise, resulting in an array of socio-economic impacts (Kingsford et al., 2018, Crowley-Cyr and Gershwin, 2021). This is especially true for the Australian box jellyfish, *Chironex fleckeri*, which has been responsible for more than 200 deaths in the Indo-Pacific region (Gershwin et al., 2013). Despite this, considerable knowledge gaps exist surrounding the ecology of this species and most other cubozoans. These gaps are a result of the challenges associated with detecting and subsequently studying these animals in their natural environment, resulting from their spatial and temporal variability, transparency, cryptic nature, and the fact that they commonly reside in waters of low visibility (Kingsford and Mooney, 2014). Multiple techniques have been utilised to detect jellyfish, including *in-situ* visual observations (Brown, 1973, Pitt and Kingsford, 2000), various styles/sizes of netting (Hartwick, 1991a, Gordon et al., 2004, Bordehore et al., 2011, Kingsford et al., 2012), light attraction techniques (Barnes, 1966, Kingsford et al., 2012, Llewellyn et al., 2016), acoustics (Zhang et al., 2019, Lee et al., 2007), and most recently drones (Rowley et al., 2020, Schaub et al., 2018, Rowe et al., 2022). Each method has benefits and limitations, depending on the application and ambient conditions. However, all require considerable effort and resources to be undertaken and sometimes have low levels of detection where the target organisms are well dispersed. It has been highlighted (Kingsford et al., 2018) that new techniques and technologies are needed to study cubozoans and to improve levels of detection; genetic detection techniques may be the solution (Beng and Corlett, 2020).

Environmental DNA (eDNA) has gained considerable interest in recent years as a method for detection of rare and cryptic organisms in terrestrial, aquatic, and marine settings (Rees et al., 2014, Wilcox et al., 2013, Jerde et al., 2011). eDNA refers to extra-

organismal genetic material which has been shed by organisms through various physiological, behavioural, reproductive, and defensive responses into their surrounding environment (Shaw et al., 2017). This technique works by utilising species-specific DNA identifiers to isolate the genetic material, eDNA, of a particular species from collected water, air, or soil samples (Clare et al., 2021, Rees et al., 2014, Thomsen et al., 2012). This allows for the determination of the presence/absence and potentially abundance of a target species based on the presence/absence of its genetic signature (Rees et al., 2014, Thomsen et al., 2012). The major interest in the technique as a detection tool is due to not needing specialised skills of identification based on the morphology of species (Evans et al., 2017, Jerde et al., 2011, Sigsgaard et al., 2015, Barnes and Turner, 2016, Smart et al., 2016). eDNA has been used successfully for the detection of numerous cryptic organisms (Cooper et al., 2021, Budd et al., 2021, Villacorta-Rath et al., 2022, Rose et al., 2020) and more recently has been applied to jellyfish (Gaynor et al., 2017, Minamoto et al., 2017, Bolte et al., 2021, Ames et al., 2021, Mychek-Londer et al., 2020).

The eDNA technique has been used to successfully detect jellyfish. Minamoto et al. (2017) and Gaynor et al. (2017) were the first to examine its use on scyphozoans and found the technique could detect both medusa and free-swimming larval stages (planula larvae and ephyra). Minamoto et al. (2017) reported that the distribution of the jellyfish's eDNA in surface waters corresponded with the presence of the jellyfish, highlighting the technique's potential as an ecological survey tool. The technique has recently been used to detect cubozoans with Bolte et al. (2021) reporting they could detect the medusoid stage of three species (*Copula sivickisi, Carybdea xaymacana* and *C. fleckeri*). Further, the polypoid stage of *C. sivickisi* was detected near the substratum outside of the jellyfish season, when medusae are not found. Thus, eDNA has passed the proof-of-concept phase for the detection of jellyfish and the study of their ecology (Kingsford et al., 2021, Bolte et al., 2021). To ensure both accurate detection and interpretation of the technique for answering ecological questions, the best available procedures and processes should be utilised.

Probe-based assays are the most specific and accurate means to detect single species via the eDNA approach (Klymus et al., 2020a, Coster et al., 2021). This is due to the use of an additional eDNA identifier, a TaqMan probe (ThermoFisher, 2018, Klymus et al., 2020a). Currently, only a SYBR green assay exists for *C. fleckeri* (Bolte et al., 2021). Probe-based assays allow for quantification of the amount of eDNA present in a collected sample which can aid in interpreting detection results (Klymus et al., 2020a). Probes also allow for multiplexed quantitative Polymerase Chain Reactions (qPCR) and hence the use of internal positive controls (IPC's) to test for inhibitors within

samples, or better yet, use of an endogenous control assay (Furlan and Gleeson, 2016). This positive control monitors for the presence of non-target eDNA in collected water samples, through use of a generic primer assay, to ensure robustness of methodological procedures (Darling and Mahon, 2011, Furlan and Gleeson, 2016). If non-target eDNA is absent from a collected water sample, it highlights potential issues with the sample. Either methodological errors have occurred in one of the workflow steps of the technique, PCR inhibitors are present in the sample, or, in the case of target species detection, contamination has occurred. This positive control hence examines for false negative/false positive detection while allowing for enhanced quality control of the entire eDNA workflow (Furlan and Gleeson, 2016, Vuong et al., 2013). On top of utilising the best available procedures and processes, an understanding of *C. fleckeri*'s eDNA decay is also needed for accurate interpretation of detection results (Barnes and Turner, 2016).

Understanding the persistence of eDNA in the environment is critical for its use as a proxy of species presence (Harrison et al., 2019, Collins et al., 2018). Persistence times ranging from hours/days (Seymour et al., 2018, Ely et al., 2021) to months (Strickler et al., 2015) have been found for differing species and as a result is needed to be investigated on a per species basis (Andruszkiewicz Allan et al., 2021, Harrison et al., 2019). Persistence of eDNA in an environment is influenced by both microbial degradation and transportation, with degradation being the primary cause (Harrison et al., 2019). Multiple abiotic factors (temperature, salinity, UV radiation, water turbidity and pH) have also been reported to both promote and/or slow eDNA decay (Barnes and Turner, 2016, Barnes et al., 2014, Collins et al., 2018, Lamb et al., 2022). As a result, an understanding of the decay of a species eDNA is required for the precise interpretation of detection results (Rees et al., 2014).

The objective of this study was to develop a specific and sensitive eDNA assay for the detection and study of the ecology of the Australian box jellyfish, *Chironex fleckeri*. Specifically, this was undertaken through (1) developing and optimising a *C. fleckeri* specific detection assay, (2) multiplexing the assay with an endogenous control assay for enhanced quality control, (3) *in-situ* testing of the multiplexed detection assay, and (4) determination of the decay rate of *C. fleckeri* eDNA at different temperatures.

2.3 Materials and Methods

2.3.1 Sequence Database Creation

A database of all currently available mitochondrial DNA 16S ribosomal RNA gene (16S) sequences for *C. fleckeri* and sympatric species (Table 2.1), from the GenBank nucleotide database (National Centre for Biotechnology Information, NCBI: Coordinators 2015), were assembled using Geneious Prime 2021.2.2 (http://www.geneious.com). The 16S gene was selected due to it consisting of highly conserved sequences interspersed with species-specific variable regions, making it ideal for species-specific assay design (Deagle et al., 2014). To supplement available sequences, genomic DNA (gDNA) was extracted from eight *C. fleckeri* medusae specimens collected from four geographically distinct locations across northern Australia (Figure 2.1) and from sympatric jellyfish species known to occur within *C. fleckeri*'s range (Table 2.1). Specimens were collected as part of previous work undertaken by the Reef and Ocean Ecology Laboratory (ROEL), James Cook University (JCU), shared by collaborators, or were captured and provided by Surf Life Saving Queensland (SLSQ) (Table 2.1). Genomic DNA was extracted through use of the Qiagen DNeasy Blood and Tissue Kit (Qiagen, Venlo, Netherlands) as per manufacturers protocol (exception of conducting overnight tissue lyse). An end-point PCR was then performed to isolate a 584 bp length of the 16S gene, through use of universal jellyfish 16S primers, as per Bolte et al. (2021) (Table S1.1). PCR product, confirmed to be the desired sequence via visualisation on an agarose gel (1.5% gel, 60 V for 40 min against an Easy Ladder 1 and negative control), was then sent to the Australia Genome Research Facility (AGRF) for clean-up and bidirectional Sanger sequencing. Returned sequences were BLASTn searched, through use of the NCBI database, to verify their taxonomic identity, and finally all *C. fleckeri* and sympatric species sequences were aligned (MUSCLE with 10 iterations) with single nucleotide polymorphisms (SNPs) between individual *C. fleckeri* sequences being noted for consideration in assay design. A tree was also assembled utilising IQ-TREE v1.6.12 (Trifinopoulos et al., 2016) (ultrafast bootstrap analysis; Hoang et al. (2018)) to examine the intraspecific similarity of *C. fleckeri* sequences and interspecific divergence between *C. fleckeri* and sympatric species for the 16S gene. This tree displayed both sufficient intraspecific sequence similarity and interspecific divergence, confirming appropriate use of the 16S gene for primer and probe design (Figure S1.1).

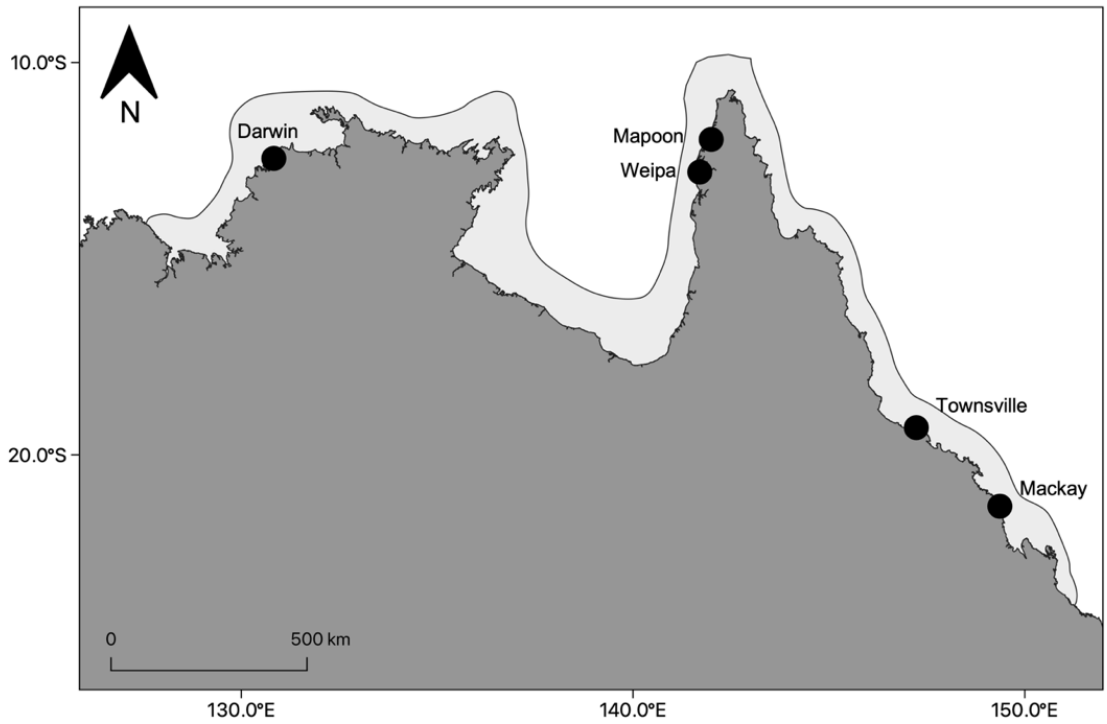

Figure 2.1. Map displaying the range of *C. fleckeri* in northern Australia (light grey) and collection locations of reference specimens (Darwin - n = 1, Weipa - n = 3, Townsville - n = 2 & Mackay - n = 2).

Table 2.1. Cubozoan sequence database utilised for assay design, with NCBI accession numbers and country of origin, for both existing (E) and new (N) 16S sequences (ROEL – Reef and Ocean Ecology Laboratory, SLSQ – Surf Life Saving Queensland).

Species	Accession No.	Country of Origin	Source
Chironex fleckeri	GQ849101 (E)	Darwin, Australia	Bentlage et al. (2010)
	GQ849102 (E)	Weipa, Australia	Bentlage et al. (2010)
	GQ849103 (E)	Weipa, Australia	Bentlage et al. (2010)
	OP877024 (N)	Townsville, Australia	SLSQ
	OP877025 (N)	Townsville, Australia	SLSQ
	OP877026 (N)	Weipa, Australia	Mooney and Kingsford (2012)
	OP877027 (N)	Weipa, Australia	Mooney and Kingsford (2012)
	OP877028 (N)	Weipa, Australia	SLSQ
	OP877029 (N)	Mackay, Australia	Mooney and Kingsford (2012)
	OP877030 (N)	Mackay, Australia	Mooney and Kingsford (2012)
	OP877031 (N)	Darwin, Australia	Mooney and Kingsford (2012)
Carukia barnesi	GQ849097 (E)	Cairns, Australia	Bentlage et al. (2010)
	GQ849098 (E)	Cairns, Australia	Bentlage et al. (2010)
	OP877033 (N)	Palm Cove, Australia	Sample provided by Jamie Seymour to ROEL
Alatina alata	GQ506980 (E)	Osprey Reef, Australia	Bentlage et al. (2010)
	OP877035 (N)	Waikiki, Hawaii	Sample provided by Lisa Gershwin to ROEL
Carybdea xaymacana	KT288254 (E)	Puerto Rico, Caribbean	Acevedo et al. (2019)
	GQ849114 (E)		Bentlage et al. (2010)
	GQ849115 (E)	Panama, Caribbean	Bentlage et al. (2010)
	GQ849118 (E)	Panama, Caribbean	Bentlage et al. (2010)
	OP877034 (N)	Panama, Caribbean Townsville, Australia	ROEL
Copula sivickisi	GQ849113 (E)	Cairns, Australia	Bentlage et al. (2010) Bolte et al. (2021)
	OP877032 (N)	Townsville, Australia	ROEL
Tamoya ohboya	HQ824528 (E)	Bonaire, Caribbean Netherlands	Collins et al. (2011)

2.3.2 Assay Design

To design candidate primers and probes (eDNA identifiers), the sequence database was analysed through use of Geneious Prime and Allele ID (Version 2021.2.2; Premier Biosoft, California, USA). These bioinformatic software's suggest candidate identifiers, based upon several criteria (base-pair length, melting temperature, GC content, presence/absence of hairpins and GC clamps, occurrence of self- and hetero-dimer formation), which may be suitable for species-specific eDNA detection (Klymus et al., 2020a, Prediger, 2013). These suggested identifiers were also further assessed through use of OligoAnalyzer (Owczarzy et al., 2008) and the Sequence Manipulation Suite (Stothard, 2000). Once candidate identifiers passed the above selection criteria, intraspecific sequence similarity, incorporating previously identified SNPs, was examined and inappropriate candidate identifiers were excluded from further assessment. Interspecific sequence divergence was then examined and candidate identifiers with significant mismatches in the 3' end for primers and the 'middle' for probes to sympatric species sequences were kept for further *in-silico* analysis. Finally, remaining candidate identifiers were checked for specificity to *C. fleckeri* through use of NCBI's Primer-Blast (Ye et al., 2012) and Blast N (Altschul et al., 1990) assessment tools. Candidate primers and a minor groove binding (MGB) TaqMan probe, which passed all *in-silico* assessments, were ordered (Applied Biosystems, ThermoFisher Scientific Pty Ltd) for *in-vitro* validation and optimisation.

2.3.3 Assay Validation and Optimisation

The candidate *C. fleckeri* assay was examined to ensure *in-silico* specificity occurred *in-vitro*. This was conducted via use of an exclusion qPCR. The assay was tested against ~80 ng of *C. fleckeri* and sympatric species gDNA in triplicate reactions using a QuantStudio 3 Real-Time PCR system (Applied Biosystems, ThermoFisher Scientific Pty Ltd, Victoria, Australia). Each reaction contained gDNA template, 5 µl of TaqMan Environmental Master Mix 2.0 (Life Technologies, Carlsbad, California, United States), 0.7 µM sense and anti-sense primers, 0.25 µM TaqMan probe and adjusted with MilliQ water to a final volume of 10µl. A two-step cycling profile was utilised; 95 °C for 10 min, followed by 50 cycles of 95 °C for 15 s and 60 °C for 1 min. Any amplicons from sympatric species were sent to AGRF for clean-up and bidirectional Sanger sequencing for verification.

To ensure optimal working conditions of the *C. fleckeri* assay, optimisation trials concerning annealing temperature (T_a) and primer concentrations were undertaken. The optimal T_a of the assay was determined through use of an end-point temperature gradient PCR (primers only). This was centred ~5 °C below the predicted nearest neighbour melting temperature of the sense and anti-sense primers (65 °C), ranging from 50 °C to 65 °C, in triplicate reactions using an Eppendorf Mastercycler Nexus GSX1 (Eppendorf Pty Ltd, Hamburg, Germany) (thermocycling conditions: 95 °C for 3 min, followed by 50 cycles of 95 °C for 30 s, 60 °C for 30 s and 72°C for 1 min, with final extension step of 72 °C for 10 min). Each reaction contained gDNA template, 2.5 µl X10 PCR buffer, 0.5 µl of dNTP's, 1.5 µl of $MgCl_2$, 0.5 µM sense and anti-sense primers, 0.1 µl Taq polymerase and adjusted with MilliQ water to a final volume of 25 µl. Optimal T_a was chosen based on presence and intensity of a single band (no primer-dimer) as visualised on a 1.5% agarose gel (60 V for 40 min against an Easy Ladder 1 and a negative control). Optimal primer concentrations, which allowed for quickest amplification, were determined via a concentration gradient qPCR. Concentrations ranging from 200 to 900 nM at 100 nM increments, with a constant probe concentration of 250 nM, were tested in triplicate. Reaction and cycling conditions were as per the above exclusion qPCR. The optimal primer concentration was chosen based on reliable target amplification at the lowest number of quantification cycles.

2.3.4 Assay Efficiency and Sensitivity

Efficiency and sensitivity of the *C. fleckeri* specific TaqMan assay was assessed through use of standard curves. Synthetic DNA (sDNA; gBlocks – Integrated DNA Technologies Pty Ltd, New South Wales, Australia) of 200 bp in length, and designed with a 7 bp reverse complemented region (cross contamination control), was utilised as the standard for this assessment due to the ease of calculating copy number. The sDNA fragment was designed based on the 16S *C. Fleckeri* consensus sequence utilised for assay design (See 2.3.1). sDNA was resuspended as per manufacturer instructions and concentration was quantified via use of a Quantus Fluorometer (Promega; Madison, Wisconsin, United States). Copy number was calculated through conversion of the determined concentration (10 ng μl^{-1}) utilising Avogadro's constant, double-stranded molecular weight and the known bp length of the sDNA sequence (as per manufacturer instructions). A 10-point standard curve using 10-fold serial dilutions of sDNA, ranging from a theoretical 1 billion to one copy μl^{-1} was utilised to examine assay efficiency. This was calculated using the QuantStudio Design and Analysis Software (Version 2.6.0). Standards ranging from a theoretical 1000 to 0.01 copies μl^{-1}, again using 10-fold serial

dilutions of sDNA, were further utilised to examine the assay's Limit of Detection (LoD) and Quantification (LoQ). Nine replicates per standard were run. The Generic qPCR LOD calculator R script (Klymus et al., 2020b) was utilised to calculate these metrics. A Weibull type II two parameter function which an upper limit of one was selected as the best fitting model (lack of fit test: $F(52, 4) = 0.056$, $p = 0.99$) and was subsequently used to determine the effective LoD for each quantity of qPCR replicate.

2.3.5 Endogenous Control Assay and Multiplexed qPCR

To enhance quality control of this genetic tool, an endogenous control assay was multiplexed with the *C. fleckeri* assay. A 16S endogenous control assay developed by Furlan and Gleeson (2016) (generic fish assay) was selected for use (Table 2.2). *In-silico* testing was undertaken to ensure the assay would also work for marine samples. It was tested against 16S and complete genome sequences of common marine fish species (obtained from the GenBank nucleotide database; NCBI: Coordinators 2015) which are known to reside in North Queensland, Australia (Table S1.2). The endogenous assay was further checked for hetero-dimer formation with the *C. fleckeri* assay and was assessed using NCBI's Primer-Blast tool (Ye et al., 2012) to ensure it would not amplify *C. fleckeri* DNA. Following these *in-silico* assessments, the *C. fleckeri* and endogenous control assays were multiplexed, and *in-vitro* trials were undertaken.

Specificity of the species-specific assay was re-examined in the presence of the endogenous control assay to ensure it did not promote the amplification of other cubozoan species. This was again undertaken using a QuantStudio 3 Real-Time PCR system, in triplicate reactions. Each reaction contained gDNA template, 10 µl of TaqMan Environmental Master Mix 2.0, 0.7 µM sense and anti-sense *C. fleckeri* specific primers, 0.25 µM *C. fleckeri* specific TaqMan probe, 0.525 µM sense and anti-sense endogenous control primers, 0.25 µM endogenous control TaqMan probe and adjusted with MilliQ water to a final volume of 20 µl. A two-step cycling profile was again utilised (See 2.2.3). Any amplicons from sympatric species were sent to AGRF for clean-up and bidirectional Sanger sequencing for verification. The species-specific assay's efficiency was also re-examined to ensure addition of the endogenous control assay had no influence upon it. Two five-point standard curves using 10-fold serial dilutions of sDNA, ranging from a theoretical 500 million to 50 thousand copies μl^{-1}, with three replicate samples of each dilution, were undertaken. The first of the two standard curves examined efficiency of the *C. fleckeri* assay alone with the second examining its efficiency when multiplexed with the endogenous control assay. The endogenous assay was used at 0.75X the

concentration of the *C. fleckeri* specific assay to ensure it did not compete with the *C. fleckeri* assay for available reagents. Each assay also utilised differing fluorophores (*C. fleckeri*; FAM – 495-520 nm. Endogenous; VIC – 538-554 nm) which fluoresce at different wavelengths to ensure each was easily distinguishable from the other.

2.3.6 eDNA Collection and Preservation

Collection and handling of eDNA is needed to be undertaken with utmost care due to risk of both false negative and false positive detection (Roussel et al., 2015, Darling and Mahon, 2011, Rees et al., 2014, Xiong et al., 2016). Prior to entering the field, all equipment utilised to collect and filter eDNA samples were both bleached (10% solution for 24 h), thoroughly rinsed with reverse osmosis (RO) water, and exposed to UV (1 h). This was to ensure any potential DNA contaminates were removed. The equipment was also appropriately packaged to further remove risk of contamination while sampling in the field.

For each collection site, duplicate 2 L water samples were collected. The 2 L volume was chosen due to previous successful detection (Bolte et al., 2021). This was undertaken through utilising sterile 2 L jars which were held windward of the boat with use of a 'jar holder', as a sort of advection current to limit potential contamination. Water samples were collected from the top 0.5 m of the water column and were filtered in the field immediately after collection (to limit potential eDNA decay and loss of sample). Samples were filtered through use of 300 ml MicroFunnel Filter Funnels (Pall, New York, United States), 5 µm nylon net filters (Merck, New Jersey, United States) and a peristaltic pump. Filters were rolled and cut in half, with each filter half being placed into separate 2 ml microtubes containing 1.5 ml of Longmires buffer. Longmires buffer is ideal for lengthy eDNA storage and transport at tropical ambient temperatures (Edmunds and Burrows, 2020, Cooper et al., 2022). An equipment control, prior to sample collection, was also undertaken. This consisted of filtering 250 ml of MilliQ water to ensure equipment used (collection cup, forceps, and scissors) to collect the subsequent sample was not contaminated. New sterile equipment (collection cup, filter funnel, forceps, and scissors) and gloves were used/worn for every replicate sample to inhibit potential contamination. All samples upon completion of field work were stored in temperatures of 4 °C until processed.

2.3.7 eDNA Extraction and Purification

To extract the collected eDNA from the filters, the PPLPP (preserve, precipitate, lyse, precipitate, and purify) method, developed by Edmunds and Burrows (2020) and adapted for extraction from filter papers stored in 2 ml microtubes by Cooper et al. (2021), was utilised. The PPLPP method first precipitates extracellular DNA via NaCl and isopropanol, a lysis step (chemical and mechanical) is then undertaken, followed by a second precipitation step utilising polyethylene glycol, and finally the extracted eDNA is washed twice in 75% ethanol and eluted in elution buffer (TE buffer). This extraction technique was utilised as, combined with the use of Longmires buffer to store samples, it was found to yield a significantly higher concentration of eDNA in comparison to the widely used Qiagen DNeasy extraction kit (Cooper et al., 2021). Following extraction of collected eDNA, it was purified through use of the Zymo One Step PCR Inhibitor Removal kit (Zymo IR; Zymo Research; Irvine, California, United States), as per manufactures instructions. Purified DNA was stored in -20°C conditions until quantified.

2.3.8 Quantitative PCR

The multiplexed assays were utilised to detect, quantify, and interpret presence of *C. fleckeri* eDNA via qPCR. QuantStudio 3 and 5 Real-Time PCR systems (Applied Biosystems, ThermoFisher Scientific Pty Ltd, Victoria, Australia) were utilised for this purpose. Each reaction consisted of 2 µl of eDNA template, 10 µl of TaqMan Environmental Master Mix 2.0, 0.7 µM sense and anti-sense *C. fleckeri* primers, 0.525µM sense and anti-sense endogenous control primers, 0.25 µM of both *C. fleckeri* and endogenous control TaqMan MGB probes, and adjusted with MilliQ water to a final volume of 20 µl. A two-step cycling profile was utilised; 95 °C for 10 min, followed by 50 cycles of 95 °C for 15 s and 60 °C for 1 min. Each replicate water sample had six technical replicates, and each plate contained at least three negative controls, extraction blanks (negative control monitoring contamination during extraction procedures), a positive control (*C. fleckeri* gDNA template) and sDNA standards (10 thousand to one copy μl^{-1}) which acted to ensure consistency among plates and allowed for quantification. qPCR plates were prepared in a laminar flow hood to minimise potential contamination. Positive detection of *C. fleckeri* for a water sample was reported with minimum amplification of a single technical replicate. Replicate samples were combined for reporting purposes. Any positive detection was confirmed to be *C. fleckeri* through clean up and bidirectional Sanger sequencing of PCR product (undertaken by AGRF) and cross checking against reference sequences.

2.3.9 In-situ Validation of Multiplexed Assays

The aim of *in-situ* sampling was to detect *C. fleckeri* medusae where they were known to be present, and therefore, to validate the multiplexed assay method. eDNA samples were collected from Horseshoe Bay on Magnetic Island (19°07'03"S, 146°51'09"E) and outside the Port of Weipa, Queensland, Australia (12°41'01"S, 141°48'14"E) during the 2020 jellyfish season (15th of December and 1st of December, respectively) (Figure 2.2). Sampling sites were chosen based on locations known to have, or have had, *C. fleckeri* medusae. At Horseshoe Bay, three shore sites (sites 1, 3, 4) were sampled, along with a site within the creek located within the bay (site 2). Two additional sites, one along the shore (site 6) and one within a creek (site 5), in neighbouring Maud Bay, were also sampled. SLSQ reported the presence of *C. fleckeri* medusae within Horseshoe Bay in the week prior to sampling. Near Weipa, two sites were sampled along the beach at the entrance to the Port. Seine netting to catch medusae was utilised to ground truth any positive detections, with a single individual (2.5cm IPD) being caught (site 2).

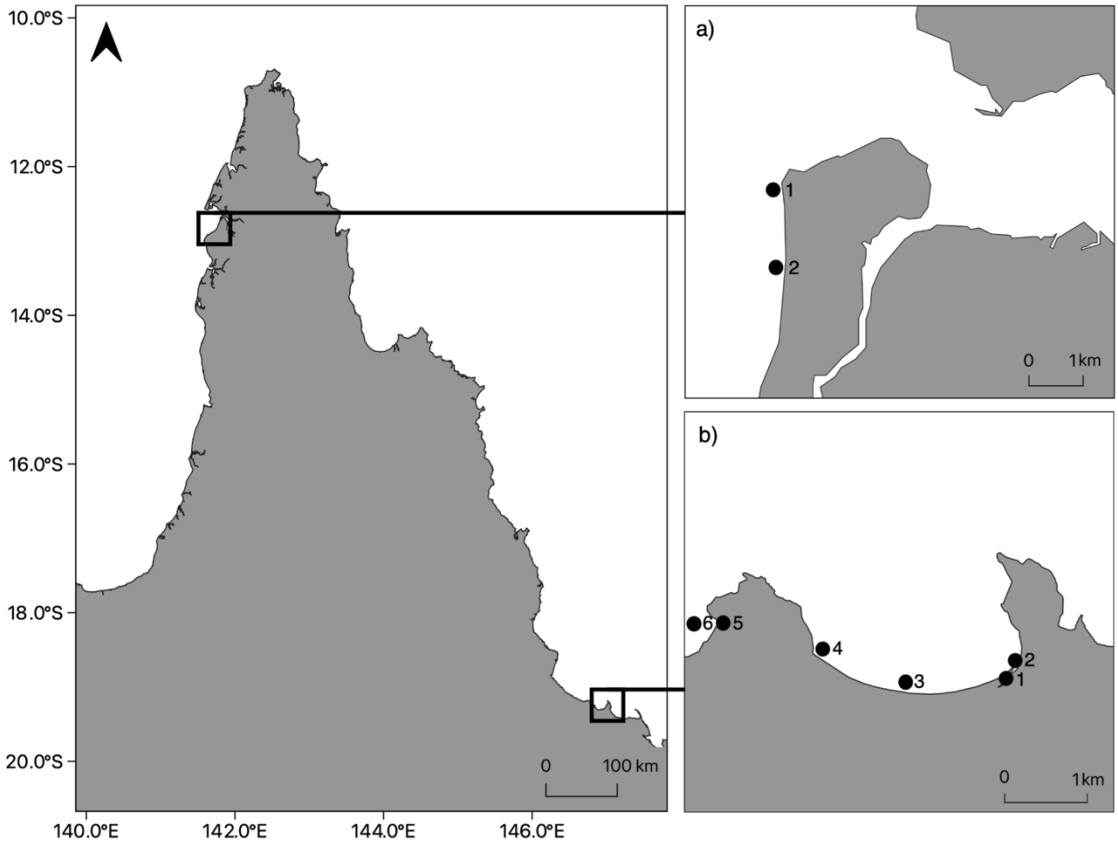

Figure 2.2. Map displaying *in-situ* sampling sites (black dots) located at Weipa, Queensland (a) and Horseshoe Bay, Magnetic Island (b).

2.3.10 Effect of Temperature upon *Chironex fleckeri* eDNA Decay

2.3.10.1 Collection of Study Organisms

C. fleckeri medusae were collected at Port Musgrave, Mapoon in December of the 2020 jellyfish season (Figure 2.1). The animals (n = 18 medusae) were collected with a 40 m beach seine. All animals were collected in accordance with permit number 207602 (Department of Agriculture and Fisheries, Queensland State Government) and captured medusae were transported in large drums to Weipa, Queensland, where experimentation was undertaken.

2.3.10.2 Experimental Design

Seawater from a ~1800 L tank containing *C. fleckeri* medusae (n=18, average inter-pedalia distance (IPD) of 3.2 cm) was utilised to examine the species eDNA decay rate. All medusae were placed into the tank for a 2 h acclimation/shedding period to ensure sufficient and well dispersed *C. fleckeri* eDNA throughout the tank. The tank was continuously circulated (closed system), maintained at an ambient temperature (27 °C) and 30 L of seawater, post acclimation, was then transferred into 24 individual tanks (closed systems). Four replicate 2 L samples were also taken from the large tank at this point to serve as time zero samples. The 24 tanks were randomly assigned a sampling time, approximately one (27 h), three (73 h) or six days (140 h) after removal from the tank (n=8 samples per time), and half (n=4 samples) were further randomly assigned to one of two temperature-controlled water baths. These baths were maintained at 26 °C and 28 °C, through use of Aqua Medic Titan 2000 cooling units. Tinytag TG-3100 data loggers were placed within each bath to confirm stable temperatures for the duration of the experiment (Figure S1.2), and the temperature of each experimental water replicate was checked daily until sampled. A positive control sample, from the large holding tank containing *C. fleckeri* individuals, was taken to confirm detection, or lack thereof, of *C. fleckeri* at each sampling time within the experimental water. All equipment used to transport and house experimental water was sterilised (10% bleach and UV) prior to use to ensure potential DNA contaminates were removed. A two-way analysis of variance (ANOVA) was utilised to examine differences in eDNA quantities with the factor's times (a=2) and temperature (b=2); all factors were treated as fixed. Tukey's a-posteriori test was used to detect differences between treatments. Data was log transformed to satisfy the assumptions of the statistical test. Tests were conducted using R (Version 4.1.2).

2.4 Results

2.4.1 Assay Design and in-vitro Validation

Sense and anti-sense primers and a 5' FAM labelled TaqMan probe with a 3' MGB nonfluorescent quencher molecule (NFQ) were identified and designed between base pairs 165 and 311 on the *C. fleckeri* 16S gene consensus sequence (Table 2, Figure 2.3). Specific selection characteristics of the identifiers are provided (Table S1.3). Numerous base pair mismatches were observed between the identifiers and sympatric species 16S gene sequences (Figure 2.3). A minimum of 17 (*A. alata*), and a maximum of 33 (*T. ohboya*) total base pair differences were noted (Table S1.4). Subsequently, all exclusion qPCR tests revealed the TaqMan assay to amplify only the 147 bp 16S *C. fleckeri* target sequence, hence confirming the assay's specificity.

All tests showed strong amplification of *C. fleckeri* synthetic DNA and genomic DNA from all four geographically distinct locations, covering the range of the species across northern Australia (Figure 2.1). Optimisation trails indicated an optimal T_a of 60 °C, which matched the T_a of the endogenous control assay (60 °C), and optimal primer concentrations of 700 nM with a probe concentration of 250 nM. All negative controls showed no amplification of either *C. fleckeri* or marine fish DNA in all *in-vitro* trials.

Table 2.2. Species-specific and endogenous control assays used to detect *Chironex fleckeri* and as technical controls.

Assay	Label	Sequence (5'-3')	Target Sequence Length (bp)
Species-specific Assay	Chironex_16S_F Chironex_16S_R Chironex_16S_P	ATCTTCCACTGTCTCAGCTTTACC CCTCAGTACTCGTGTCTCCCTA (FAM)-CTCGTCCTTCCAAGTATAAG-(MGB)	147
Endogenous Control Assay	Fish_16S_F Fish_16S_R Fish_16S_probe	GACCTCGATGTTGGATCA CTCAGATCACGTAGGACTTTA (VIC)-ACATCCTAWTGGTGC-(MGB)	87-88

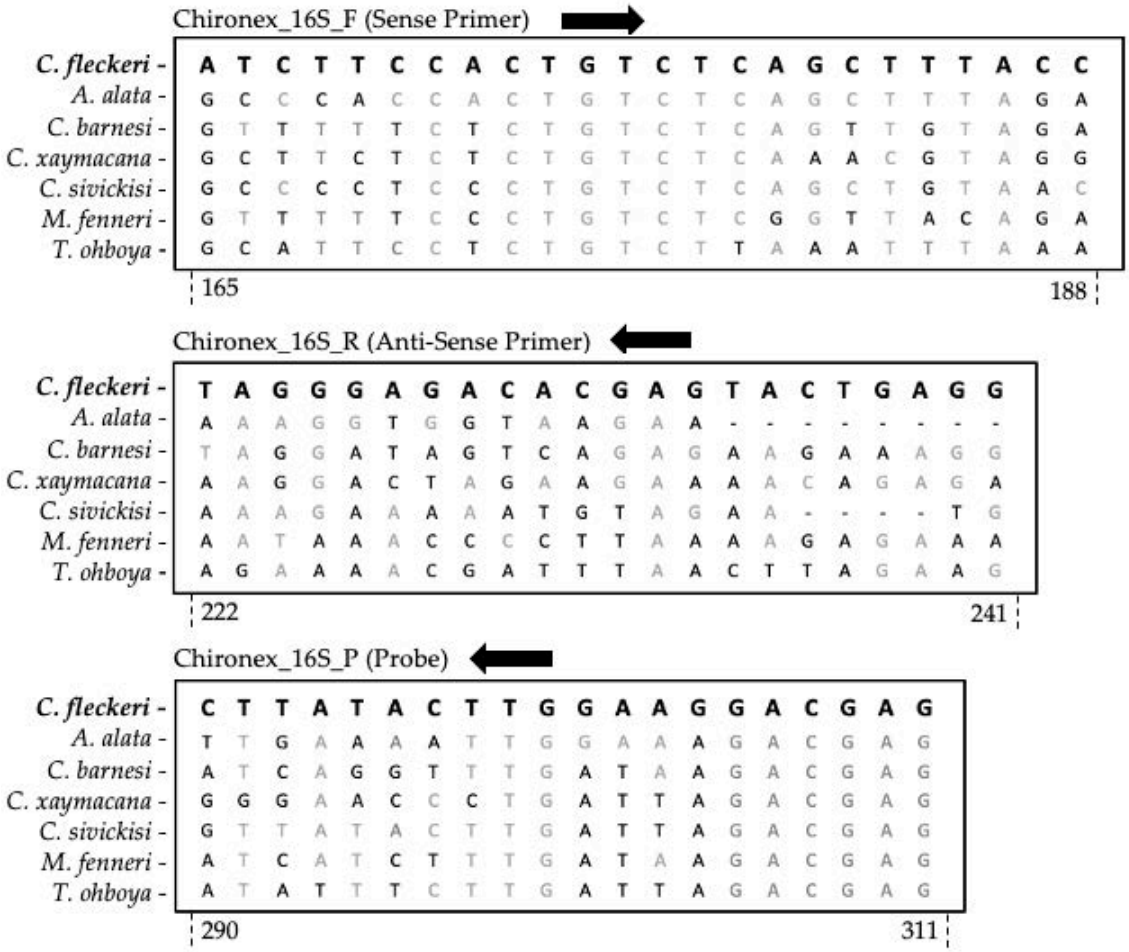

Figure 2.3. Alignment highlighting base pair similarities (grey) and differences (black) between the *Chironex fleckeri* eDNA identifiers and sympatric jellyfish species. The anti-sense primer is illustrated as the reverse complement of the sequence. (*Chironex fleckeri*; OP877024, *Copula sivickisi*; OP877032, *Carukia barnesi*; OP877033, *Alatina alata*; OP877035, *Carybdea xaymacana*; OP877034, *Tamoya ohboya*; HQ824528). Base pair start and end position of each identifier on the consensus sequences is indicated below each alignment.

2.4.2 Assay Efficiency and Sensitivity

The *C. fleckeri* assay was found to have both a high efficiency and sensitivity. A 10-point standard curve using 10-fold serial dilutions of sDNA, ranging from a theoretical 1 billion to one copy μl^{-1} showed the *C. fleckeri* assay to have an appropriate efficiency (efficiency = 93.9%, slope = -3.47**,** R^2 = 0.994**)**. The assay was also found to have a high sensitivity with an effective LoD, using six technical replicates for each replicate water sample, of 0.45 copies/reaction, and a modelled LoQ of 6 copies/reaction (Figure 2.4).

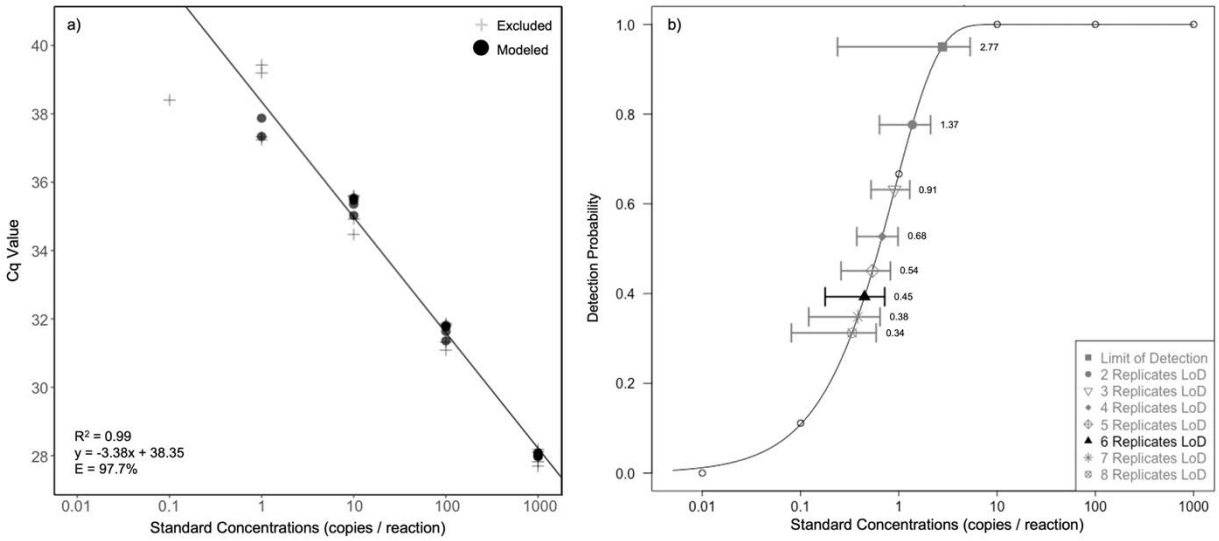

Figure 2.4. *Chironex fleckeri* assay sensitivity. A) Standard curve plot from a six point ten-fold serial dilution of synthetic DNA, from theoretical 1000 to 0.01 copies μl^{-1}. Grey pluses (+) are replicates which fall outside the middle two quartiles for standards or are standards with less than 50% detection; both of which were excluded from linear regression calculations. B) Effective Limit of Detection (LoD) for each quantity of technical replicates as determined via a Weibull type II two parameter function which an upper limit of one. Effective LoD's are plotted with 95% confidence intervals (n=8) and open circles represent the detection rates of each standard.

2.4.3 Multiplexed qPCR Assays

The endogenous control assay was found to multiplex successfully with the *C. fleckeri* assay (Figure S1.3). Although the endogenous assay was designed to amplify freshwater fish species of Australia, *in-silico* bioinformatic investigations found it to also detect numerous marine fish species common to northern Australia (Table S1.2). In addition, the endogenous assay was found to not amplify any cubozoan DNA (as per NCBI's Primer-Blast tool, and subsequently *in-vitro* testing), hence not effecting the specificity of the *C. fleckeri* assay. The efficiency of the *C. fleckeri* assay was also found to not be influenced by presence of the endogenous control assay (Table S1.5).

2.4.4 In-situ Validation

Detection of *C. fleckeri* was found at all four sampling locations within Horseshoe Bay, Magnetic Island, and at both sites outside the Port of Weipa (Table 3). The amplified target sequences matched the *C. fleckeri* consensus sequence, hence validating the use of the developed *C. fleckeri* assay to detect the jellyfish *in-situ*. Further, all samples displayed amplification of non-target eDNA via use of the endogenous control assay, ensuring appropriate methodological procedures, lack of inhibitors, and further validation of the detection technique. The Maud Bay sample sites at Magnetic Island showed no presence of *C. fleckeri* eDNA, indicating absence of the animal. SLSQ and seine net drags confirmed the physical present of *C. fleckeri* at both Horseshoe Bay and Weipa hence ground truthing positive detections. No jellyfish were collected in Maud Bay.

Table 2.3. Amplification results for *in-situ* validation samples from Weipa, Queensland and Horseshoe and Maud Bay, Magnetic Island. Results for duplicate replicates combined.

Location	Site	*C. fleckeri* assay	Endogenous control assay
Weipa	1	7/12	12/12
Weipa	2	6/12	12/12
Horseshoe Bay	1	2/12	12/12
Horseshoe Bay	2	1/12	12/12
Horseshoe Bay	3	5/12	12/12
Horseshoe Bay	4	3/12	12/12
Maud Bay	5	0/12	12/12
Maud Bay	6	0/12	12/12

2.4.5 *Chironex fleckeri* eDNA Decay and the Influence of Temperature

The eDNA signal of *C. fleckeri* decayed rapidly within the first 27 h of the experiment representing a loss of 99%. This change in eDNA quantity, between time zero and the first sampling time (27 h), was found to be significant for both temperature treatments ($p = <0.0001$). In addition, eDNA quantity was found to be less than 1% of the initial quantity at this sampling time for both the 26 °C and 28 °C temperature treatments (0.94 and 0.28%, respectively) (Figure 2.5).

The decay rate constants for the two temperature treatments were estimated to be 0.068 h^{-1} and 0.057 h^{-1}. The eDNA quantities between the two temperature treatments were not significantly different ($F = 1.11$, $df = 1$, $p = 0.30$, Figure 2.5). Further, no significant difference was found between them at each sampling time ($p = 0.90$ - 27 h; $p = 0.63$ - 73h; $p = 0.74$ - 140 h). Accordingly, all data points from both treatments were combined and the decay rate constant of these points was estimated as 0.060 h^{-1}, with a corresponding half-life of 11.47 h. Positive controls ($n = 3$) from the holding tank contained *C. fleckeri* eDNA, in high quantities at each sampling time, verifying decay of the species eDNA was occurring within experimental tanks. Temperatures of the baths were consistent throughout the experiment (26 °C; 26.17 ± 0.21, 28 °C; 28.19 ± 0.31) and daily monitoring showed consistent temperatures of the experimental water.

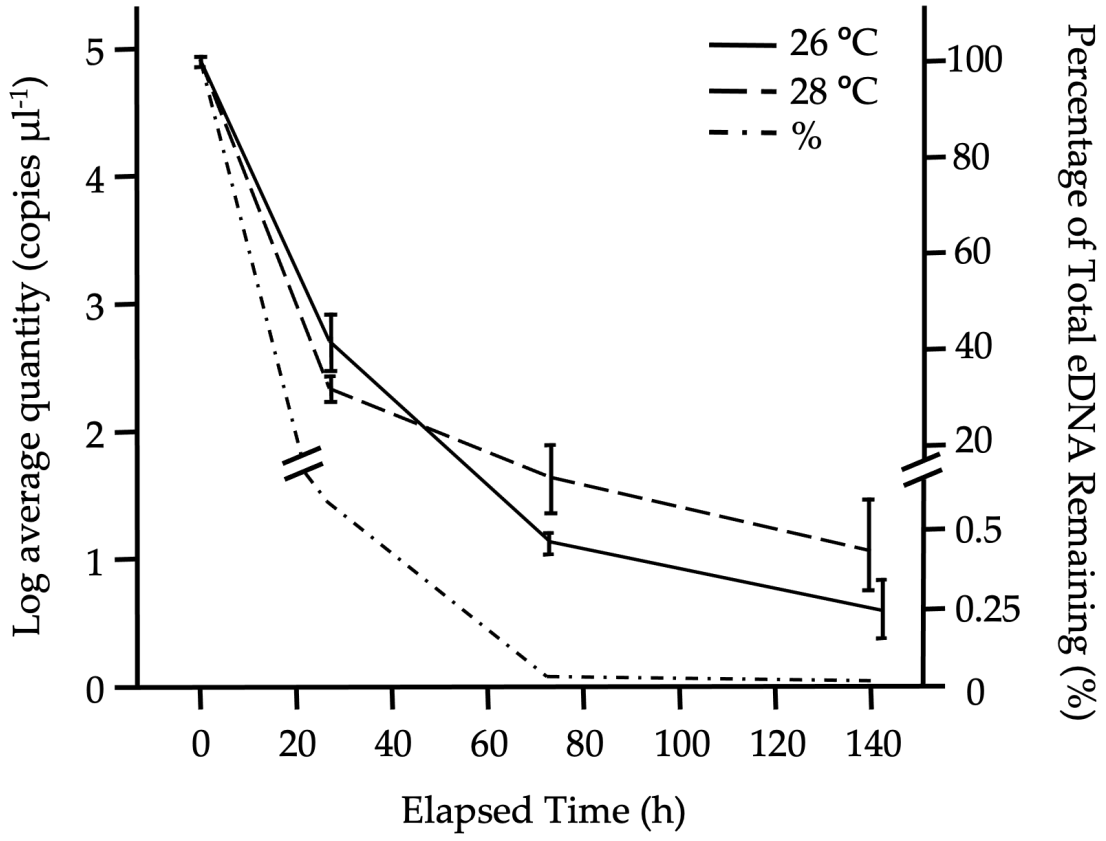

Figure 2.5. Decay of *Chironex fleckeri* eDNA under two temperature treatments (26 °C & 28 °C), displaying log average quantity (copies μl^{-1}) over a 140 hr period, and percentage (%) of total eDNA remaining.

2.5 Discussion

In this study, we have developed and successfully utilised a specific and sensitive eDNA assay for detecting *Chironex fleckeri*. This genetic tool was designed to meet development criteria, and was undertaken with optimised collection, extraction, and best practice control procedures and processes. This ensured the reliability of both positive and negative detection results. A rapid eDNA decay rate (0.060 h^{-1}), with no detectable temperature influence was also shown. Accordingly, the technique is robust to provide crucial information on the spatial and temporal presence of medusae within 3 days of the jellyfish being present. The method can help to determine the risk of envenomation and provide a tool for ecological studies.

2.5.1 Assay Development and Informative Controls

High performing eDNA assays are critical for the detection of cryptic and especially dangerous organisms due to the risk of false positive and false negative detection (Wilcox et al., 2013, Langlois et al., 2021). If an assay fails to be specific to the target species, or fails to be sensitive enough to detect low copy target DNA, errors in detection can occur (Wilcox et al., 2013, Langlois et al., 2021). As a result of this, this study undertook a through design and validation process to ensure the suitability of the developed eDNA detection assay (Klymus et al., 2020a, Klymus et al., 2020b). Firstly, the assay was designed to be specific to only *C. fleckeri* and to detect the species across its range in northern Australia. The ability of the technique to be utilised throughout its range and not just at a single study location is essential for its wide-use application as a detection tool. Locations where the species is known to occur outside of Australia were not considered in this process due to the unavailability of reference specimens. To ensure assay specificity, the eDNA primer and probe sequences were designed to contain extensive mismatches with sympatric species sequences. A minimum of five mismatches across all three eDNA identifiers, and a minimum of three specifically in the probe is sufficient for exclusion of non-target species (Cooper et al., 2021, Klymus et al., 2020a). For the developed *C. fleckeri* assay, total mismatches ranged from 17 to 33, with a minimum of five in each primer and six in the probe, exceeding the minimal recommended mismatches considerably (Cooper et al., 2021, Klymus et al., 2020a). The assay was designed to contain extensive mismatches to ensure and safeguard specificity. Secondly, the *C. fleckeri* assay was found to be highly sensitive. This is critical for detection of cryptic and spatially disperse species as their eDNA is likely to be of low quantities in the environment (Wilcox et al., 2013). Understanding assay sensitivity is

also crucial for appropriate interpretation of detection results and hence was determined through modelling detection (LoD) and quantification (LoQ) limits (Klymus et al., 2020b). The developed assay was found to be highly sensitive (0.45 copies/reaction) and of similar or greater sensitivity to numerous published eDNA detection assays (Klymus et al., 2020b, Wilcox et al., 2013, Budd et al., 2021, Cooper et al., 2021). The high specificity and sensitivity of the *C. fleckeri* assay, in addition to use of optimised collection and extraction methods for filter-based eDNA detection of cryptic species (Edmunds and Burrows, 2020, Budd et al., 2021, Cooper et al., 2021), provides assurance of a powerful and reliable tool for detection of the jellyfish.

In addition to high performing eDNA assays, multiple control measures are required to ensure accuracy of the technique (Darling and Mahon, 2011). Failure to implement adequate controls to monitor the assay's quality and success leave it susceptible to error (Darling and Mahon, 2011, Furlan and Gleeson, 2016). An endogenous control assay was multiplexed with the *C. fleckeri* assay and was critical to identify and exclude vulnerability to false negative detection. Furlan and Gleeson (2016) highlighted the inadequate use of *in-situ* positive controls subsequently leaving the technique vulnerable to false negative detection. For most studies, positive controls are only utilised to monitor the PCR amplification step leaving potential methodological errors occurring in previous workflow steps undetected (Furlan and Gleeson, 2016). Monitoring the success of all steps is crucial for confirming both positive and negative detection results. The endogenous control assay developed by Furlan and Gleeson (2016) was utilised successfully here for marine samples and is an 'all-in-one package' for monitoring method success and inhibitor presence. The benefits of this control outweigh the additional development and operational costs. Accordingly, we concur with Furlan and Gleeson (2016), and encourage the implementation of this additional assay to probe-based workflows, particularly when the technique is being utilised to inform management decisions or investigate ecological hypotheses.

2.5.2 eDNA Decay and Temperature Influence

A rapid decay rate for *C. fleckeri* eDNA (0.060 h^{-1}) was found in this study. This rate is comparable to those found for both *Chrysaora pacifica* and *Copula sivickisi* (Table 4), further adding to the evidence of rapid eDNA decay for jellyfish (Bolte et al., 2021, Minamoto et al., 2017). The rate found for *C. fleckeri* is further comparable to that of multiple marine fish species (Sassoubre et al., 2016), marine invertebrates (Kwong et al., 2021, Wood et al., 2020) and falls within the 10-50 hour half-life range found for the

majority of marine organism eDNA (Collins et al., 2018) (Table 4). The rapid eDNA decay of *C. fleckeri* suggests that positive detections of the animal represent their close proximity. This is especially because they are found in weak nearshore currents and often have limited movements (Schlaefer et al., 2018). Further, in the present study, the rate of decay was quantified in a mesocosm and is probably conservative due to additional unexamined *in-situ* factors, such as transport (Harrison et al., 2019), diffusion (Shogren et al., 2016) and geochemical adsorption (Cai et al., 2006). These are known to further decrease persistence of eDNA in the environment (Harrison et al., 2019). Ely et al. (2021) undertook a study examining this through introducing foreign eDNA (414 g of homogenised *Ctenopharyngodon idella* muscle tissue) into a protected bay and found the signal to be undetectable after 7.5 h compared to the multi-day persistence found in mesocosm studies. *Chironex fleckeri*'s rapid eDNA decay rate is ideal for the techniques use as a proxy for the jellyfish's presence and is beneficial for its potential use as a management and ecological survey tool.

Table 2.4. Reported Decay Rates of Other Jellyfish and Marine Organisms

Species	Decay Constant (h^{-1})	Time to Decay	Experimental Temperature (°C)	Source
Chironex fleckeri	0.060	27 h (99% decay)	26 - 28	Present study
Chrysaora pacifica	0.033	4 days (90% decay)	17.1 - 20	Minamoto et al. (2017)
Copula sivickisi	0.034	9 days (99% decay)	20	Bolte et al. (2021)
Engraulis mordax	0.101	3-4 days (detection limit)	22	Sassoubre et al. (2016)
Sardinops sagax	0.068	3-4 days (detection limit)	18.7	Sassoubre et al. (2016)
Scomber japonicas	0.070	3-4 days (detection limit)	18.7	Sassoubre et al. (2016)
Acanthaster spp.	0.048	96 h (99% decay)	24 - 28	Kwong et al. (2021)
Styela clava	0.104	94 h (detection limit)	19	Wood et al. (2020)
Sabella spallanzanii	0.248	42 h (detection limit)	19	Wood et al. (2020)

Understanding of the influence of abiotic factors upon eDNA persistence in the environment is critical for understanding on the technique's capabilities (Collins et al., 2018, Harrison et al., 2019). It is essential to determine which factors may promote or slow eDNA decay, especially if sampling across differing environments (Collins et al., 2018). This study examined the influence of temperature upon the decay rate of *C. fleckeri* eDNA; however, despite temperature being extensively reported to have influence (Jo et al., 2019, Barnes et al., 2014, Tsuji et al., 2017, Strickler et al., 2015), no effect was found under our experimental temperatures. Similar findings were also reported for Crown-of-Thorns starfish (*Acanthaster* spp.) where a similar temperature range was examined (Kwong et al., 2021). Minor temperature changes, as a result, are unlikely to influence eDNA persistence in the environment. As water temperature varies by only a few degrees Celsius at any one time across *C. fleckeri*'s range in northern Australia (AIMS, 2017), it is unlikely a factor to affect persistence of the jellyfish's signal and subsequently its interpretation. It should be noted, however, that if sampling in areas where water temperatures between sites/seasons vary on a larger scale (10's of degrees Celsius), temperature likely will have an influence as multiple studies (Eichmiller et al., 2016, Strickler et al., 2015) have reported significantly longer persistence times in colder (5 °C) waters. The influence of the remaining abiotic factors should also be examined to further explore the capabilities of the technique, specifically the influence of salinity as *C. fleckeri* commonly reside in estuarine environments. Higher salinities have been reported to decrease eDNA persistence times (Collins et al., 2018). In addition, understanding eDNA dynamics will play a critical role in utilising the eDNA technique in a quantitative manner, which subsequently may allow for quantification of target species abundances (Harrison et al., 2019, Deiner et al., 2017).

2.5.3 Application and Implications of Cubozoan Detection with eDNA

This study successfully detected presence and absence of the medusa stage of *C. fleckeri* at two locations and further displays the successful use of eDNA to detect jellyfish (Minamoto et al., 2017, Bolte et al., 2021, Gaynor et al., 2017, Ames et al., 2021). Further, the technique can be utilised to detect *C. fleckeri* across its range in northern Australia. The assay may also work across the species entire range (Kingsford et al., 2021), however further *in-silico* testing is needed. eDNA provides a means to overcome the challenges associated with jellyfish detection while being both cost effective and less labour-intensive. Additionally it allows for an increased probability of detection as eDNA

disperses into the local surrounding environment (Beng and Corlett, 2020). There is potential to determine eDNA dispersal, hence the distance of detection from the target species, with biophysical modelling (Ellis et al., 2022). In addition to traditional methods, eDNA provides an extra tool for detection, which can be utilised for all species and in any environment. The technique also removes the need to handle highly venomous jellyfish species and makes sampling in environments where other dangerous organisms (e.g., estuarine crocodiles) reside safer. eDNA overall allows for a more efficient and sensitive detection tool.

eDNA clearly has the potential to be utilised as a tool to address critical knowledge gaps surrounding cubozoan ecology and other plankton. It provides a spatially comprehensive detection tool which is key for investigating and exploring species distributions and multi spatial scale population structures (Kingsford et al., 2021). Considerable evidence exists showing cubozoan stocks being of small spatial scales (Schlaefer et al., 2018, Kingsford et al., 2021, Mooney and Kingsford, 2017, Mooney and Kingsford, 2016a) and eDNA may be utilised to further explore this, in addition to traditional genetic approaches (Kingsford et al., 2021). Minamoto et al. (2017) found a correlation between eDNA distribution and visual presence of *C. pacifica* medusae. This and mounting evidence of limited eDNA dispersal (Port et al., 2016, Kelly et al., 2018, Ellis et al., 2022) and rapid jellyfish eDNA decay rates, display eDNA's potential to examine cubozoan spatial and temporal distributions. Further, the potential use of eDNA as a proxy for species abundance/biomass has been demonstrated in the literature for numerous species (Takahara et al., 2012, Pilliod et al., 2013, Lacoursière-Roussel et al., 2016a, Wilcox et al., 2016, Yamamoto et al., 2016, Thomsen et al., 2012). Minamoto et al. (2017) found a direct correlation between eDNA concentration and the number of *C. pacifica* individuals in surface waters highlighting the potential of this for jellyfish. To determine if eDNA can be utilised in this matter for cubozoans, *in-situ* trials and mesocosm experimentation are needed. The ability to gain both distribution and abundance data on cubozoans via eDNA would significantly increase our ability to investigate cubozoan population dynamics.

As most cubozoan jellyfish have both benthic and pelagic life history stages, occurring at differing times, it may be possible to identify source locations (polyp beds) when medusae are absent from waters. Bolte et al. (2021) used eDNA to successfully detect *C. sivickisi* polyps when medusae were absent and the only source of eDNA could be from polyps. The ability to locate sources of polyp beds would be 'game-changing'. Both understanding on ecology and subsequent management of cubozoans would significantly benefit. There is also potential for the technique to be utilised to monitor

potential range expansions of deadly species, which is a concern held by scientists with the oncoming of climate change (Richardson et al., 2009, Madin et al., 2012, Orellana and Collins, 2011, Kingsford and Mooney, 2014). The technique has also already been utilised to determine species responsible for stings via swabbing sting sites on victims, and so may also increase accuracy in data surrounding jellyfish envenomation's (Sathirapongsasuti et al., 2021). Further, Ames et al. (2021) demonstrated the ability of this technique to be utilised in a field-based manner and so there is potential for its use to provide real-time detection data to better inform coastal managers.

2.6 Conclusion

Cubozoan jellyfish are medium to large zooplankters that often pose a threat to humans and business; furthermore, crucial gaps exist in our understanding of their ecology (Kingsford et al., 2018, Kingsford and Mooney, 2014, Crowley-Cyr and Gershwin, 2021). This study has demonstrated the potential for a highly specific and sensitive detection tool, environmental DNA (eDNA), for the dangerous cubozoan species, *C. fleckeri*. Utilisation of optimised methodologies (Budd et al., 2021, Cooper et al., 2021), a species-specific probe-based and endogenous control assay (Furlan and Gleeson, 2016) provided an effective means for cubozoan detection. The study also assessed critical aspects of *C. fleckeri* eDNA dynamics and found a rapid eDNA decay rate (99% in 27 h) that was consistent at different temperatures. This indicates that medusae would have to be in close proximity for detection, and with knowledge of decay rates, biophysical calculation on the sources of eDNA could be undertaken. The eDNA technique is an effective means for cubozoan detection, to address knowledge gaps in their ecology, and has broad applicability to other types of zooplankton.

Chapter 3.

Use of eDNA to Test Hypotheses on the Ecology of *Chironex fleckeri* (Cubozoa)

3.1 Abstract

Considerable gaps in our understanding of cubozoan ecology exist due to challenges associated with their detection. Environmental DNA (eDNA) removes the need for physical identification, offering a new approach to detect and study these elusive taxa. The objective of this study was to utilise eDNA as an ecological tool to test hypotheses surrounding the ecology of *Chironex fleckeri*, through examining the presence of both polyp and medusae life history stages. Additionally, the utility of eDNA as a proxy of abundance was explored. This study was conducted within and outside of Port Musgrave, a semi-enclosed estuarine system in northern Australia. eDNA proved successful in detecting both life history stages. Polyps were detected during winter when medusae were absent. This detection allowed investigation into potential polyp habitat. Polyps were exclusively detected in habitats characterised by nearby patches of rocky substrata and shallow carbonate reefs, with no detection occurring in mangrove habitats. The highest frequency of medusae detections occurred within Port Musgrave, while detections outside were more sporadic. Through comparing the distributions of both life history stages, evidence suggests that Port Musgrave is likely a population stock of the species, aligning with predictions from biophysical models. Finally, eDNA's use as a proxy of abundance showed a poor relationship which can be attributed to likely higher variance in eDNA concentrations resulting from the jellyfish's spatially dispersed nature. We conclude that eDNA provides a new approach to study cubozoan ecology and will provide critical information needed to mitigate against their threat of envenomation.

3.2 Introduction

Cubozoan jellyfish are a class of marine taxa which contain members described as the most venomous organisms on the planet (Chung et al., 2001, Kintner et al., 2005, Bentlage et al., 2010, Gershwin et al., 2013, Kingsford and Mooney, 2014). Stings from these members can result in severe reactions, hospitalisation of the recipient, and potentially death (Fenner et al., 1996, Fenner and Harrison, 2000, Gershwin et al., 2013). The ability to manage this risk of envenomation is a global challenge faced by stakeholders and decision makers (Kingsford et al., 2018). To overcome this challenge, an increased understanding of the ecology of these organisms is needed (Kingsford and Mooney, 2014). Significant knowledge gaps exist surrounding cubozoan jellyfish life histories and population structures/dynamics (Kingsford and Mooney, 2014). These gaps result from challenges associated with their detection due to the elusive nature of cubozoans, their transparency, and their spatial and temporal variability (Kingsford and Mooney, 2014, Kingsford et al., 2018). Environmental DNA (eDNA) has emerged as a formidable tool in ecological research which significantly enhances our ability to detect and monitor elusive species (Beng and Corlett, 2020). Subsequently, it has been highlighted as a detection tool to overcome some of the above mentioned challenges as the approach removes the need to physically capture and morphologically identify individuals (Jerde et al., 2011, Evans et al., 2017, Sigsgaard et al., 2015, Barnes and Turner, 2016, Smart et al., 2016). This genetic detection tool has recently been applied successfully to detect multiple jellyfish species (Minamoto et al., 2017, Gaynor et al., 2017, Ames et al., 2021, Bolte et al., 2021, Bayha and Graham, 2008) and most recently, has been developed for *Chironex fleckeri* (Morrissey et al., 2022).

Chironex fleckeri is considered the most notorious cubozoan species (Kingsford and Mooney, 2014, Gershwin et al., 2013). It is responsible for over 200 deaths in the Indo-Pacific region and contains the most potent venom of any organism on the planet (Gershwin et al., 2013). In an effort to understand more about this jellyfish's life history, presence and abundance, Morrissey et al. (2022) developed a highly sensitive and specific eDNA detection assay for *C. fleckeri*. Morrissey et al. (2022) further showed that an eDNA approach is able to detect *C. fleckeri* medusae when they have been confirmed as present in an ecosystem. Additionally, as jellyfish eDNA has been found to decay rapidly (Minamoto et al., 2017, Bolte et al., 2021, Morrissey et al., 2022), comparable to that of multiple marine fish and invertebrate species (Sassoubre et al., 2016, Kwong et al., 2021, Wood et al., 2020), eDNA detection likely reflects the close proximity of an individual of the targeted species (Morrissey et al., 2022). eDNA, therefore, allows for

location-specific detections which is significantly advantageous for studying cubozoan jellyfish ecology (Morrissey et al., 2022). In addition to detection, eDNA has previously been utilised as a proxy for species abundance (Pilliod et al., 2013, Lacoursière-Roussel et al., 2016a, Wilcox et al., 2016, Yamamoto et al., 2016, Thomsen et al., 2012). Although this relationship has not been validated for cubozoan jellyfish, if the relationship was established then, in addition to detection, eDNA may allow for further investigation surrounding spatial and temporal abundance variation of cubozoan jellyfish (Morrissey et al., 2022). Thus, such a robust methodology would allow ecological hypotheses related to cubozoan population ecology to be tested (Kingsford et al., 2021, Morrissey et al., 2022).

Research focus has largely been placed upon detecting the medusae stage of cubozoan jellyfish due to their direct threat to human health and enterprise (Fenner et al., 1996, Fenner and Harrison, 2000, Gershwin et al., 2013, Kingsford et al., 2018, Bolte et al., 2021, Bordehore et al., 2011). However, cubozoans have a polymorphic life history consisting of two major stages, the medusae and polyp stages (Kingsford et al., 2018, Kingsford and Mooney, 2014). Polyps, due to their tiny size (1-2 mm), are a challenge to detect and study in their natural environment, and to date, cubozoan polyps have only been located twice; Cutress and Studebaker (1973) located *Carybdea xaymacana* polyps in Puerto Rico, within mangrove channels, and Hartwick (1991a) reported a few *C. fleckeri* polyps in Australia, within an estuarine river. An example of how difficult cubozoan polyps have been to find, Hartwick (1991a) reported that they spent seven years undertaking intense and timely *in-situ* searches within multiple Australian estuarine systems to locate polyps of *C. fleckeri*. Even after this intense surveying only a few polyps were ever found. Consequently, to understand fully the ecology of *C. fleckeri*, a more effective approach to identify and narrow-down areas of the habitat where *C. fleckeri* polyps reside is needed (Morrissey et al., 2022). eDNA has potential here, as Bolte et al. (2021) demonstrated the successful use of eDNA to detect habitat putatively holding polyps of *Copula sivickisi* near the substratum in seasons when medusae were absent. As *C. fleckeri*'s medusae are generally found in defined seasons (Kingsford et al., 2018, Kingsford and Mooney, 2014), there is the potential to identify source locations of polyps when medusae are absent. Accordingly, this would provide information on a critical component of cubozoan jellyfish's population dynamics.

The detection of *C. fleckeri* polyps in an estuary by Hartwick (1991a) led to the assumption that polyps of *C. fleckeri* reside within estuarine environments. Recent evidence however, from the use of statolith microchemistry profiles (Mooney and Kingsford, 2012), has questioned this assumption. Mooney and Kingsford (2012)

concluded that suitable habitat for *C. fleckeri* polyps may extend beyond estuaries to marine environments. The thermo/osmotic tolerances of cubozoans highlight the ability of these organisms to endure a large range of conditions and hence their potential to reside in a range of environments (Rowley et al., 2023, Mooney and Kingsford, 2016b, Courtney et al., 2016a). eDNA may provide more information beyond general environmental conditions to that of specific habitat types, such as mangroves, reefs and seagrass. This would advance our understanding of the requirements of polyps which can be quite specific (Cargo, 1979, Svane and Dolmer, 1995, Brewer, 1984, Zang et al., 2023). Understanding habitat requirements and hot spots of polyps would significantly aid in furthering understanding on cubozoan ecology, specifically sources of medusae and their stock boundaries (Kingsford et al., 2021).

Mesopopulations, or stocks nested within metapopulations are the population units of greatest interest to ecologists as they are largely self-sustained, and are relevant to understanding an organisms ecology (Sinclair, 1988). Despite the general assumption that jellyfish are planktonic and therefore should have high levels of connectivity, there is growing evidence that cubozoan stock boundaries are often at small spatial scales (Kingsford et al., 2021). Data on cubozoan distributions (Kingsford et al., 2021), statolith morphometrics (Mooney and Kingsford, 2017) and statolith microchemistry profiles (Mooney and Kingsford, 2012, Morrissey et al., 2020a, Mooney and Kingsford, 2016a) support restricted distributions and discrete populations for some of the ~50 cubozoan species (Collins and Jarms, 2018). Recently, biophysical modelling has also been utilised to make predictions on the dispersal of medusae and likely stock boundaries (Schlaefer et al., 2020, Schlaefer et al., 2018, Schlaefer et al., 2021). Schlaefer et al. (2020) reported *C. sivickisi* medusae are likely to stay within 2 km of a bay, and that populations were at the scale of hundreds of metres to kilometres wide. Schlaefer et al. (2018) further reported that *C. fleckeri* medusae were retained within a semi-enclosed estuarine bay, when both modelled as passive and swimming, and therefore concluded that cubozoan stocks may often be at the scale of estuaries and bays with unlikely connectivity to other populations. As eDNA can provide spatially comprehensive detection data, it allows for the testing of these model's predictions.

The objective of this study was to employ the box jellyfish (*C. fleckeri*) eDNA assay (Morrissey et al., 2022) to test hypotheses surrounding this species' ecology. Specifically, this study examined the following; (1) *in-situ* testing of eDNA concentrations as a proxy for *C. fleckeri* abundance, (2) the distribution of *C. fleckeri* medusa throughout and outside of a semi-enclosed estuarine system, (3) the use of eDNA to detect the species' elusive polypoid stage, (4) how the distribution of medusae, based on eDNA,

compares with that of the eDNA putatively detected from polyps, and (5) identifying habitats in which polyps are detected.

3.3 Materials and Methods

3.3.1 Study Area

This study was conducted in Port Musgrave, Cape York Peninsula, Australia (11.99°S, 141.91°E). The area is a semi-enclosed shallow estuarine system with a ~3.5 km wide mouth (Figure 3.1). Two major rivers feed directly into Port Musgrave including the Wenlock and Ducie Rivers. Knowledge of *Chironex fleckeri* medusae abundance 'hotspots' (Red Beach) and strong ecological information of the species exists for this area (Schlaefer et al., 2018). Qualitatively, water clarity nearshore in Port Musgrave ranged from 0.2 to 3 m, while along beaches outside of the port, water clarity was greater and estimated to be 3 to 10 m. At each sampling site a conductivity, temperature, and depth device (CTD; Seabird SBE 19 Plus) was utilised to measure both salinity and temperature among sites, and to examine the level of stratification. A high level of stratification could indicate that eDNA would be trapped below a halocline/thermocline (Gray and Kingsford, 2003, Bolte et al., 2021, Littlefair et al., 2021). Given the absence of stratification, the temperature and salinity measurements for each site were reported as average values taken across the water column.

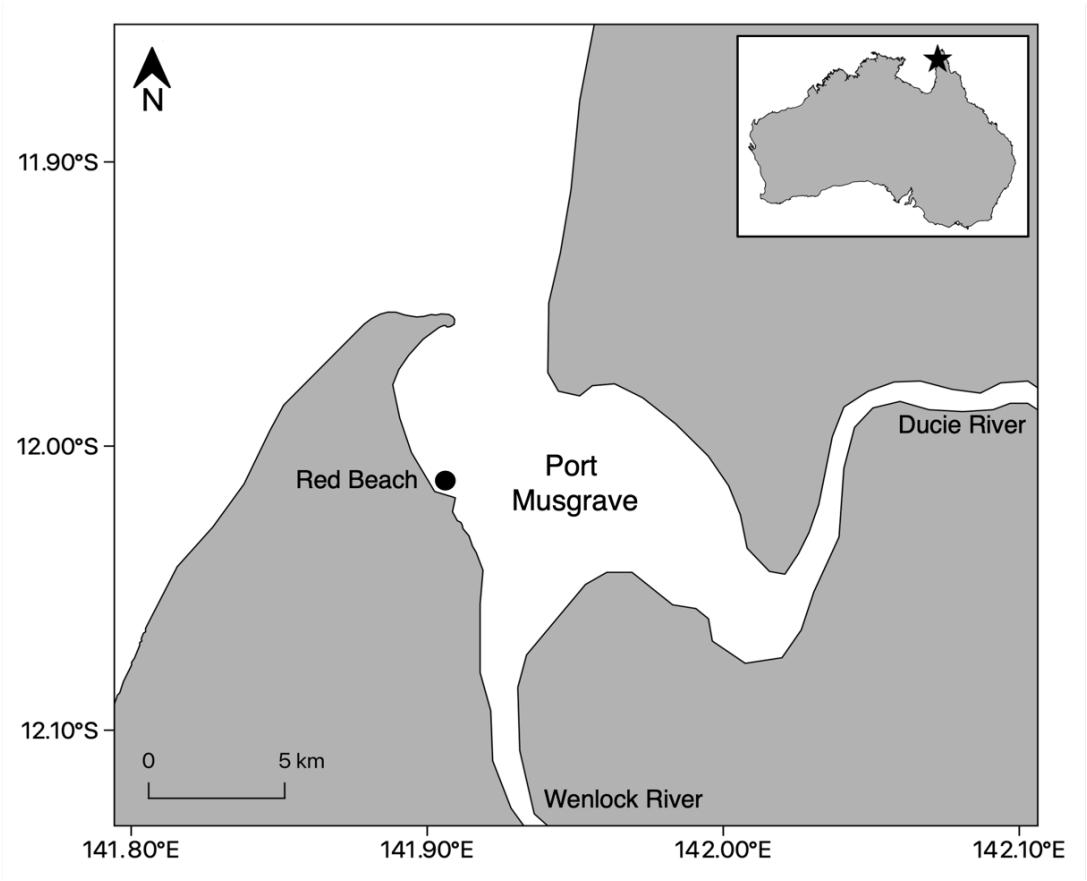

Figure 3.1. Map of the study location, Port Musgrave, Cape York Peninsula in Northern Australia.

3.3.2 Field Sampling

Schlaefer et al. (2018) studied the behaviour and likely dispersal of *C. fleckeri* in Port Musgrave and predicted that medusae were likely to remain in the Port due to favourable oceanography, strong swimming behaviour and an orientation to nearshore environments. We hypothesised, therefore, that abundance and eDNA detections would decrease towards the mouth of the Port and with distance along the coast to the north and south. Sampling for eDNA was undertaken along a gradient leading from a known hotspot, Red Beach, to the mouth of the Port, approximately eight kilometres away (Figure 3.2). Sampling sites were located ~ 600 m apart, including both beach and mangrove habitats. In addition, samples were collected up both major river systems, the Wenlock and Ducie rivers, to examine the extent of which the species occurred within the semi-enclosed estuary. Outside of the Port was also sampled, along a gradient leading into the Gulf of Carpentaria and at sites located along the shore both north and south of the mouth.

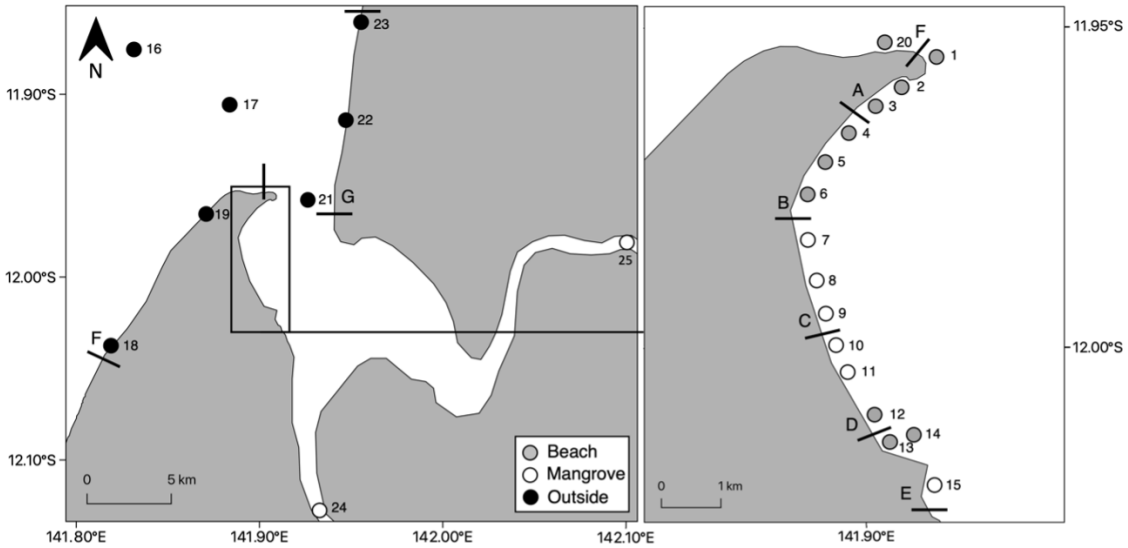

Figure 3.2. Sampling location and sites located inside and outside of Port Musgrave, North Queensland, covering both beach (grey dots), mangrove (white dots) and marine (black dots) habitats. Sampling sites are numbered. Black lines indicate location boundaries which are labelled (A – G).

We also hypothesised that any detection of eDNA in the study area during the Austral winter, when *C. fleckeri* medusae are absent (no detection via seine netting or casual observations) (Hartwick, 1991a, Mooney and Kingsford, 2012, Mooney and Kingsford, 2016b, Gordon and Seymour, 2012), could only be explained by the presence of benthic polyps. Accordingly, sampling was undertaken both during Summer (December 2020) and mid-winter (July 2021) when medusae were respectively present or absent. To confirm the absence of medusae in mid-winter, sampling using seine net drags (mesh size of 3 cm) was undertaken at each sampling site. This ensured no medusae individuals were still present following the previous Australian box jellyfish season as water temperatures were within their tolerance levels (Hartwick, 1991a). The sampling during mid-winter had the potential to identify local hotspots of polyps.

For each sample site, two replicate 2 L water samples were collected. These samples were filtered (10 µm pore size) in the field immediately after collection and were stored in Longmires buffer in temperatures of 4 °C until processed. An equipment control, prior to the collection of each replicate, was also undertaken. Specific details surrounding collection, handling, and storage of eDNA samples can be found in Morrissey et al. (2022).

3.3.3 Jellyfish Abundance versus eDNA Concentration

The estimated abundance of *C. fleckeri* medusae in the field was correlated with copies of eDNA to determine if eDNA could provide a robust proxy for jellyfish abundance. At each site two methods, visual counts, and a beach seine, were used to estimate abundance of jellyfish and to ground truth detections using eDNA. eDNA samples were collected first to avoid potential contamination from the fragments of jellyfish that could have been in a net. Visual transects (1 – 2 m deep) involved using two trained observers located on the bow of the boat. Transect width was measured using a 3 m pole of the bow of the boat and each observer counted in a 1.5 m lane over a 100 m distance. Jellyfish were collected with a 100 x 30 m beach seine net drag (mesh size of 3 cm) with an estimated sample volume of 1200m^3. Seines and visual counts were centred where eDNA samples were collected. Both visual and seine net transects were undertaken for beach sampling sites, while only visual transects were undertaken for mangrove sites where obstacles prevented the use of nets. Number and sizes (IPD – inter-pedalia distance) of all captured *C. fleckeri* medusae were noted.

3.3.4 eDNA Extraction and Purification

The PPLPP (preserve, precipitate, lyse, precipitate, and purify) method (Edmunds and Burrows, 2020), adapted for extraction from filter papers (Cooper et al., 2021), was utilised to extract collected eDNA. Following extractions, the eDNA sample was purified through use of the Zymo One Step PCR Inhibitor Removal kit (Zymo IR; Zymo Research; Irvine, California, United States), as per manufacturer's instructions. Purified eDNA was stored at -20°C until quantified. Specific details surrounding eDNA extractions and purifications can be found in Morrissey et al. (2022).

3.3.5 Quantitative PCR

A multiplexed TaqMan assay developed by Morrissey et al. (2022); Table S2.1) was utilised to detect, quantify, and interpret presence of *C. fleckeri* eDNA via qPCR. QuantStudio 3 and 5 Real-Time PCR systems (Applied Biosystems, ThermoFisher Scientific Pty Ltd, Victoria, Australia) were utilised for this purpose. Each reaction consisted of 2 µL of eDNA template, 10 µL of TaqMan Environmental Master Mix 2.0, 0.7 µM sense and anti-sense *C. fleckeri* primers, 0.525µM sense and anti-sense endogenous control primers, 0.25 µM of both *C. fleckeri* and endogenous control TaqMan MGB probes, and adjusted with MilliQ water to a final volume of 20 µL. A two-

step cycling profile was utilised; 95 °C for 10 min, followed by 50 cycles of 95 °C for 15 s and 60 °C for 1 min. Six technical replicates were run per sample and each plate contained at least three negative controls, extraction blanks (negative control monitoring contamination during extraction procedures), a positive control (*C. fleckeri* gDNA template) and synthetic DNA (sDNA) standards (10 thousand to one copy μL^{-1}) which acted to ensure consistency among plates and allowed for quantification. Positive detection of *C. fleckeri* for a water sample was reported with minimum amplification of a single technical replicate. Any positive detection was confirmed to be *C. fleckeri* through clean up and bidirectional Sanger sequencing of PCR product (undertaken by the Australian Genome Research Facility, Brisbane) and cross checking against reference sequences. Further, use of the endogenous control assay assures appropriate use of methods, their success, lack of false negative detection and PCR inhibitors (Furlan and Gleeson, 2016).

3.3.6 Statistical Analysis

Two measures of positive detection of *C. fleckeri* eDNA from a water sample were used. Each sampling site was utilised as the unit of measure for eDNA concentration (copies L^{-1}). Replicate filters (n = 2) were treated as sub-samples with positive technical replicates being averaged to represent eDNA concentration (copies L^{-1}) at each sample site (Goldberg et al., 2013, Thomsen et al., 2012, Congram et al., 2022). Additionally, detections were also reported as number of positive technical replicates out of 12 per sampling site.

A robust comparison of eDNA concentrations inside and outside of the Port was obtained by analysing clusters of sites that were close together as the factor location. Accordingly, our sampling design addressed variation among locations (a = 7) separated by ~600 metres to 20 km and sites within locations (Table 1 & 3), by hundreds of metres to kilometres (b = 3). The data were tested with a fully hierarchical nested Analysis of Variance that provided a critical test for each level of the design. Furthermore, the proportion of the variation explained for each level of the design was estimated with variance components using the raw data (Kingsford, 1998). Following the recommendations of Underwood et al. (1997) the data were log transformed to satisfy the assumptions of the statistical test. A Kendall's Tau correlation test was utilised to investigate the relationship between eDNA quantity/positive technical replicates and *C. fleckeri* medusa abundance. In all statical analyses critical P was < 0.05. All statistical

analyses were undertaken using R (Version 4.1.2, R Core Team, Vienna, Austria) and SYSTAT (Version 13).

3.4 Results

3.4.1 eDNA Quantity as a Proxy of *Chironex fleckeri* Medusa Abundance

There was a poor relationship between abundance of *C. fleckeri* medusa in seines and eDNA concentration (copies L^{-1}) ($tau_{(n = 17)}$ = 0.15, p = 0.45) and number of positive technical replicates ($tau_{(n = 17)}$ = -0.11, p = 0.59) via this *in-situ* trial (Figure 3.3). eDNA detection of *C. fleckeri* occurred at all sites where medusae were observed in counts and/or were captured in seines. A total of 34 *C. fleckeri* medusa were captured, in beach seines within Port Musgrave during summer. Further, densities of *C. fleckeri* medusa ranged from 0.7 to 21 individuals/1000m^3 (Table S2.2). The poor relationship between densities in seines and eDNA quantity was attributed to the Red Beach sites where large numbers of medusae were collected in nets, but concentrations of eDNA were low (Figure 3.3). Only three individuals were observed with visual transects in the low visibility waters; interestingly only one individual was found in and around mangrove habitats (site 10).

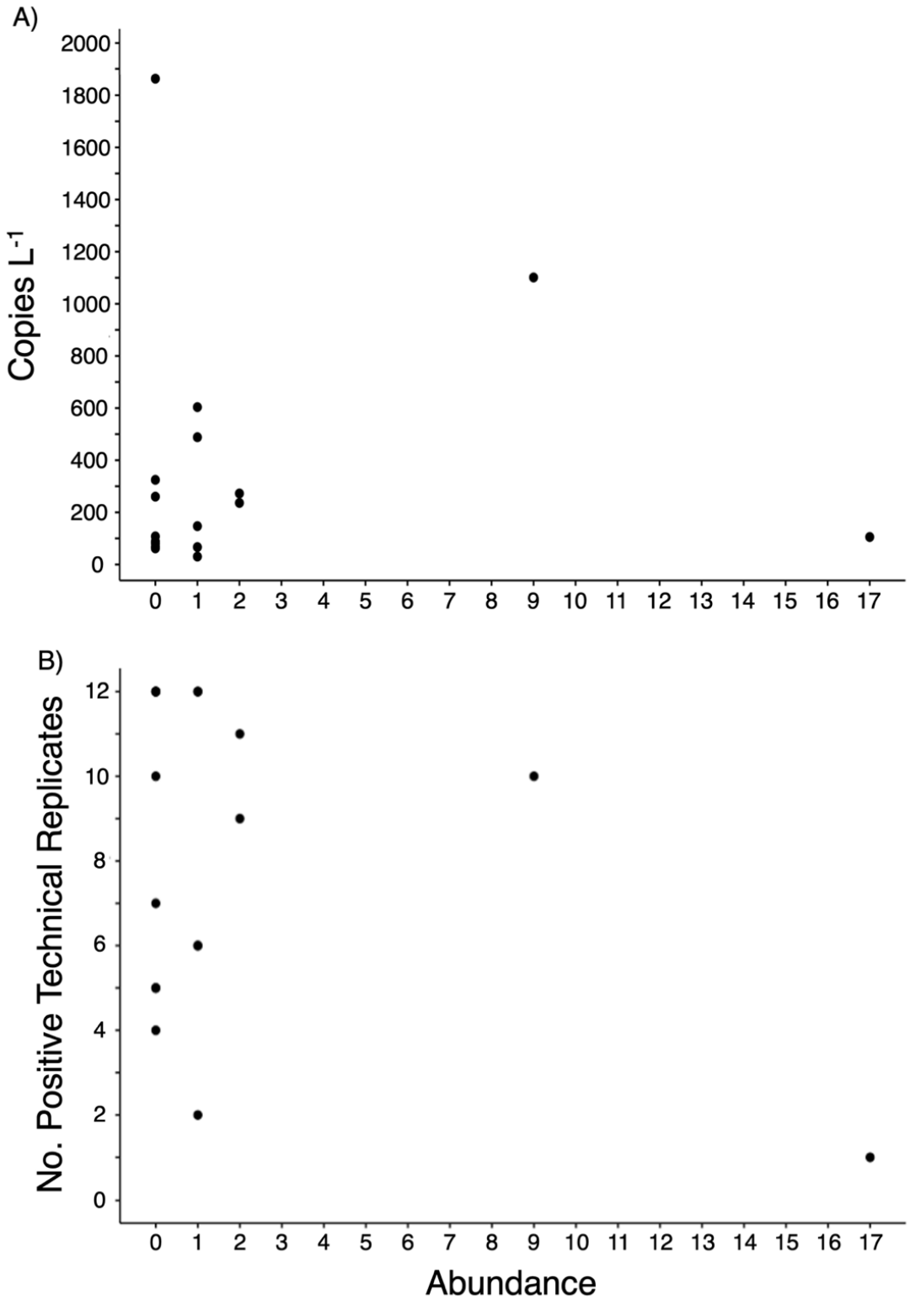

Figure 3.3. Relationship between A) eDNA copies (L^{-1}) and *Chironex fleckeri* medusae abundance, and B) number of positive technical replicates and *Chironex fleckeri* medusae abundance, caught via seine net drags, at each sampling site.

3.4.2 Detection and Distribution of *Chironex fleckeri* Medusae during the Australian Box Jellyfish Season

Chironex fleckeri eDNA was found throughout the study area, both inside and outside of the Port, in summer. eDNA was detected at all 25 sampling sites and in 93 % of field replicates (Figure 3.4 & 3.5). 63.9 % of the technical replicates displayed detection and eDNA copies L^{-1} ranged from 21.91 – 2374.01 copies L^{-1} (Table 1). All equipment controls confirmed the absence of contamination, and the endogenous control demonstrated appropriate use and success of collection, handling, and extraction methods, as well as the absence of PCR inhibitors in analysed samples. There were large differences in eDNA concentrations among sites, however, there were no significant differences among close or widely separated locations, in both the concentration of eDNA (Table 2), and number of positive detections (out of the 12 technical replicates). Differences among locations only represented 0 – 3.5 % of the variation (Table 2), this was especially obvious outside of the Port and near beaches where many copies were recorded at some sites and very little at others. Variation at the replicate level was also very high and explained 43-82% of the variation. Additional detections found outside of the Port were generally low and away from beaches. Detections were also found 21.5 – 28.5 kms from the Port Musgrave mouth up the Ducie and Wenlock River, where salinities were 30 to 29.5 ppt, matching the other sample sites.

CTD profiles did not detect stratification of the water column in temperature or salinity where it was possible that eDNA could have been trapped below a thermocline/halocline even in water columns of up to 12m deep. Temperatures and salinities were similar throughout the study area and along the estuarine gradient. Temperature and salinity were therefore unlikely to influence the patterns of eDNA described in this study (Collins et al., 2018, Barnes et al., 2014, Lamb et al., 2022). Temperatures generally ranged from 35.3 – 36.3°C, exceptions were low temperature readings (26 and 30.5°C) in shallow waters of the Wenlock and Ducie Rivers. Salinities ranged from 27.9 – 31.6 ppt (Table 1).

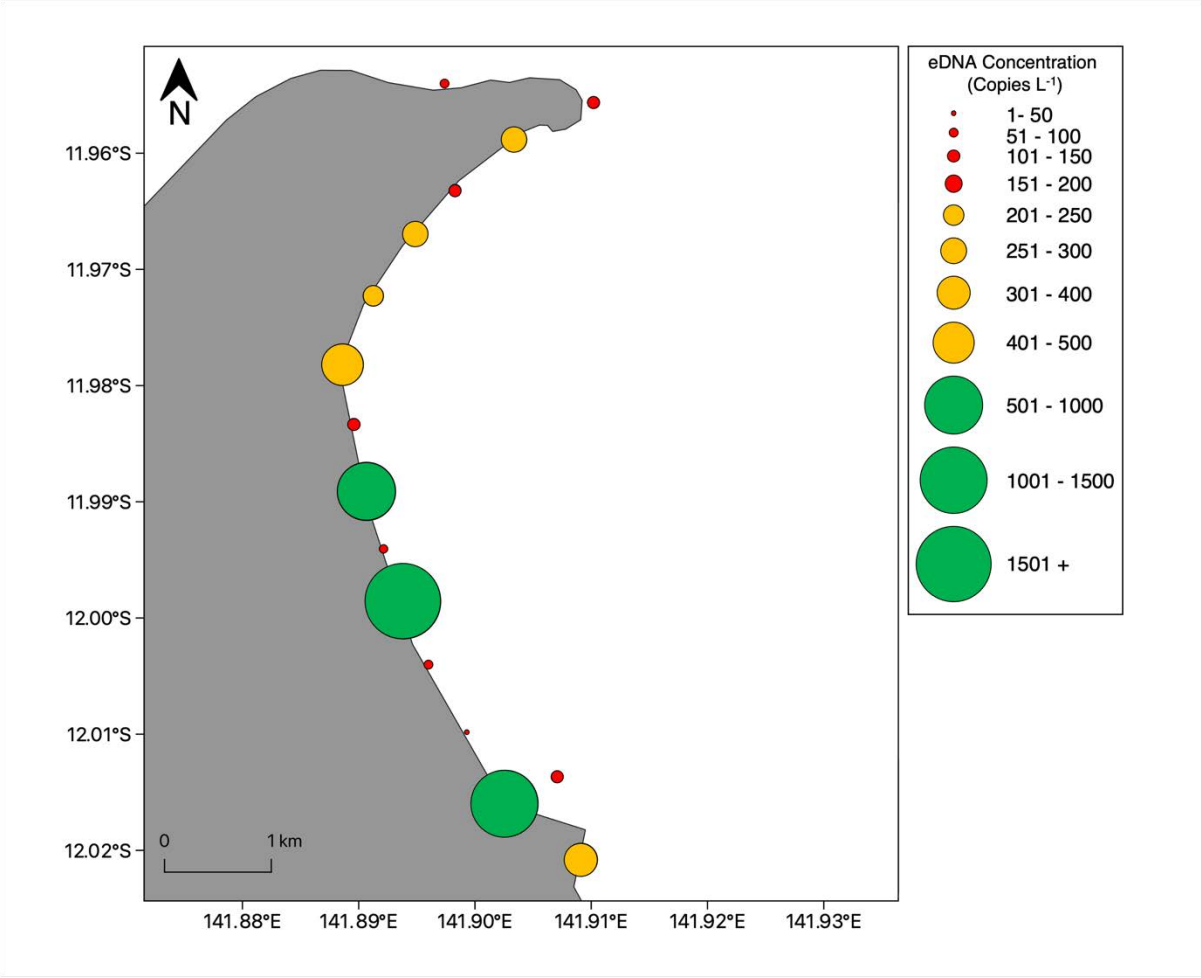

Figure 3.4. Bubble map plot displaying sampling sites within Port Musgrave with positive detections of *Chironex fleckeri* medusa eDNA. Bubbles indicate eDNA concentrations (copies L^{-1}).

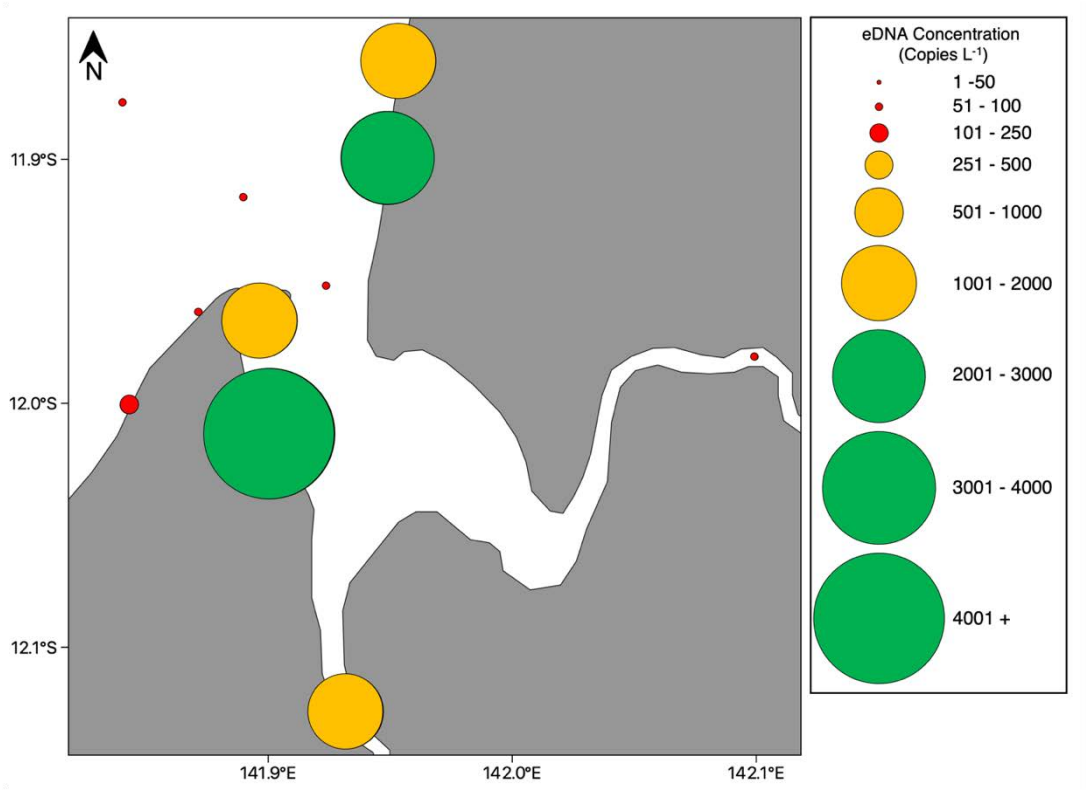

Figure 3.5. Bubble map plot displaying sampling sites outside and within (sites grouped) Port Musgrave with positive detections of *Chironex fleckeri* medusae eDNA. Bubbles indicate eDNA concentrations (copies L^{-1}).

Table 3.1. eDNA sample collection locations and sites with, site description, depth (m), depth integrated temperature and salinity, number of positive technical replicates and eDNA quantity (Copies L^{-1}) during the summer sampling period.

Site no.	Location	Habitat description	Water depth (m)	Temp (°C)	Sal. (ppt)	No. of +ve detections / 12	Copies L^{-1}
1	A	Sandy Beach with carbonate reef – mouth of the Port	5.53	35.7	30.3	6	120.7
2	A	Sandy beach with carbonate reef	0.9	35.7	30.3	12	260.3
3	A	Sandy beach with carbonate reef	0.93	35.7	29.2	6	147
4	B	Sandy beach with carbonate reef	0.95	35.8	29.2	9	272.4
5	B	Sandy beach with carbonate reef	1.08	35.7	29.1	11	236.1
6	B	Sandy beach with carbonate reef, ~600m from mangroves	1.14	35.8	29.1	12	488.1
7	C	Mangroves	0.87	35.7	27.9	5	107.8
8	C	Mangroves	1.08	35.6	28.4	12	603.5
9	C	Mangroves	0.83	35.3	29.6	4	61.9
10	D	Mangroves	0.69	35.7	29.4	12	1863.5
11	D	Mangroves	0.7	35.5	29	10	80.8
12	D	Sandy beach with rocky substrate, bordering mangroves – Red Beach	0.9	35.5	28.7	2	30.4
13	E	Sandy beach with rocky substrate, bordering mangroves – Red Beach	0.67	35.8	31.6	1	1101.1
14	E	Sandy beach with rocky substrate, bordering mangroves – Red Beach	0.85	35.5	29	7	105.5
15	E	Mangroves	0.73	35.6	28.5	12	324.5
16	–	Open Ocean outside of the Port	11.4	35.6	30	1	32.4
17	–	Open Ocean outside of the Port	5.19	35.6	29.9	5	36.9
18	F	Sandy beach, with reef, ~10km south of the Port mouth	0.97	35.7	30.3	9	225.3
19	F	Sandy beach, with reef, ~5km south of the Port mouth	0.87	35.7	30.5	3	21.9

Table 3.1. Continued.

20	F	Sandy beach with rocky substrate, ~1km south of the Port mouth	1.07	35.6	30.2	5	88.5
21	G	Open Ocean outside of the Port	19.99	35.6	29.6	11	65.6
22	G	Sandy beach, ~5km north of the Port mouth	1.14	36	29.6	12	2374
23	G	Sandy beach, ~10km north of the Port mouth	0.91	36.3	29.7	12	1340.4
24	–	Estuarine river	0.5	26	30	12	1187.9
25	–	Estuarine river	4.4	30.5	29.5	1	34.3
Average	–	–	–	35.1	29.5	–	–

Table 3.2. Nested ANOVA, *Chironex fleckeri* eDNA copies (L^{-1}); data transformed (log x +1) and number of positive technical replicates (raw data) among locations and between sites nested in locations (ns = not significant, *** denotes $p < .001$); all variance components (% var) were calculated from untransformed data.

		Copies (L^{-1})			Positive Technical Replicates		
Source	df	MS	F	% var	MS	F	% var
Location	6	82.675	0.987 ns	3.6	5.802	0.776 ns	0
Site (Location)	14	83.75	26.49 ***	53.1	7.476	3.75 ***	18.1
Residual	231	3.161		43.3	2.048		81.9

3.4.3 Detection and Distribution of *Chironex fleckeri* Polyps

Positive detection of *C. fleckeri* was found within the Port outside of the recognised medusae season (Figure 3.6). These detections, therefore, could only be due to the presence of the species benthic polyp stage. No medusae were collected in beach seines at the time of sampling. If medusae were present, eDNA detection would also be expected on the open coast, in higher concentrations across the study area, and within mangrove habitat, as was observed during summer sampling. *C. fleckeri* eDNA was detected in nine out the 24 sampling sites and in 50 % of field replicates. 10.2 % of technical replicates displayed detection and eDNA copies L^{-1} ranged from 0.46 – 73.38 copies L^{-1} (Table 3). eDNA concentrations were found to be considerably lower in comparison to those found in summer when the medusae stage was present (polyp mean eDNA quantity = 14.38 copies L^{-1}, medusa mean eDNA quantity = 504.86 copies L^{-1}) in addition to a lower detection rate in technical replicates (Tables 1 & 3). The equipment controls provided assurance of contamination-free conditions, while the endogenous control affirmed the proper application of collection, handling, and extraction methods, with absence of PCR inhibitors in analysed samples.

Polyp detection was only found at habitats with hard substratum present, these habitats being sandy beaches with carbonate reefs (Figure 3.7). Detection of polyps did occur at one site outside of the Port that was close to the mouth (< 1 km); it was also characterised by hard substrate (site 1). Interestingly, polyps were not detected in mangrove habitat.

CTD data showed that waters at all sample sites were uniform in temperature and salinity with exception of within the Wenlock River where salinity was considerably lower (16 ppt). Temperatures ranged from 26.4 – 29.2°C and salinities from 16 – 34.3 ppt.

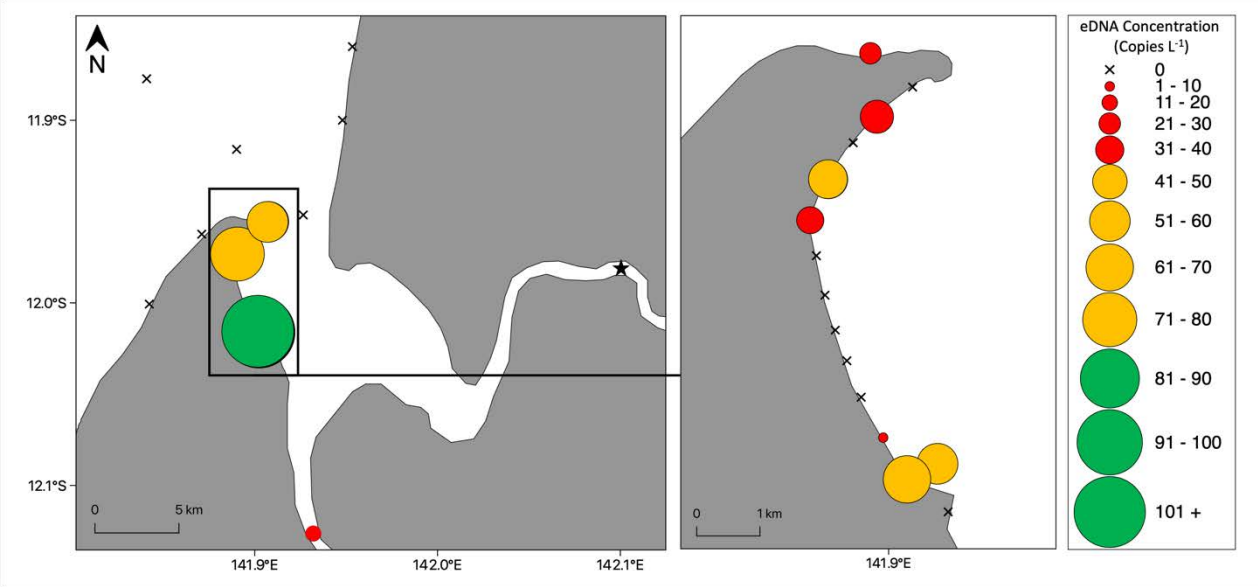

Figure 3.6. Bubble map plot displaying sampling sites within and outside of Port Musgrave with positive and negative detections of *Chironex fleckeri* polyp eDNA when medusae were absent. Bubbles indicate eDNA concentrations (copies L^{-1}). Star indicates that no data was collected at the site during this sampling time.

Table 3.3. eDNA sample collection locations and sites with, site description, depth (m), depth integrated temperature and salinity, number of positive technical replicates and eDNA quantity (Copies L^{-1}) during the winter sampling period.

Site no.	Location	Habitat description	Water depth (m)	Temp (°C)	Sal. (ppt)	No. of +ve detections / 12	Copies L^{-1}
1	A	Sandy Beach with carbonate reef – mouth of the Port	2.9	26.4	32.3	1	73.4
2	A	Sandy beach with carbonate reef	0.31	29.2	31.7	0	-
3	A	Sandy beach with carbonate reef	0.38	27.6	32	1	33
4	B	Sandy beach with carbonate reef	0.64	27.2	31.9	0	-
5	B	Sandy beach with carbonate reef	0.45	26.8	31.9	1	48.4
6	B	Sandy beach with carbonate reef, ~600m from mangroves	0.27	29.1	31.8	1	29.7
7	C	Mangroves	0.15	27.1	31.7	0	-
8	C	Mangroves	0.3	27.3	31.7	0	-
9	C	Mangroves	0.65	27	31.2	0	-
10	D	Mangroves	0.65	27.2	31.4	0	-
11	D	Mangroves	0.47	27.1	31.3	0	-
12	D	Sandy beach with rocky substrate, bordering mangroves – Red Beach	0.38	28.1	31.5	1	0.5
13	E	Sandy beach with rocky substrate, bordering mangroves – Red Beach	0.54	27.4	31.5	1	52.6
14	E	Sandy beach with rocky substrate, bordering mangroves – Red Beach	0.51	28.3	31.6	1	48.4
15	E	Mangroves	0.77	26.8	31.7	0	-
16	–	Open Ocean outside of the Port	4.83	26.7	33.7	0	-
17	–	Open Ocean outside of the Port	5.2	26.6	33.6	0	-
18	F	Sandy beach, with reef, ~10km south of the Port mouth	0.74	27.5	34.2	0	-
19	F	Sandy beach, with reef, ~5km south of the Port mouth	0.88	27.2	34.2	0	-

Table 3.3. Continued.

20	F	Sandy beach with rocky substrate, ~1km south of the Port mouth	0.41	28.3	33.4	3	18.2
21	G	Open Ocean outside of the Port	18.09	27	33.4	0	-
22	G	Sandy beach, ~5km north of the Port mouth	1.23	26.3	33.4	0	-
23	G	Sandy beach, ~10km north of the Port mouth	1.14	26.6	33.5	0	-
24	–	Estuarine river	0.5	28.5	16	1	12.4
Average	–	–	–	27.4	31.7	–	–

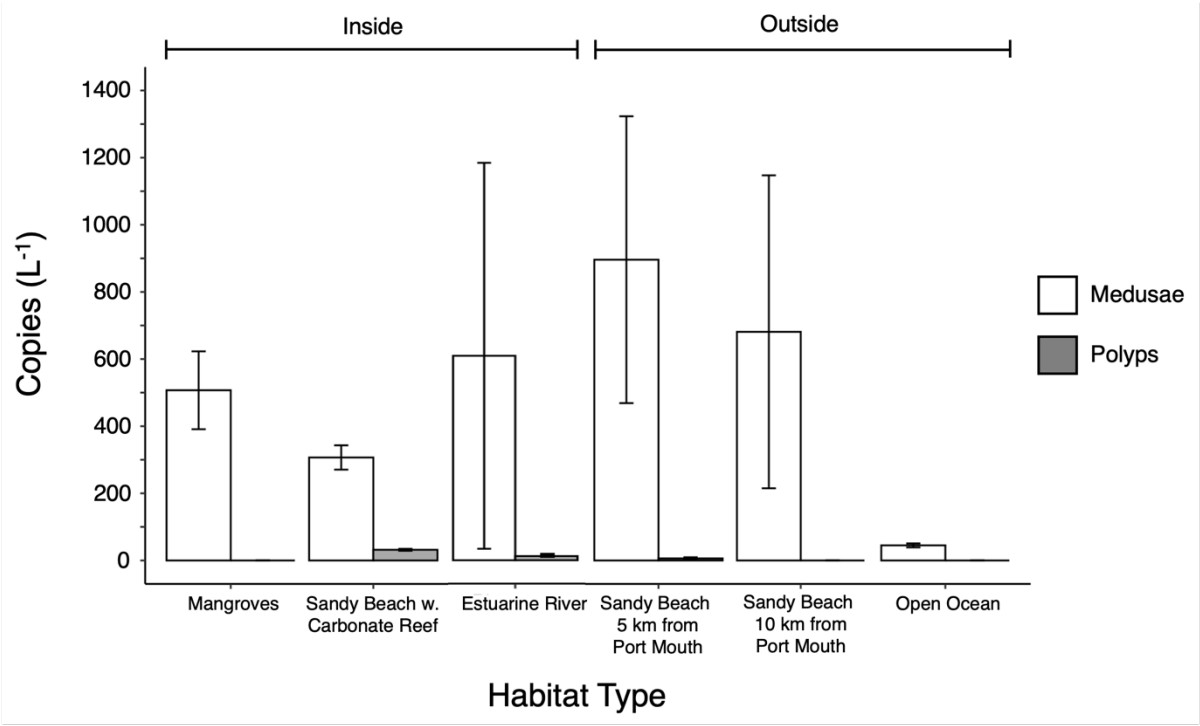

Figure 3.7. Histogram displaying average eDNA copies (L^{-1}) for different habitat types, inside (Mangrove, Sandy beach w. carbonate reef) and outside (Sandy beach >6 km from Port mouth, sandy beach <6 km from Port mouth, Open Ocean) of Port Musgrave, for both *Chironex fleckeri* medusa and polyp stages. The detection of polyps outside of the Port was within 1 km of the Port mouth.

3.5 Discussion

3.5.1 eDNA as a Proxy of Abundance for Cubozoan Jellyfish

A poor association between eDNA concentration/number of positive technical replicates and *C. fleckeri* medusa abundance was found to occur. This contrasts the strong positive associations between these two factors reported for numerous other taxa (Takahara et al., 2012, Pilliod et al., 2013, Lacoursière-Roussel et al., 2016a, Wilcox et al., 2016, Yamamoto et al., 2016, Thomsen et al., 2012, Rourke et al., 2023), including scyphozoan jellyfish (Minamoto et al., 2017). The poor association may, however, be resultant from the spatially dispersed nature of *C. fleckeri* medusa. *C. fleckeri* medusa were spatially dispersed with densities of medusa ranging from 0.7 – 1.7 individuals/1000 m^3 (as per this study), with highest densities occurring at known medusae 'hotspots' (20.9 individuals/1000 m^3). The lower abundances of *C. fleckeri* medusa, in comparison to other taxa (i.e., *Chrysaora pacifica* – 10s to 100s of individuals/150 m^2), would result in lower eDNA concentrations, as found in this study (in comparison to those reported by Minamoto et al. (2017) for *C. pacifica* medusae; 7.05 x 10^2 – 2.60 x 10^5 copies L^{-1}). It is known that the precision of eDNA-based abundance estimates are influenced by the amount of eDNA present (Pilliod et al., 2013). Additionally, as eDNA concentrations were lower, influence by both biotic and abiotic factors upon eDNA presence and persistence would drive higher variation in eDNA concentrations, hence, further impacting upon this association (Harrison et al., 2019, Barnes and Turner, 2016, Collins et al., 2018, Barnes et al., 2014, Lamb et al., 2022, Jo et al., 2019, Huerlimann et al., 2020, Stewart, 2019).

Considerable variation in eDNA concentrations between neighbouring sample sites were found in this study. eDNA concentrations were significantly different between neighbouring sites within locations. This is prominently displayed at Red Beach, where samples (sites 13, 14 & 15) were taken 10s to 100s of metres apart. Varying abundances of *C. fleckeri* medusae were present at this location (1, 17 & 9 individuals, respectively) with associated eDNA concentrations (30, 105 & 1101 copies L^{-1} respectively) which did not align with our estimates of abundance from seines. This variation is suggested to result from the above-mentioned factors, specifically, variability in release of eDNA from individuals (shedding rate), related dispersion from source individuals, transport of eDNA via oceanographic processes, and rates of decay. Therefore, the use of eDNA as a proxy for species abundance is likely to be more complex and variable for spatially disperse and rare taxa due to complex eDNA dynamics (Harrison et al., 2019), and more so in

tropical environments (Huerlimann et al., 2020). We have, however, demonstrated that use of the method for detection is robust.

Mesocosm trials, controlling for the influence of biotic/abiotic factors should be undertaken to examine this use of eDNA within a controlled environment. Further, we require a greater understanding of variability in release of eDNA (shedding rates) from individuals of varying sizes and life history stages (Klymus et al., 2015), and understanding on the related dispersion of eDNA in marine environments (Harrison et al., 2019). Increased replication would allow for less variation in eDNA concentrations (Pilliod et al., 2013). Further, clustering sites and utilising a nested sampling design provides more accurate measures of spatial variation in eDNA. Finally, there was potential for tiny medusae (< 3 cm IPD) to avoid detection via seine netting, and the authors suggest that plankton tows, in areas where possible (lack of net snags and estuarine crocodiles), be utilised for further in-field exploration of this potential relationship. It should be noted however, that *C. fleckeri* medusa smaller than the mesh size were on occasion captured likely due to the pinching of the mesh, making it functionally smaller, and due to debris, that was commonly found in the net.

3.5.2 Distribution of *Chironex fleckeri* Medusae

We found great spatial variation in eDNA over spatial scales of 100s of metres to 10s of kilometres which has not been documented for cubozoans. Within Port Musgrave, there was a high frequency of detection of eDNA and there was considerable variation in eDNA concentrations among sites within locations. The high detection rate of medusae within the Port is likely attributed to the presence of mangrove habitat. Post-larval, juvenile and small adult fish, along with juvenile crustaceans, which are common medusa prey (Carrette et al., 2002), are known to be of higher abundance within mangroves (Robertson and Duke, 1987). Gordon and Seymour (2009) have previously suggested that *C. fleckeri* medusa may remain in these areas of higher prey abundance to maximise energy expenditure. These findings further align with and provide support to Schlaefer et al. (2018)'s biophysical models. Schlaefer et al. (2018)'s models found medusa to aggregate in shallow waters within 10s to 100s of metres from Red Beach which is where presence of medusae was highest and where highest concentrations of eDNA, within the Port, were found. Additionally, the models showed a decrease in medusa moving towards the Port mouth, further aligning with what was found *in-situ*, suggesting that medusa are largely retained within the Port. *C. fleckeri* medusa were also detected with eDNA up both major river systems. As these areas are of suitable

conditions for medusae (Mooney and Kingsford, 2016b), it is likely that the species is present throughout the entire estuarine system. As a result, this study's findings align with the predictions made by Schlaefer et al. (2018)'s biophysical models regarding the distribution of *C. fleckeri* medusae within the Port.

C. fleckeri medusa were also detected at sample sites outside of the Port, with strong detections along the beach north of the Port's mouth. However, the frequency of detection was lower among sites outside of the estuary. It is suggested that these detected medusas are undertaking excursions outside and along these beaches. This suggestion is supported by Gordon and Seymour (2009) who, via acoustic tracking, observed a large adult medusa individual to move from estuarine habitat to open coastal beaches and then return. Further, Schlaefer et al. (2018)'s biophysical models showed high retention within the Port with less than 2.5% of modelled medusa being advected from the Port, hence aligning with this suggestion. Regarding the transect leading from the Port mouth out into the Gulf of Carpentaria, it should be noted that were was some detection of eDNA at these sample sites. This detection however may result from the afore mentioned excursion, dispersal of eDNA from the Port via tidal currents, or even from fragments of dead jellyfish from predation by turtles and other predators (the frequency of predation however is unknown). To further investigate these suggestions, models are required that combine diffusion of eDNA, accounting for the influence of tides and currents, and decay rates to determine likely distances of detection from the source. Further, the genetics of populations found in different estuaries are required to determine if the low ecological connectivity argued in this study and that of Schlaefer et al. (2018) contrasts with multi generation time scales.

3.5.3 Cubozoan Polyp Detection with eDNA

3.5.3.1 Ability of eDNA to Detect Cubozoan Polyps

This study successfully detected the putative presence of *C. fleckeri* polyps outside of the jellyfish season, when their medusae stage was absent. Therefore, eDNA has the potential to provide an effective and resource-efficient technique to locate the source locations (polyp beds) of this and other cubozoan jellyfish species (Bolte et al., 2021). Despite the lack of visual confirmation of polyps in their natural habitat, the utilisation of the eDNA technique aims to remove the necessity for such confirmation, considering the challenges involved in detecting cubozoan polyps. Subsequently, the found positive detections putatively confirm the presence of *C. fleckeri*'s polyp stage. The

accuracy and confidence in these detections are ensured by the sensitivity and specificity of the detection assay, the utilisation of optimised methodologies for elusive species, and the implementation of best practice control measures (Morrissey et al., 2022). Arguments suggesting that the detection has arisen from the medusae stage are invalid. This is because sampling took place in July, two months after the end of the Australian box jellyfish season when medusae are known to be absent due to their seasonality (Kingsford and Mooney, 2014, Hartwick, 1991a, Mooney and Kingsford, 2012, Gordon and Seymour, 2012). Additionally, *C. fleckeri*'s eDNA is known to decay rapidly (Morrissey et al., 2022), and no medusae were collected in seines or observed during the sampling period. The detected eDNA concentrations and presence were found to be significantly lower and highly localised compared to when medusae were present. This aligns with the understanding that medusae are free-swimming, while polyps reside on the benthos with likely minimal movement once they establish themselves in a suitable habitat, providing further evidence for the diminished and more localised eDNA detections. Bolte et al. (2021) also successfully utilised eDNA to detect the polyp stage of the harmless cubozoan species *Copula sivickisi*. In their study, they reported detecting *C. sivickisi* exclusively in near substrate samples outside of the Australian box jellyfish season, where the species' polyp stage would be expected to reside due to its diurnal swimming behaviour (Garm et al., 2012). This current study has successfully demonstrated the use of eDNA to detect this elusive life history stage. The ability to do so is 'game-changing' for the field and our understanding on the ecology of this and other cubozoan species, and subsequent management of them, will greatly benefit.

3.5.3.2 Implications of Cubozoan Polyp Detection

The ability to detect and locate cubozoan polyps has numerous benefits for our ability to advance understanding on this elusive and dangerous taxon. Current understanding of cubozoan polyp ecology is based upon laboratory investigations (Boco et al., 2019, Courtney et al., 2016a). eDNA opens the door to studying the basic ecology of this life history stage in its natural environment which, until now, was logistically challenging to undertake. The potential to examine the environmental conditions which this life history stage endures, impacts of weather events i.e., freshwater pulses due to rain events, potential habitat (see 4.5), and investigation into potential abiotic (temperature, salinity, pH) and biotic (food availability) drivers of polyp metamorphosis, and subsequent seasonality of medusae, in *in-situ* conditions, are now within reach of ecologists. Further, a more in-depth understanding surrounding the population dynamics

and population stock boundaries (see 4.4) of these taxa are now possible with understanding of polyp locations due to their role as the major driver of medusa abundance and periodicity (Kingsford and Mooney, 2014, Arai, 1997). This will further allow for the identification of jellyfish 'hotspots' which is of great benefit for furthering understanding on cubozoan ecology and for management of the risk of envenomation to water users. Additionally, the ability to locate cubozoan polyps allows for further investigation into how cubozoan jellyfish will respond to future oceanic conditions resulting from climate change (Klein et al., 2014). With the development and emergence of environmental RNA (eRNA) detection (Yates et al., 2021), which enables finer resolution detection of organisms due to the rapid decay of eRNA, a higher resolution of polyp occurrence may be possible and assist physical *in-situ* locating of this life history stage. eDNA hence is the best available tool to advance understanding on key aspects of cubozoan ecology and, due to the ubiquitous nature of DNA, can be applied to all cubozoan species including other dangerous species such as Irukandji jellyfish (~16 species within the Carybdeidae order) (Gershwin et al., 2013).

3.5.4 Comparison of *Chironex fleckeri* Medusae and Polyp Distributions to Inform Stock Boundaries

As both life history stages can be detected with eDNA, it allows, for the first time, a comparison of their distributions which may inform the spatial extent of population stocks. Polyps are the source of medusae and likely play a key role in the dispersal patterns of medusae. Their location, combined with the swimming behaviours of medusae, would influence the likelihood of retention. Currently, for cubozoans, growing evidence suggests that some species have population stocks of small spatial scales, to the extent of bays and estuaries, however, the locality of the polyp stage has yet to be incorporated and considered (Kingsford et al., 2021). As polyps have been putatively detected with eDNA in this study, it allows for us to explore this for *C. fleckeri*, and to subsequently test predictions on the species stock structure for this area inferred from biophysical models (Schlaefer et al., 2018).

C. fleckeri medusae were found to occur extensively within the Port and were detected at some beaches outside. However, *C. fleckeri* polyps were only detected within the Port or close to the mouth, indicating that medusae in the study area likely originated from within the Port. These detections of polyps provide further evidence that medusae found outside were likely undertaking excursions from within the Port. Additionally, Red Beach, which is a known 'hotspot' of medusae, was utilised as a seeding location for

Schlaefer et al. (2018)'s biophysical models. This, aligned well with where the largest concentrations of polyp eDNA were found and so subsequently reflects a real-world medusae source location. Medusae distributions reported in this study subsequently align with and provided support to those predicted by Schlaefer et al. (2018)'s biophysical models. Furthermore, from the extensive presence of medusae within the Port and within both river systems which flow into the Port, the authors agree with Schlaefer et al. (2018)'s suggestion that the estuarine system likely contains numerous local populations with high connectivity.

Enclosed bays and associated estuarine conditions are ideal for retention based on favourable currents, the behaviour of medusae, and with presence of polyp source locations. To further explore the spatial scales of cubozoan stocks, the spatial robustness of populations needs to be investigated from coastal environments of different geomorphologies from those that are enclosed to open.

3.5.5 Cubozoan Polyp Habitat

As eDNA can be utilised to detect *C. fleckeri* polyps, it provides a new approach to further our understanding on the species ecology and assess hypotheses surrounding cubozoan polyp habitat. This study looked for the presence of *C. fleckeri*'s polyp stage in both beach, mangrove and estuarine river habitats and is the first study to directly examine this. Prior to eDNA, inferences on cubozoan polyp habitat have largely been limited to broad environment categories such as estuaries. These inferences have resulted from the *in-situ* reporting of *C. fleckeri* polyps (Hartwick, 1991a), statolith microchemistry profiles (Mooney and Kingsford, 2012), and understanding on cubozoan thermo/osmotic tolerances (Rowley et al., 2023, Mooney and Kingsford, 2016b, Courtney et al., 2016a). In this study, detection of the polyp stage was found majorly at beach habitats containing hard substratum within the Port. Detection was also found within the Wenlock River and at a single site outside of the Port which was in close proximity to the Port mouth (1km south, containing rocky substrata). These findings hence support those of Mooney and Kingsford (2012)'s analysis of *C. fleckeri* statolith microchemistry profiles, which suggested suitable habitat of *C. fleckeri* polyps to extend beyond estuaries to marine environments. Most interestingly, no detection of polyps was found at mangrove habitats within this study. This finding aligns with Hartwick (1991a)'s *in-situ* reporting of *C. fleckeri* polyps, where polyps were only located under stones and shells and not on nearby mangrove structures. As a result, it is possible that mangroves are not suitable habitat for *C. fleckeri* polyps. The findings of this study hence highlight

the ability of *C. fleckeri* polyps to reside in environments of varying environmental conditions, as is supported by known cubozoan thermal/osmotic tolerances (Rowley et al., 2023, Mooney and Kingsford, 2016b, Courtney et al., 2016a). Additionally, it showcases the need of eDNA to directly locate this life history stage as suggestions based on physical tolerances provide only general environmental conditions. Currently, no other technique is able to efficiently detect polyps.

Regarding specific substrata, the authors note that expansive carbonate reefs often associated with oysters were present at sites where *C. fleckeri* polyps were detected. Oyster reef, due to its structural complexity, likely makes for suitable habitat where polyps can reside in crevices with appropriate water flow (bringing in food, removing waste) (Holst and Jarms, 2006, Chapman, 1973), while also providing shelter from predators and sedimentation (Svane and Dolmer, 1995). This suggestion is supported by the *in-situ* finding of *C. fleckeri* polyps by Hartwick (1991a), which were located on the underside of rocks, and from knowledge on scyphozoan polyp habitat preferences which are often quite specific (Holst and Jarms, 2007, Svane and Dolmer, 1995, Brewer, 1984, Zang et al., 2023). Multiple species of scyphozoan polyps have been reported to reside on the underside of rocks, below overhangs and in concealed habitats (Brewer, 1976, Cargo and Schultz, 1966, Svane and Dolmer, 1995, Kikinger, 1992, Pitt, 2000), which additionally provides further support to the suggestion of mangroves being unsuitable habitat. We suggest, therefore, that any hard substrata (carbonate oyster reef, coral reef, rocky substrata, and potentially artificial substrates), in both estuarine and marine environments, are likely suitable habitat for the polyp stage of this, and other cubozoan species. Additionally, largest concentrations of polyp eDNA in this study were found at the medusa ‘hotspot’ (Red Beach), suggesting that these ‘hotspots’ may be good predictors for the presence of polyps. This makes logical sense as, due to the nature of cubozoan sexual reproduction (broadcast spawning or internal development), and understanding on scyphozoan jellyfish planula larvae behaviour, which initially show a geopositive reaction once developed (Holst and Jarms, 2007), cubozoan planula likely settle promptly following release from medusae and reside in close proximity to these areas. It is therefore suggested that eDNA sampling efforts to locate the polyp stage of cubozoan jellyfish should target areas of higher medusae abundances and areas where hard substratum is present.

3.6 Conclusion

Significant knowledge gaps surrounding the ecology of cubozoan jellyfish exist as a result of the challenges associated with their detection (Kingsford and Mooney, 2014, Kingsford et al., 2018). This study has demonstrated the use of eDNA as an ecological tool to investigate and address these critical gaps surrounding the ecology of this dangerous cubozoan taxa. Through an *in-situ* trial, the potential of eDNA as a proxy for species abundance was explored, but the relationship between abundance in seines and eDNA was weak for *C. fleckeri*. This highlights the need for a deeper understanding on eDNA dynamics in marine environments, especially for rare/spatially disperse taxa. Distributions of the medusoid stage of *C. fleckeri* were found to be of spatially broad occurrence within the Port, with a lower frequency of detection being found outside of the Port suggesting medusae may be undertaking excursions from the Port. Additionally, eDNA proved successful in detecting the elusive benthic polyp stage of the species, hence revealing their locality, which is 'game-changing' for the field. The seasonality of the species medusae stage, being absent during the Austral winter (Kingsford and Mooney, 2014, Hartwick, 1991a, Mooney and Kingsford, 2012, Gordon and Seymour, 2012), enabled this detection. This, for the first time, allowed investigation into potential cubozoan polyp habitat. Polyps were only detected in habitats that had nearby patches of rocky substrata and shallow carbonate reefs, not at mangrove habitats. Therefore, any hard substructure adjacent to the shore which provides appropriate shelter and water flow, likely is suitable habitat for cubozoan polyps. Comparison of the distributions of both life history stages allowed for investigating the spatial extent of *C. fleckeri* population stocks, with evidence found to support predictions made by biophysical models (Schlaefer et al., 2018), that Port Musgrave likely represents a population stock of the species. This subsequently validates the use of biophysical models to examine the movements and population structures of cubozoan jellyfish. Accordingly, eDNA offers a novel ecological tool to investigate hypotheses surrounding the ecology of dangerous cubozoan taxa which will subsequently benefit coastal managers to better understand and mitigate the threat these species pose to both human health and enterprise.

Chapter 4.

Use of eDNA to Determine Source Locations of Deadly Jellyfish (Cubozoa) in an Open Coastal System

4.1 Abstract

Challenges associated with cubozoan jellyfish detection and limitations of current detection techniques limit the ability of scientists to fill critical knowledge gaps surrounding their ecology. Environmental DNA (eDNA), however, has proven useful as an ecological survey tool to detect and study these deadly jellyfish. This study aimed to leverage the power of eDNA to detect and explore the distribution of the Australian box jellyfish (*Chironex fleckeri*), encompassing both its medusae and polyp life history stages, within an open coastal bay (Horseshoe Bay) of Magnetic Island, Queensland, Australia. Our investigation focused on a hypothesis concerning source locations of the jellyfish within Horseshoe Bay and, through a comparison of both life history stage distributions, aimed to determine potential population stock boundaries. eDNA results aligned with the predicted nearshore distribution of medusae. Further, the elusive benthic polyp stage was also detected. These findings confirmed Horseshoe Bay as a source location of the jellyfish. Moreover, our evidence supported a model that the area likely represents a population stock of the species. This adds to growing evidence suggesting some cubozoan jellyfish have population stocks of small spatial scales in both open and relatively closed ecosystems such as estuaries. In conclusion, this study serves as a notable example of eDNA's ability to resolve critical knowledge gaps surrounding cubozoan ecology and to enhance the management ability of these deadly jellyfish to reduce envenomation's.

4.2 Introduction

Stinging jellyfish pose a global issue due to their threat to human health and their subsequent economic impacts (Kingsford et al., 2018, Graham et al., 2014, Lucas et al., 2014, Gershwin et al., 2010, De Donno et al., 2014, Cegolon et al., 2013, Bosch-Belmar et al., 2020, Bordehore et al., 2016, Rodrigues et al., 2020, Kennerley et al., 2022). Cubozoan jellyfish, known for their potent venom, are the class of most concern (Kingsford and Mooney, 2014, Gershwin et al., 2010, Fenner and Harrison, 2000, Currie and Jacups, 2005). Of these taxa, the Australian box jellyfish, *Chironex fleckeri*, is the most notorious, and is responsible for more than 200 recorded deaths in the Indo-Pacific region (Gershwin et al., 2013). The presence of these stinging jellyfish leads to extensive beach closures, which significantly impacts upon local tourism industries and consequently, local economies (Lucas et al., 2014). However, due to their elusive nature and the challenges associated with their detection, mitigating and managing their threat is a 'wicked' problem (Gershwin et al., 2010, Gershwin and Crowley-Cyr, 2021, Crowley-Cyr and Gershwin, 2021). To enhance the ability of stakeholders to effectively manage these taxa, it is important to gain a greater understanding of their ecology (Kingsford and Mooney, 2014). The more that is known surrounding these taxa, more informed and appropriate management solutions can be applied.

Considerable knowledge gaps, namely understanding of population dynamics, distribution limits, and the locality of benthic life history stages (polyps), exist surrounding the ecology of cubozoan jellyfish (Kingsford and Mooney, 2014, Gershwin et al., 2013, Kingsford et al., 2021). Importance is placed upon understanding the locality of polyps as they are the source of stinging medusa and, given their asexual characteristics (Courtney et al., 2016b), play a major role in the population dynamics and distributional limits of cubozoans (Kingsford and Mooney, 2014, Arai, 1997, Kingsford et al., 2021, Courtney and Seymour, 2013, Courtney et al., 2016b). Additionally, growing evidence suggests that population stocks of some of the ~50 cubozoan species (Collins and Jarms, 2018) are of small spatial scales and therefore the locality of the polyp stage is central to this understanding (Kingsford et al., 2021, Mooney and Kingsford, 2017, Schlaefer et al., 2021, Schlaefer et al., 2020, Schlaefer et al., 2018, Mooney and Kingsford, 2016a). The ability to study these aspects of cubozoan ecology, however, are logistically challenging to undertake. This is due to limitations of current detection/sampling techniques which hinder the ability of scientists to fill these critical knowledge gaps. Environmental DNA (eDNA), however, provides a new approach to

investigate the ecology of these dangerous taxa (Bolte et al., 2021, Morrissey et al., 2024a, Morrissey et al., 2022, Sathirapongsasuti et al., 2021, Azama et al., 2023).

A highly specific and sensitive eDNA detection assay has recently been developed for *Chironex fleckeri* (Morrissey et al., 2022). This detection tool was successfully utilised as an ecological survey tool and successfully detected the elusive benthic polyp life history stage of the species (Morrissey et al., 2024a). Morrissey et al. (2024a) putatively detected the polyp stage of *C. fleckeri* and further examined this life history stage's potential habitat. Polyps were detected in habitats with rocky substrata and shallow carbonate reefs. Further, Morrissey et al. (2024a) utilised the genetic tool to contribute to an understanding of population stock boundaries of the jellyfish in a relatively enclosed estuarine system (Port Musgrave, Australia) (Schlaefer et al., 2018). The results from eDNA largely concurred with the results of a biophysical modelling and jellyfish behaviour study indicating low connectivity from Port Musgrave and a source of polyps that were only found in the estuary (Morrissey et al., 2024a).

In contrast, *C. fleckeri* is also found in a relatively open coastal system at Magnetic Island, situated off the coast of North Queensland, Australia. The island is not only of interest ecologically, but it is a tourism hotspot where cubomedusae co-occur with swimmers and are responsible for beach closures during the Australian box jellyfish season (October – May) (pers. comms. Surf Life Saving Queensland, SLSQ). Multiple coastal bays on the island are monitored and patrolled by local management authorities (SLSQ) due to the threat posed by cubozoan jellyfish. Horseshoe Bay, which is located on the northern side of the island, is a recognised hotspot for *C. fleckeri* medusae and it has been hypothesised as a source location of the jellyfish (Brown, 1973). This hypothesis arose from a multi-year study undertaken by Brown (1973) who examined the distribution and movements of the species medusae stage on Magnetic Island. Brown (1973) made visual surveys around the entire island and noted that *C. fleckeri* medusa appeared firstly within the vicinity of Horseshoe Bay during November, the start of the Australian box jellyfish season, and from December onwards individuals were encountered in neighbouring bays; however, in considerably lower abundance. Furthermore, Brown (1973) noted that small juvenile *C. fleckeri* individuals were only found within Horseshoe Bay whereas larger specimens were found at multiple locations around the island. From these findings, Brown (1973) hypothesised that Horseshoe Bay was the source location of the species for the island and, therefore, most likely contains the polyp life history stage of the species. eDNA, as it can detect putative presence of nearby polyps, therefore allows for the testing of components of this hypothesis (Bolte et al., 2021, Morrissey et al., 2022). Further, as *C. fleckeri* medusae have primarily been

observed along the north side of Magnetic Island, primarily within Horseshoe Bay, it is possible that the area may represent a local stock of the jellyfish. This provides an opportunity to test a developing paradigm that *C. fleckeri* commonly have population stocks of small spatial scales (Morrissey et al., 2024a, Kingsford et al., 2021, Schlaefer et al., 2018, Mooney and Kingsford, 2016a).

The objective of this study was to utilise eDNA to detect and study the Australian box jellyfish, *C. fleckeri*, in an open coastal system, contrasting that of Morrissey et al. (2024a) (semi-enclosed system). Specifically, we aimed to determine the following (i) the presence and localised distribution of the species medusae stage, (ii) the source of medusae by examining the distribution of polyps in the absence of medusae, and (iii) compare medusae and polyp distributions to infer likely population concentrations and boundaries. Further, the results of our sampling will allow us to contribute to knowledge on the spatial scales of *C. fleckeri* populations.

4.3 Materials and Methods

4.3.1 Study Area

This study was conducted within and near a group of open coastal bays at Magnetic Island, Australia (19.11°S, 146.85°E). Horseshoe is the largest bay and, to the west and outside of Horseshoe Bay is Maud Bay (Figure 4.1). Horseshoe Bay, in particular, is a hotspot for tourism where *Chironex fleckeri* medusa are known to reside during the Australian box jellyfish season (October to May). Surf Life Saving Queensland (SLSQ) monitor this area through undertaking daily beach tows, within and outside of the local stinger net (preventative measure to provide a safe, jellyfish free swimming area), covering ~150 m of shoreline. These beach tows provided information on the presence or absence of *C. fleckeri* medusae in the study area. Additionally, oceanographic data exists for Horseshoe Bay (pers. comms JA Schlaefer). Both Horseshoe and Maud bays have some freshwater inflows which become isolated during low tide (tidal range 3.4 m).

4.3.2 Field Sampling

The sampling was divided into two temporal windows as follows; October to May, when medusae are present (Australian box jellyfish season), and July to September, when medusae are absent. Sampling took place between 2020 and 2022. It has been

predicted from other studies that medusae will be most abundant close to shore (Brown, 1973, Kingsford et al., 2012) and that sites with freshwater inflows may be a source of medusae from benthic polyps (Hartwick, 1991a, Cutress and Studebaker, 1973). Within the Australian box jellyfish season, spatial variation in the distribution of medusae was determined by sampling for eDNA. Initially, sampling was undertaken at sites located along the shores of Horseshoe and Maud Bays resulting from their known nearshore distribution (Figure 4.1, December 2020 and February 2021). Samples were also collected within and at the mouths of freshwater inflows into these bays (The flora and landform of Horseshoe Bay is shown in Figure S3.1). It was possible that medusae could move outside the open coastal bays. To detect this potential scenario, samples were collected in a grid-design across the bays and positioned at three distances from shore (Figure 4.1, March 2021 and December 2021). This sampling design also allowed us to examine whether any eDNA signal was being transported out of the bay. eDNA sampling for medusae detection was conducted concurrently with SLSQ's detection of *C. fleckeri* medusae using beach tows.

The distribution of polyps could only be determined in the absence of medusae (Morrissey et al., 2024a). The seasonality of *C. fleckeri* medusae is well established with medusae only being present during summer months (October to May) (Hartwick, 1991a, Gordon and Seymour, 2012, Mooney and Kingsford, 2012, Kingsford and Mooney, 2014). Accordingly, sampling for eDNA was undertaken in the austral winter (July 2020, Figure 4.1). Polyps were detected in winter, and a modified sampling design, in July 2022, gave greater sampling effort in and near sites where polyps had been detected, and some emphasis was given to sites with freshwater inflows within Horseshoe Bay. Samples were collected within the freshwater inflow when connected (sites 6, 8 and 10) and, when isolated (sites 7, 9 and 11) by the tide and along the shore of Horseshoe Bay. An offshore site (site 14) acted as an *in-situ* negative control and a 100 x 16 m beach seine net drag (mesh size of 3 cm) was utilised at all sites along the shore of Horseshoe Bay to further confirm the absence of medusae.

For each site, replicate 2 L water samples were collected and filtered immediately in the field, and were stored in Longmires buffer in temperatures of 4 °C until processed. An equipment control, prior to sample collection, was also undertaken for each replicate sample to ensure sampling equipment was not contaminated. Specific details surrounding collection, handling, and storage of eDNA samples can be found in Morrissey et al. (2022). Further, a conductivity, temperature, and depth device (CTD; Seabird SBE 19 Plus) was utilised at each sample site to examine the level of

stratification as this is known to have influence upon eDNA within the water column (Jeunen et al., 2020, Gray and Kingsford, 2003).

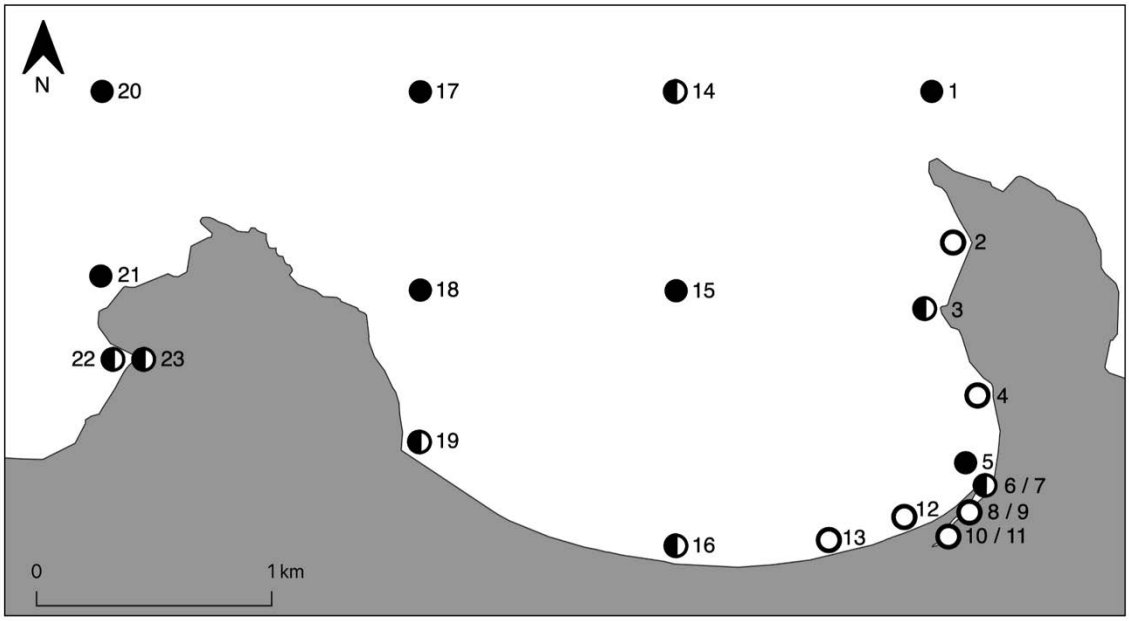

Figure 4.1. Sampling sites in Horseshoe and Maud Bays, Magnetic Island. Sampling sites are numbered. The circle colours indicate the sampling design: black circles for the grid sampling design, white circles for the modified winter sampling design, and half-white, half-black circles for sites included in both designs. Sites 5, 6, 16, 19, 22 and 23 reflect the nearshore sampling design. Sites 6/7, 8/9, 10/11 and 23 reflect the freshwater inflows. design: black circles for the grid sampling design, white circles for the modified winter sampling design, and half-white, half-black circles for sites included in both designs. Sites 5, 6, 16, 19, 22 and 23 reflect the nearshore sampling design.

4.3.3 eDNA Extraction and Purification

Collected eDNA samples were extracted using the PPLPP method, initially developed by Edmunds and Burrows (2020) and subsequently modified for filter-based extractions by Cooper et al. (2021). Following extraction, the eDNA underwent purification utilising the Zymo One Step PCR Inhibitor Removal kit (Zymo IR; Zymo Research; Irvine, California, United States) in accordance with the manufacturer's instructions. The resulting eDNA, now purified, was then stored under -20°C conditions until the quantification process. Specific details surrounding eDNA extractions and purifications can be found in Morrissey et al. (2022).

4.3.4 Quantitative PCR

This study utilised a multiplexed assay, developed by Morrissey et al. (2022), for the identification, quantification, and interpretation of *Chironex fleckeri* eDNA. To assess method success and for potential PCR inhibitors, an endogenous control assay was multiplexed with the *C. fleckeri* specific assay (Morrissey et al., 2022). qPCR reactions were composed of 2 µl of eDNA template, 10 µl of TaqMan Environmental Master Mix 2.0, 0.7 µM sense and anti-sense *C. fleckeri* primers, 0.525µM sense and anti-sense endogenous control primers, 0.25 µM of both *C. fleckeri* and endogenous control TaqMan MGB probes (Assay sequences listed in Table S3.1). MilliQ water was added to adjust the final volume to 20 µl. Utilising the QuantStudio 3 and 5 Real-Time PCR systems, each reaction followed a two-step cycling profile (95 °C for 10 min, succeeded by 50 cycles of 95 °C for 15 s and 60 °C for 1 min). Six technical replicates were performed for each sample to ensure precision. Additionally, each plate included at least three negative controls, extraction blanks, a positive control, and synthetic DNA (sDNA) standards (10 thousand to one copy μl^{-1}) to ensure lack of contamination and consistency among plates. The criteria for confirming positive detection of *C. fleckeri* involved the amplification of a single technical replicate. The decision to consider a single positive technical replicate as indicative of species presence is common for eDNA detection of cryptic and low abundance species (Cooper et al., 2021, Budd et al., 2021, Trujillo-González et al., 2019, Bessell et al., 2023, Villacorta-Rath et al., 2022). Additionally, zeroing single technical replicate detections in an ad hoc manner may introduce uncertainties, biases, or type II errors into subsequent analyses (Lahoz-Monfort et al., 2016). Any positive findings were substantiated through clean up and bidirectional sanger sequencing of PCR product, undertaken by the Australian Genome Research

Facility. The results were cross-checked against reference sequences to ensure accuracy.

4.3.5 Statistical Analysis

Replicate filters (n = 2) were treated as sub-samples. Positive technical replicates, from each replicate water sample, were averaged to represent the eDNA concentration (copies L^{-1}) at each sample site (Goldberg et al., 2013, Thomsen et al., 2012, Congram et al., 2022). The average provided a more representative snapshot of *Chironex fleckeri* presence in the study area. Additionally, detections were also reported as number of positive technical replicates out of 12 per sampling site, hence, two measures of positive detection of *Chironex fleckeri* eDNA are presented.

4.4 Results

4.4.1 Seasonality of *Chironex fleckeri* Medusae within Horseshoe Bay

The detection of *Chironex fleckeri* in beach tows is known to be highly seasonal within Horseshoe Bay. Medusae were only present in summer months (October - May) in each year of sampling (pers. comms. Surf Life Saving Queensland). The seasonality of *C. fleckeri* medusae is additionally well established (Kingsford and Mooney, 2014, Hartwick, 1991a, Gordon and Seymour, 2012, Mooney and Kingsford, 2012). The absence of observations of medusae and stings confirmed the absence of the taxa's medusae stage during winter months (June - September). Additionally, no medusae were captured in beach seine net drags at any Horseshoe Bay sites that were sampled during the July 2022 sampling time. This sampling regime ground-truthed that *C. fleckeri* medusae are only present during summer months and thus any detections during winter months are most likely eDNA shed from *C. fleckeri* polyps, rather than medusae.

4.4.2 Detection and Distribution of *Chironex fleckeri* Medusae

4.4.2.1 Nearshore Detection of Chironex fleckeri Medusae

Chironex fleckeri eDNA was detected along the shores of both Horseshoe and Maud Bays in 2020 and 2021 (Figure 4.2 and 4.3). In the summer of 2020 (December), eDNA was exclusively found within Horseshoe Bay, with increasing concentrations observed along the shore to the western end of the bay (Figure 4.2, sites 16 - 19). Detection was noted in 22.9 % of technical replicates from positive sample sites, with eDNA copies L^{-1} ranging from 32 – 275.6 copies L^{-1} (Table S3.2).

Two months later (i.e. February 2021) *C. fleckeri* eDNA was again detected within Horseshoe Bay. Additionally, eDNA was detected in Maud Bay close to this bay's freshwater inflow (Figure 4.3). During this sampling time, detection was found in 15 % of technical replicates from positive sample sites, with eDNA copies L^{-1} ranging from 17.8 – 92.4 copies L^{-1} (Table S3.2). Notably, eDNA concentrations were generally lower during this sampling period (exception of site 6). Equipment controls for both sampling times verified lack of contamination, while the endogenous control affirmed method success.

Temperatures and salinities were similar throughout the study area within each sampling period (December 2020; 29 – 31.1 °C and 36.2 – 36.4 ppt, February 2021; 29.8 – 30.3 °C and 32.4 – 33.8 ppt). Lower salinities were observed in February, likely due to rainfall in the week preceding sampling. No stratification of the water column in temperature or salinity was detected in water depths of 0.4 – 3.1 m (Figure S3.2 and S3.3).

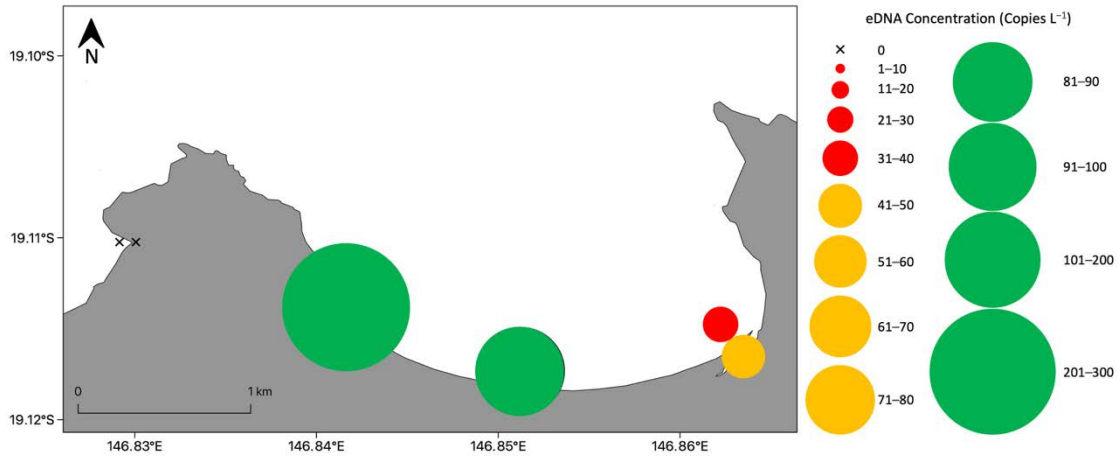

Figure 4.2. Bubble map plot displaying sampling sites along the shore of Horseshoe and Maud Bays with positive detections of *Chironex fleckeri* medusae, in December 2020. Bubbles indicate eDNA concentrations (copies L^{-1}), colours are for visualisation purposes only.

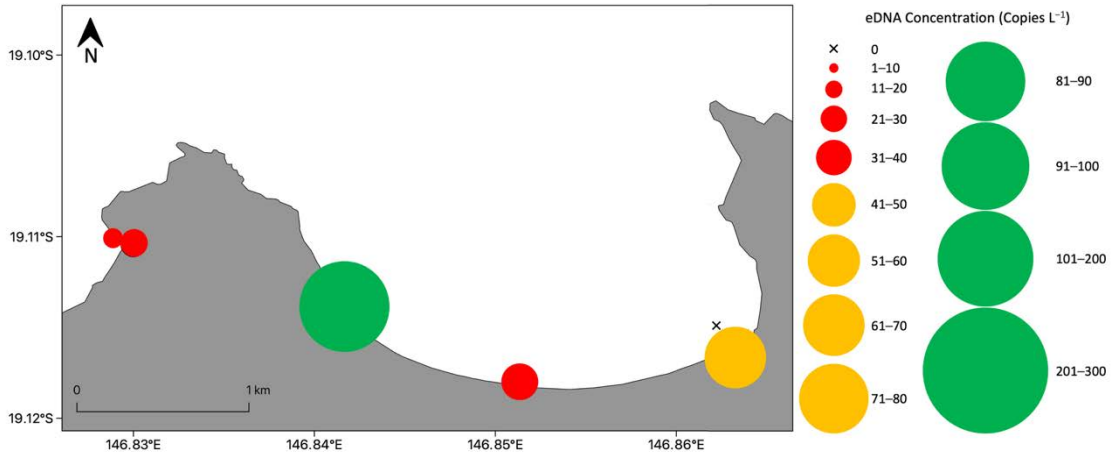

Figure 4.3. Bubble map plot displaying sampling sites along the shore of Horseshoe and Maud Bays with positive detections of *Chironex fleckeri* medusae, in February 2021. Bubbles indicate eDNA concentrations (copies L^{-1}), colours are for visualisation purposes only.

4.4.2.2 Bay Wide Sampling Design for Chironex fleckeri Medusae

The eDNA of *Chironex fleckeri* was only detected at nearshore sample sites in Horseshoe Bay and there were some detections in Maud Bay (Figure 4.4 and 4.5). Interestingly, no *C. fleckeri* eDNA was detected at mid-shore or offshore sites. In March 2021, the highest eDNA concentrations were observed near the freshwater inflow in Horseshoe Bay (Figure 4.4). Notably, this specific site consistently exhibited positive detections at all sampling times within the 2020/21 box jellyfish season (Figure 4.2, 4.3 and 4.4). Detection was observed in 22.2 % of the technical replicates from positive sample sites, and eDNA copies L^{-1} ranged from 18.8 – 134.8 copies L^{-1} (Table S3.3). At the end of 2021 (December), at the beginning of the next Australian box jellyfish season (2021/22), detection of eDNA was confined to sites with freshwater inflow in both Horseshoe and Maud Bays (Figure 4.5). Thus, only two sites have positive detections during this sampling time with eDNA copies L^{-1} ranging from 22.5 – 33.5 copies L^{-1} (Table S3.3). All controls again verified lack of contamination and method success for both sampling times.

Stratification of the water column is known to restrict eDNA dispersal within the water column (Jeunen et al., 2020, Gray and Kingsford, 2003). However, CTD profiles did not detect stratification of the water column in temperature or salinity at all sites ranging in depths of 0.4 to 13 m (Figure S3.4 and S3.5). Temperatures and salinities were consistent throughout the study area for each sampling period (March 2021; 27.2 – 29.6 °C and 31.4 – 32.9 ppt, December 2021; 30 – 30.7 °C and 35.2 – 35.5 ppt). It should be noted however that nearshore waters in March 2021 were 2°C higher than all other sites.

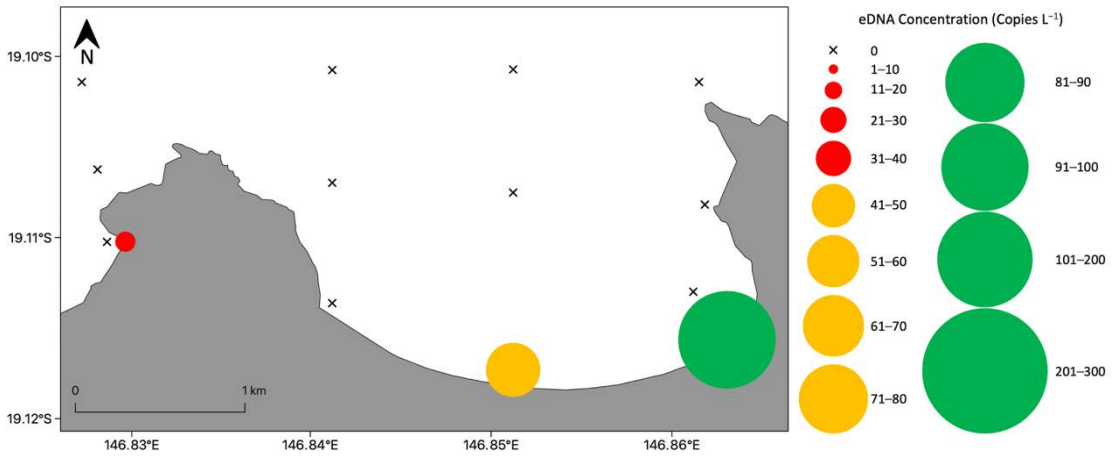

Figure 4.4. Bubble map plot displaying sampling sites within Horseshoe and Maud Bays with positive detections of *Chironex fleckeri* medusae, in March 2021. Bubbles indicate eDNA concentrations (copies L^{-1}), colours are for visualisation purposes only.

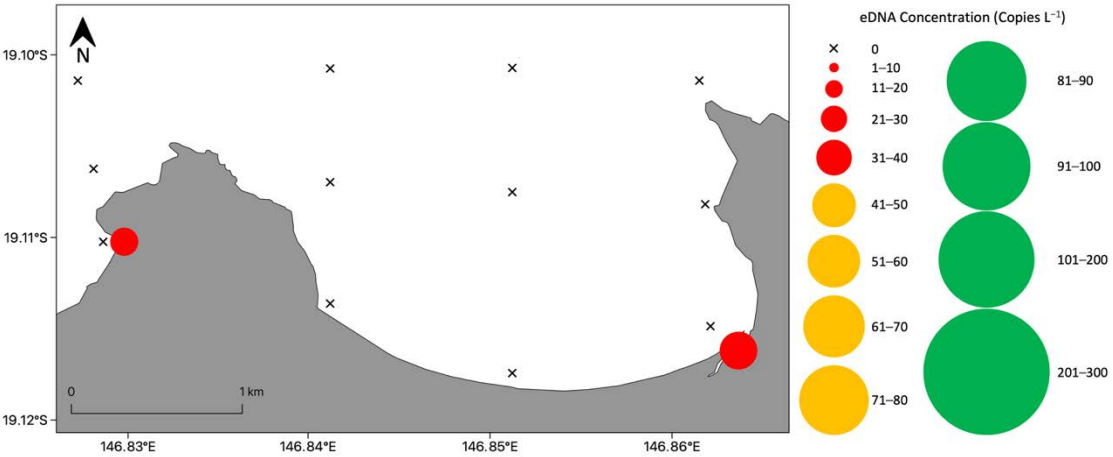

Figure 4.5. Bubble map plot displaying sampling sites within Horseshoe and Maud Bays with positive detections of *Chironex fleckeri* medusae, in December 2021. Bubbles indicate eDNA concentrations (copies L^{-1}), colours are for visualisation purposes only.

4.4.3 Detection and Distribution of *Chironex fleckeri* Polyps

4.4.3.1 Bay Wide Sampling Design for Chironex fleckeri Polyps

Chironex fleckeri eDNA was positively detected within Horseshoe Bay outside of the established medusae season (Figure 4.6). The detections, therefore, could only be attributed to the presence of the species benthic polyp stage as no medusae were reported to be present during this sampling period and as medusae are not usually found at this time of the year. Positive detections were only found near the freshwater inflow within Horseshoe Bay and along the eastern side of the bay. eDNA copies L^{-1} ranged from 62.5 – 63.5 copies L^{-1} (Table S3.4). All controls provided assurance of contamination-free conditions and method success.

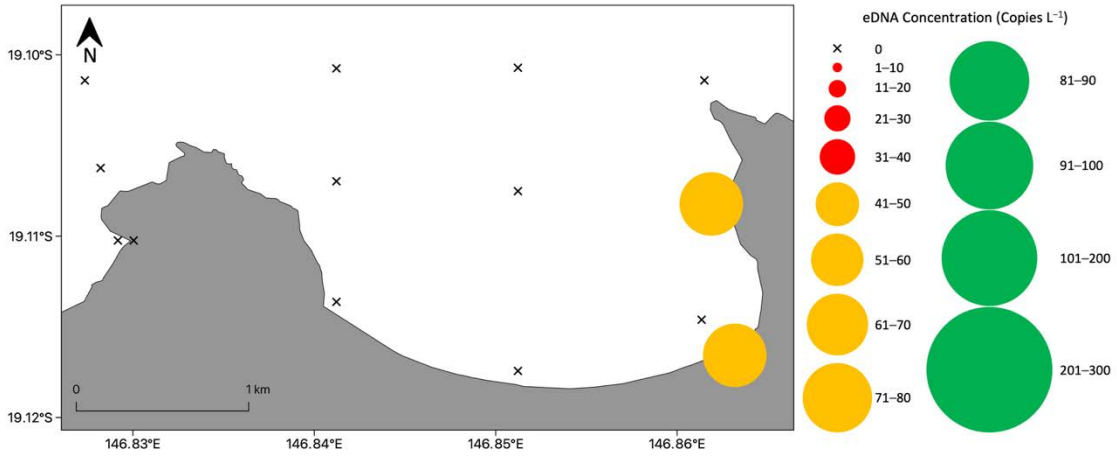

Figure 4.6. Bubble map plot displaying sampling sites within Horseshoe and Maud Bays with positive detections of *Chironex fleckeri* polyps, in July 2020. Bubbles indicate eDNA concentrations (copies L^{-1}), colours are for visualisation purposes only.

4.4.3.2 Targeted Sampling to Determine Chironex fleckeri Polyp Hotspots

The targeted sampling design only detected *Chironex fleckeri* eDNA at sites with freshwater inflows within Horseshoe and Maud Bays (Figure 4.7). These detections were outside of the established medusae season, and therefore, could only be attributed to the presence of benthic polyps. Additionally, there were no reports of medusae being present during this sampling period and none were caught in our beach seines. eDNA copies ranged from 73.6 – 82.3 copies L^{-1} (Table S3.5), and positive detection was observed in 12.5% of technical replicates from positive sample sites. All controls ensured the absence of contamination and validated the success of the applied methods.

CTD profiles did not reveal any stratification of the water column in terms of temperature or salinity in shallow water. Variation in temperature was found to occur between inshore and offshore sites, with highest temperatures being recorded nearshore. Temperatures and salinities were 20 – 22.7 °C and 31.5 – 34.4 ppt within Horseshoe and Maud Bays. Salinities were, however, found to decrease considerably when moving further within Horseshoe Bay's freshwater inflow (34.1 – 2.5 ppt) (Table S3.5). Further, salinities were observed to fluctuate (± 3.7 ppt) midway along this freshwater inflow. This lower salinity may be resultant from rainfall occurring two weeks prior to sampling resulting in recent mixing of freshwater with saltwater.

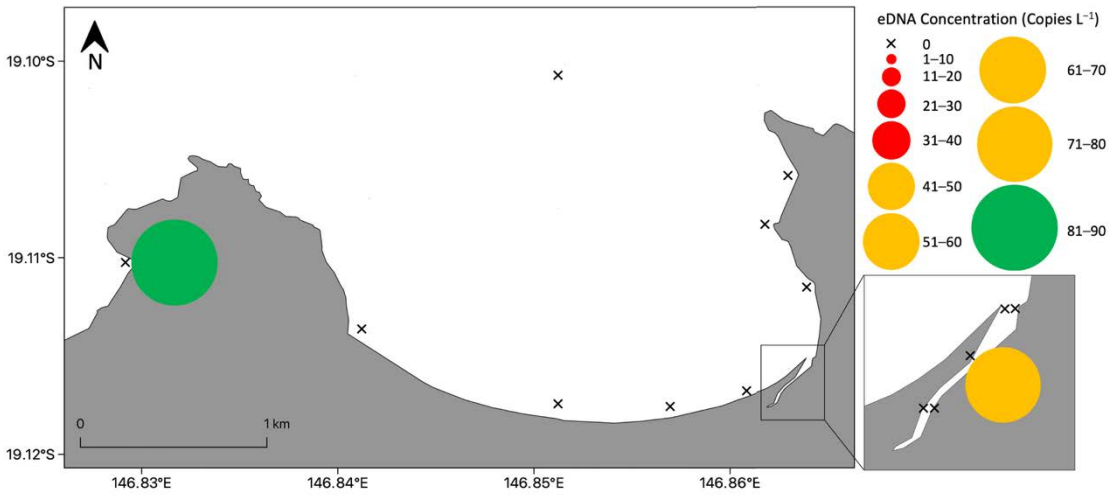

Figure 4.7. Bubble map plot displaying sampling sites within Horseshoe and Maud Bays with positive detections of *Chironex fleckeri* polyps, in July 2022. Bubbles indicate eDNA concentrations (copies L^{-1}), colours are for visualisation purposes only.

4.4.4 Detection of *Chironex fleckeri* Near Shore at all Times

There were consistent spatial patterns of eDNA detection among times (Figure 4.8). Within Horseshoe Bay's freshwater inflow, eDNA was detected at all times of sampling (site 6). This detection must be due to the presence of both medusae and polyp life history stages. Similarly, *Chironex fleckeri* eDNA was detected in Maud Bay and near a freshwater inflow (site 23) at all but one time of sampling. Regarding nearshore sample sites within Horseshoe Bay, to the west of sites 5, detection was only found during the medusae season (sites 16 and 19).

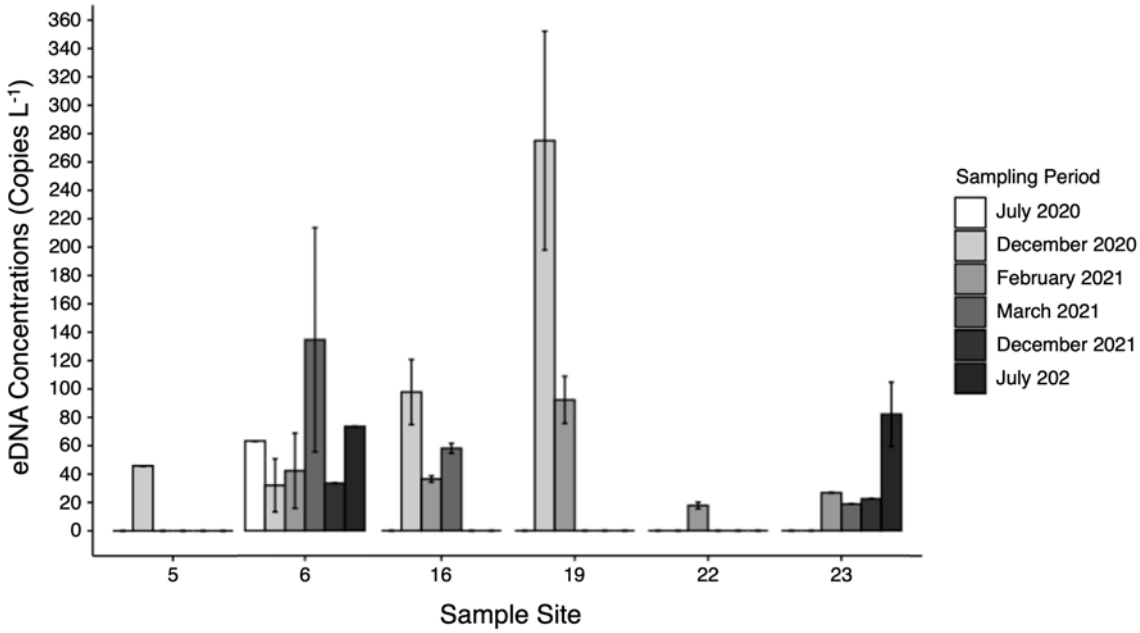

Figure 4.8. Bar plot of *Chironex fleckeri* eDNA concentrations (copies L^{-1}) (1 ± SE) at nearshore sample sites across all time of sampling in and out of the jellyfish season.

4.5 Discussion

4.5.1 Distribution of *Chironex fleckeri* Medusae

During the Australian box jellyfish season, *Chironex fleckeri* medusae were consistently detected nearshore and not in waters that were hundreds of meters to kilometres from shore. This eDNA detection aligned with Brown (1973)'s observations on *C. fleckeri* medusae distributions surrounding Magnetic Island and with previous studies indicating nearshore distributions of *C. fleckeri* (Kingsford et al., 2012, Brown, 1973, Hartwick, 1991a, Mooney and Kingsford, 2016a). This finding was predicted based on previous studies (Kingsford et al., 2012, Brown, 1973, Hartwick, 1991a, Mooney and Kingsford, 2016a); however, eDNA's use for exploring *C. fleckeri* distributions have only been undertaken in semi-enclosed waters rather than open coastal waters. eDNA, being a passive particle, can be influenced by oceanographic processes (Harrison et al., 2019, Jeunen et al., 2020, Shogren et al., 2019, Stoeckle et al., 2021, Shogren et al., 2018, Pont et al., 2018), such as transport by currents (Harrison et al., 2019) and isolation from surface waters due to water column stratification (Jeunen et al., 2020). However, in this study, despite daily persistence of *C. fleckeri* eDNA (99% decay within 27 hrs) (Morrissey et al., 2022), the dispersion of eDNA appeared to be limited. Water is known to ebb out of Horseshoe Bay along both sides of the bay (Wolanski et al., 2024a), however no detection was ever found at mid and offshore sites along these currents. Furthermore, no stratification was observed in mid and offshore water columns that could have potentially isolated eDNA below an isocline (Jeunen et al., 2020). It was also clear that despite 32 replicate samples being taken at samples sites that were hundreds of meters to kilometres from shore, *C. fleckeri* eDNA was never detected. The combined evidence, therefore, suggested restricted *C. fleckeri* eDNA dispersal, and the absence of *C. fleckeri* at mid and offshore sites. To validate this further, biophysical modelling of eDNA dispersion may be employed (Ellis et al., 2022, Andruszkiewicz et al., 2019). Additionally, because we employed a highly specific and sensitive detection assay, best practice control measures and optimised techniques for elusive species detections that ensures precision, we are confident in the accuracy of our findings (Morrissey et al., 2022). Thus, eDNA has successfully identified the nearshore distribution of the taxa for the area, further highlighting the ability of eDNA to expose elusive taxa distributions (West et al., 2021, Albonetti et al., 2023, Fukumoto et al., 2015).

Studying medusae detection throughout the 2020/21 Australian box jellyfish season may offer insights into the species' movements. In December, *C. fleckeri* was

solely detected in Horseshoe Bay. However, in February and March, *C. fleckeri* was additionally detected in neighboring Maud Bay, suggesting potential movement. This aligns with Brown (1973)'s observations that initially, *C. fleckeri* medusae were only present in Horseshoe Bay, but later appeared in adjacent bays as large medusae. Brown (1973) proposed this movement may be due to strong northerly winds causing medusae to seek calmer waters. However, we consistently found nearshore detections at multiple times of sampling, so more data are required on movements as there are few data on the movements of cubomedusae (Morrissey et al., 2020b, Kingsford and Mooney, 2014, Mooney and Kingsford, 2012, Morrissey et al., 2020a).

Persistent medusae eDNA detection was observed in the freshwater inflow of Horseshoe Bay during summer months. This may be a result of polyps being putatively present in this area (see section 4.2), as they are the source of medusae. Alternatively, or perhaps in combination, medusae may opt to remain in areas with higher/appropriate prey abundance to minimise energy expenditure (Gordon and Seymour, 2009). Mangrove habitats are known for harboring a higher abundance of post-larval, juvenile and small adult fish, along with juvenile crustaceans, which serve as common medusae prey (Carrette et al., 2002, Robertson and Duke, 1987). However, medusae were clearly venturing to nearshore waters without mangroves where perhaps prey are still available. The detection of *C. fleckeri* eDNA in Maud Bay maybe due to a combination of some leakage of medusae from Horseshoe Bay as suggested by Brown (1973), and/or recruitment from a local source of polyps.

4.5.2 Detection of *Chironex fleckeri* Polyps

Outside of the Australian medusae box jellyfish season, *Chironex fleckeri* eDNA was detected. Since *C. fleckeri* medusae are absent from waters during winter months due to their seasonality (Mooney and Kingsford, 2016b, Kingsford and Mooney, 2014, Gordon and Seymour, 2012, Hartwick, 1991a), confirmed via SLSQ for Magnetic Island (no detection or reported stings), these detections must arise from benthic polyp life history stages. Additionally, detections were of a lower frequency in comparison to those during summer months when medusae were present. Polyps of multiple scyphozoan jellyfish species have been observed to have restricted distributions, such as *Cyanea* sp. within the Niantic River, USA (Colin and Kremer, 2002) and *Aurelia aurita* within Mikawa Bay, Japan (Toyokawa et al., 2011). *C. fleckeri* polyps likely follow suit which subsequently explains the lower frequency and restricted eDNA detection of *C. fleckeri* found during winter months. Further, the reliability and confidence in these detections

are ensured through use of a highly sensitive and specific *C. fleckeri* detection assay and use of eDNA methods optimised for elusive species (Morrissey et al., 2022). This study reinforces the validity of the eDNA technique to detect the putative presence of *C. fleckeri* polyps (Morrissey et al., 2024a).

During the first sampling time within the Austral winter (July 2020), *C. fleckeri* polyps were putatively detected in Horseshoe Bay's freshwater inflow and along the eastern side of the bay. Later, in July 2022, *C. fleckeri* polyps were detected within the freshwater inflows of both Horseshoe and Maud Bays. As suggested in a previous study undertaken by Morrissey et al. (2024a), rocky substrata is likely suitable habitat for cubozoan polyps, and medusae 'hotspots' may be good indicators of their presence. All sites where polyps were detected have rocky substrata, with Horseshoe and Maud Bay's freshwater inflows containing mangroves and granite boulders, and the eastern side of Horseshoe Bay containing granite boulders, coral reef, and coral rubble. These detections subsequently align with the study of Morrissey et al. (2024a) where polyp habitat was identified. Additionally, polyp presence within Horseshoe Bay's freshwater inflow was unsurprising as it consistently showed medusae presence in summer months. This subsequently aligns with Morrissey et al. (2024a) suggestion of medusae 'hotspots' being good indicators of polyp presence. These findings additionally provide support to Brown (1973)'s hypothesis of Horseshoe Bay being a source location of *C. fleckeri*.

As cubozoan polyps are difficult to find in their natural environment, resulting from their tiny size, eDNA provides the most efficient technique for their detection (Morrissey et al., 2022, Morrissey et al., 2024a, Bolte et al., 2021). Subsequently, the technique opens the door to studying this life history stage and the filling of critical knowledge gaps surrounding *C. fleckeri*'s ecology (Morrissey et al., 2024a, Kingsford and Mooney, 2014). As previously suggested (Morrissey et al., 2024a), environmental RNA (eRNA), which may enable a finer resolution of detection due to its rapid decay (Yates et al., 2021), may assist physical *in-situ* locating of *C. fleckeri* polyps. Recent advancements in this technique, however, may completely remove this need. Parsley and Goldberg (2023) successfully utilised the technique to distinguish between amphibian life history stages. eRNA hence, in addition to the known seasonality of medusae (Mooney and Kingsford, 2016b, Kingsford and Mooney, 2014, Gordon and Seymour, 2012, Hartwick, 1991a) and lack of their presence during winter months, would undoubtably confirm detections of *C. fleckeri* polyps. The authors hence suggest exploration of this application for cubozoan and scyphozoan jellyfish, whose medusae stage is not seasonal.

4.5.3 Evaluating Distributions of *Chironex fleckeri* Medusae and Polyps for Informed Stock Boundary Assessment and the Generality of eDNA for this Application

Through utilising eDNA to detect both *Chironex fleckeri* medusae and polyp life history stages, we gain insights into the spatial extend of *C. fleckeri*'s population for the area. Polyps, as they are the benthic source of medusae (Arai, 1997, Kingsford and Mooney, 2014), likely play a key role in the spatial boundaries of the species population stocks (Kingsford et al., 2021). Studies exploring the role of scyphozoan polyps in determining the abundance and distributions of medusae have reported a strong relationship between the distribution of both life history stages (Colin and Kremer, 2002, Toyokawa et al., 2011, Shahrestani and Bi, 2018). Further, as cubozoans are gonochoristic (Siebert and Juliano, 2017), with *C. fleckeri* medusae undertaking external fertilisation (Yamaguchi, 1980), medusae need to be in close proximity to each other and in areas of suitable habitat and environmental conditions for polyps. Increasing evidence suggests that some cubozoan jellyfish, including *C. fleckeri*, have population stocks of small spatial scales, to the extent of bays and estuaries, however, polyp locations have largely not been considered or were impossible to detect (Kingsford et al., 2021). As polyps have been putatively detected within this study, it allows for exploration into potential population stock boundaries of *C. fleckeri* for the study area.

C. fleckeri medusae were exclusively detected nearshore in Horseshoe and Maud Bays, highlighting their nearshore distribution. Additionally, the benthic polyp stage of the species was consistently detected within Horseshoe Bay and once within Maud Bay. As polyps are the source of medusae, and as medusae were found to reside within these bays across the Australian box jellyfish season, it is reasonable to infer that the northern side of Magnetic Island likely represents a population stock of the jellyfish. This is supported by the lack of *C. fleckeri* captures by SLSQ in bays located on the south side of the island (pers. comms. SLSQ) and aligns with Brown (1973)'s observations on *C. fleckeri* medusae distributions surrounding the island. This suggestion additionally aligns with evidence from other sources (Mooney and Kingsford, 2017, Schlaefer et al., 2018, Gordon and Seymour, 2009, Mooney and Kingsford, 2016a).

A biophysical modelling and jellyfish behaviour study, undertaken by Schlaefer (Schlaefer et al., 2018), found *C. fleckeri* medusae to have strong swimming behaviour and an orientation to nearshore environments. Additionally, Gordon and Seymour (2009), via use of acoustic telemetry, observed multiple *C. fleckeri* medusae (n = 11) to

not venture far from initial tagging locations, covering hundreds of metres to a few kilometers over an average duration of ~15 hrs. These studies findings suggest limited dispersal of *C. fleckeri* medusae with them staying close to home and subsequently align with evidence presented within this study. Further, Mooney and Kingsford (Mooney and Kingsford, 2017, Mooney and Kingsford, 2016a) examined both the elemental chemistry and morphometrics of *C. fleckeri* statoliths to investigate the structure and scale of the species population units. Examination of *C. fleckeri* statolith morphometrics revealed variations between sites separated by 10s of kilometers (Mooney and Kingsford, 2017). Examination of statolith elemental chemistry revealed distinct variations between individuals located within Horseshoe Bay and mainland Townsville, located ~ 10 km away (Mooney and Kingsford, 2016a). Mooney and Kingsford (2016a, 2017)'s findings subsequently suggest spatially small population units of *C. fleckeri* and provide additional support to the northern side of Magnetic Island representing a population stock of *C. fleckeri*. To validate this notion, examining the genetics of individuals in Horseshoe Bay and the nearby mainland (~10 km away) would be valuable. Furthermore, since eDNA has been successfully utilised as a population genetics tool (Adams et al., 2019, Andres et al., 2023, Zanovello et al., 2023), the use of both eDNA and eRNA may enable the linking of medusae to detected polyps, thereby confirming their origin. Leveraging genetic detection techniques for this use would significantly contribute to our understanding of cubozoan jellyfish distributions, population structures, and potential movements.

Prior to this study, eDNA was utilised to investigate a hypothesis surrounding a semi-enclosed estuarine system representing a population stock of *C. fleckeri* (Morrissey et al., 2024a). The genetic detection technique proved successful, providing evidence to support the hypothesis. However, favourable currents, medusae swimming behaviour, and presence of polyps within the estuary likely favour the retention of the jellyfish in that system. In contrast, eDNA, in this current study, was utilised to inform *C. fleckeri* stock boundaries in an open coastal environment, where oceanographic and geomorphic conditions were more likely to facilitate dispersal of jellyfish rather than retention. Accordingly, based on the evidence and discussion above, *C. fleckeri* population stocks appear common at small spatial scales, in ecosystems of varying geomorphic and oceanographic conditions.

4.6 Conclusions

An in-depth understanding on cubozoan ecology is needed for effective mitigation and management of their threat posed to both human health and enterprise (Kingsford et al., 2018, Kingsford and Mooney, 2014). This study further demonstrated the ability of eDNA to investigate and fill critical knowledge gaps surrounding cubozoan ecology. *Chironex fleckeri* medusae were exclusively detected nearshore, with eDNA identifying their expected nearshore distribution despite potential eDNA dispersal. Further, the genetic tool was again successful in detecting *C. fleckeri*'s elusive benthic polyp stage. This finding concurred with a hypothesis suggesting that Horseshoe Bay was an important source of medusae for Magnetic Island. Polyps were consistently detected near freshwater inflows, and this aligned with a previous study where polyp habitat was identified (Morrissey et al., 2024a). A comparison of these two life history stages added to existing evidence that the northern side of Magnetic Island is likely a robust population stock of the jellyfish. This adds to growing evidence (Kingsford et al., 2021) suggesting that *C. fleckeri* have population stocks of small spatial scales, in both semi-enclosed estuaries (Morrissey et al., 2024a, Schlaefer et al., 2018) and open bays. Additionally, our study and other research has demonstrated that even in an open coastal setting medusae populations of *C. fleckeri* have a very restricted distribution nearshore. Accordingly, eDNA offers a tool capable of testing ecological hypotheses and filling critical knowledge gaps surrounding cubozoan ecology.

Chapter 5.

Estimating *Chironex fleckeri* eDNA Transport in an Open Coastal Bay

5.1 Abstract

Environmental DNA (eDNA) has emerged as a powerful tool for species detection and monitoring; however, understanding of its dispersion and transport dynamics and how this influences detectability is essential to enhance accuracy of eDNA use. A biophysical model was used to investigate the spatiotemporal dispersion of *Chironex fleckeri* eDNA in an open coastal bay, in northern Australia. This model simulated the transport and decay of passively drifting eDNA particles using hydrodynamic forcing and spatially explicit particle releases to estimate the extent and detectability of *C. fleckeri* eDNA. The model revealed that local hydrodynamics, geomorphology, and environmental conditions shaped 'detection shadows', with eDNA detectability constrained to hundreds of meters to kilometres from source locations. These estimates of dispersion closely aligned with empirical detections of *C. fleckeri* medusae and polyps, demonstrating the utility of biophysical models for estimating eDNA transport and dispersal dynamics. The findings highlight the influence of eDNA decay and dilution on detectability and provide valuable insights for determining the source of targeted taxa. This study demonstrates the broader potential of combining biophysical modelling with eDNA sampling.

5.2 Introduction

Environmental DNA (eDNA) provides a non-invasive and efficient method to detect species within their environment (Beng and Corlett, 2020, Thomsen and Willerslev, 2015, Bohmann et al., 2014). It is increasingly being explored and applied as an ecological survey and management tool to further understand the presence of species and their distributions. Further, it can provide critical information to stakeholders and decision makers surrounding the risk management of problematic species (Simpfendorfer et al., 2016, Eva et al., 2016, Ishige et al., 2017, Bálint et al., 2018, Uthicke et al., 2018, Doyle et al., 2017, Villacorta-Rath et al., 2020). However, the accuracy of using eDNA as a detection method is at times questioned due to fundamental knowledge gaps surrounding the "ecology" of eDNA (Hansen et al., 2018, Barnes and Turner, 2016, Scriver et al., 2023), particularly its physical transport and dispersion within the natural environment (Harrison et al., 2019, Hansen et al., 2018). This understanding is crucial for linking eDNA-based detections to the physical presence of taxa, thereby distinguishing between true detections, and those arising from allochthonous signals. Current interpretations of eDNA-based species detections often rely on eDNA persistence times and thus temporal detection windows. However, as highlighted by Thomsen et al. (2012), eDNA has the potential to travel significant distances, ranging from 10s to 100s of kilometres before decaying. Therefore, understanding the spatiotemporal dispersion of eDNA is key for enhancing the accuracy of eDNA-based species detections and to inform efficient sampling strategies (Harrison et al., 2019, Blackman et al., 2024).

As a passive particle ranging from 0.2 μm to 180 μm in diameter (Turner et al., 2014), eDNA is at the mercy of hydrodynamic forces and environmental conditions (Murakami et al., 2019, Harrison et al., 2019), which can vary significantly across environments. Interpreting eDNA detections, therefore, requires a tailored understanding of specific environmental contexts. For instance, in still water environments horizontal eDNA transport tends to be limited (Goldberg et al., 2018, Dunker et al., 2016, Brys et al., 2021), with maximum distances reported to be only 100s of meters from source individuals (Eichmiller et al., 2014, Ghosal et al., 2018, Dunker et al., 2016). Conversely, in environments with actively flowing water, eDNA transport has been reported over distances ranging from kilometres (Jane et al., 2015, Jerde et al., 2016, Wilcox et al., 2016, Shogren et al., 2017, Nukazawa et al., 2018, Robinson et al., 2019, Shogren et al., 2019, Nevers et al., 2020, Wood et al., 2021, Jo and Yamanaka, 2022) to 10s of kilometres (Pont et al., 2018, Deiner and Altermatt, 2014). This dispersion is influenced

by numerous extrinsic factors such as flow velocity, river characteristics/morphology and substrate type (Deiner and Altermatt, 2014, Jerde et al., 2016, Shogren et al., 2017, Shogren et al., 2016, Snyder et al., 2023). This variability underscores the need for detailed understandings of eDNA transport across differing environments and conditions.

In marine environments, these complexities are further amplified due to the interplay of a multitude of factors, such as tides, water density, wind patterns, ocean currents, bathymetry and coastal geomorphology. These interactions create variable water movements, both horizontally and vertically, resulting in complex hydrodynamic conditions. As a result, the transport of eDNA in marine systems is expected to exhibit significant spatial variability. Empirical studies highlight this with a wide range of maximum detection distances from known source individuals being reported in marine settings, from hundreds of meters to a few kilometres (Ely et al., 2021, Murakami et al., 2019, Port et al., 2016, Jeunen et al., 2019, Shea and Boehm, 2024, Baetscher et al., 2024, Dugal et al., 2023, West et al., 2020, McCartin et al., 2024, Kelly et al., 2018), to more expansive transport over 10s of kilometres (Thomsen et al., 2012). This variation is likely a result of differing local hydrodynamic influences among study locations. This highlights the importance of understanding the physical transport and dispersion of eDNA across diverse environments and under varying environmental conditions. Additionally, biotic and abiotic factors which influence eDNA release and persistence (Caza-Allard et al., 2022), likely influence this dispersion.

Biophysical modelling has been successfully utilised to investigate the transport and dispersal patterns of passive particles in marine systems for numerous applications (Gallego, 2011, Swearer et al., 2019, Aleynik et al., 2018). Subsequently, it has been highlighted as a method to study the transport and dispersion of eDNA (Harrison et al., 2019, Ellis et al., 2022, Andruszkiewicz et al., 2019, Kutti et al., 2020). Recently, this approach has been utilised to examine eDNA transport and dispersal to inform and improve sampling designs (Ellis et al., 2022, Richardson et al., 2016), and to identify origin locations of eDNA (Andruszkiewicz et al., 2019, Fukaya et al., 2021), showcasing the use of this tool to model eDNA dispersal patterns. With the incorporation of known eDNA degradation rates it is possible for this approach to reveal and allow study of the spatiotemporal distribution of detectable eDNA from source individuals, termed ‘eDNA shadows’ by Ellis et al. (2022). Moreover, due to the nature of these models, the influence of varying extrinsic factors upon eDNA conveyance, hence transport mechanisms, can be explored. This approach not only serves as a valuable tool for investigating eDNA transport, but also holds potential to enhance interpretation of eDNA detections and sampling effort to maximise detections (Ellis et al., 2022). The use of

biophysical models for this purpose, however, need to be further explored in marine settings with ground truthing.

eDNA has recently been applied as an ecological survey tool to detect and study problematic jellyfish, specifically the notorious Australian box jellyfish, *Chironex fleckeri* (Morrissey et al., 2024a, Morrissey et al., 2024b, Morrissey et al., 2022). Morrissey et al. (2024a) displayed the efficacy of eDNA in detecting both major life history stages (medusae and putatively polyps) and in delineating population stock boundaries of the species (Morrissey et al., 2024a, Morrissey et al., 2024b). Using a grid sampling design within an open coastal bay (Horseshoe Bay of Magnetic Island, Australia), they determined the nearshore distribution of the species, with detections exclusively at nearshore sites with non-detections at sites located hundreds of meters to kilometres from shore across multiple sampling times (Morrissey et al., 2024b). Based on these findings, they suggested that *C. fleckeri* eDNA dispersal is likely limited and recommended use of biophysical models to test this hypothesis. This presented an opportunity to utilise biophysical models to evaluate the spatiotemporal dispersal potential of eDNA within an open coastal bay and to compare modelled eDNA transport with empirical data (Morrissey et al., 2024b). This approach could additionally provide crucial information for optimising the sampling and interpretation of eDNA-based detections of this deadly species, aiding in their monitoring and management, and offering insights into eDNA dispersal dynamics in open coastal embayments.

The objective of this study was to utilise biophysical models to examine the transport of *C. fleckeri*'s eDNA within an open coastal bay under varying conditions, to improve our understanding of the relationship between species presence and eDNA detections. Specifically, we aimed to determine the following; (i) oceanographic and environmental influences upon eDNA transport and dispersion in an open coastal bay, (ii) spatiotemporal dispersion of eDNA, to better understand how long and at what spatial scale eDNA signals remain viable for detection, and (iii) how does modelled transport and estimates of detection limits compare with empirical field detections of *C. fleckeri*.

5.3 Materials and Methods

5.3.1 Study Area

This study encompassed an open coastal bay, Horseshoe Bay, Magnetic Island, Australia (19.11°S, 146.85°E). Horseshoe Bay is an approximately 2.5 by 1.7 km shallow bay, buffered by rocky headlands, with a maximum depth of approximately 10 m (Figure 5.1). The bay has a sandy beach environment, with some fringing seagrass beds and coral reef (Brown, 1973), and a tidally isolated freshwater inflow located in its south-east corner. It has an average tidal range of ~3.4 metres and the water column is generally unstratified (Morrissey et al., 2024b). This area was selected for this study as empirical data on detections of *Chironex fleckeri* eDNA was previously undertaken (Morrissey et al., 2024b).

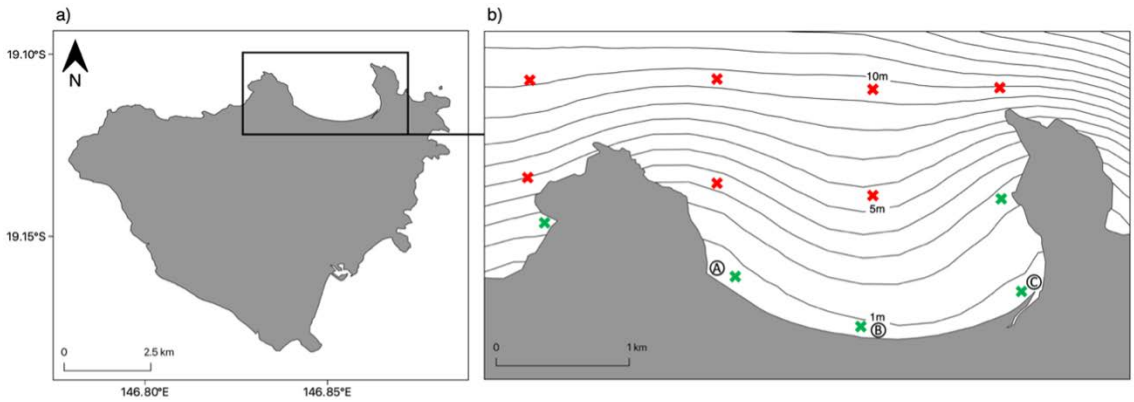

Figure 5.1. Study site. a) Magnetic Island, b) map of Horseshoe Bay with bathymetry of the bay (1m interval contour lines) and particle seeding locations (A, B, C). Outside of Australian stinger season particle seeding location is C. Green crosses are indicative of empirical positive field detections of both medusae and polyps, red crosses are indicative of negative field detections as per Morrissey et al. (2024b).

5.3.2 Study Species

C. fleckeri, the Australian box jellyfish, is a highly venomous species found along the northern coast of Australia from Gladstone to Broome, extending into the Indo-Pacific (Kingsford et al., 2021). The species has a polymorphic life history where their medusae stage is seasonal (Austral summer; October to May) (Kingsford and Mooney, 2014, Kingsford et al., 2018). This stage is known to maintain a nearshore distribution in

Australia (Kingsford et al., 2012, Gordon and Seymour, 2009, Morrissey et al., 2024b) and subsequently poses a threat to both human health and commercial enterprise (Morrissey et al., 2024a, Morrissey et al., 2022, Kingsford et al., 2018, Kingsford and Mooney, 2014, Crowley-Cyr et al., 2022, Morrissey et al., 2024b). The medusae and polyp stages of this species are known to be present within Horseshoe Bay (Morrissey et al., 2024b). Medusae were exclusively detected along the shore of the bay while polyps were putatively detected within the eastern side and a tidally isolated freshwater inflow of the bay (Figure 5.1) (Morrissey et al., 2024b). This ecological knowledge was utilised to inform our modelling approach (section 5.3.4.1).

5.3.3 Biophysical Model

5.3.3.1 Hydrodynamic Model

A hydrodynamic model simulating water movements, including tides and currents, around Magnetic Island was constructed using the two-dimensional version of the Second-generation Louvain-la-Neuve Ice-Ocean Model (SLIM, (Lambrechts et al., 2008)). The selection of the 2D iteration of SLIM was based on the shallow and well-mixed nature of the study area, where eDNA would unlikely be isolated from surface waters due to stratification (Morrissey et al., 2024b, Luick et al., 2007, Littlefair et al., 2021). Moreover, this iteration of SLIM has previously been effectively utilised in accurately simulating the hydrodynamics of shallow systems (Lambrechts et al., 2008, Schlaefer et al., 2021, Pham Van et al., 2016). In SLIM, the shallow-water equations undergo spatial discretisation using a second-order discontinuous Galerkin finite element method, and temporal integration employs a second-order implicit Runge-Kutta method (Lambrechts et al., 2008). Additionally, bottom friction dissipation is computed using a Chezy-Manning scheme, incorporating the Chezy-Manning-Strickler formulation. Finally, turbulent velocity is determined through a Smagorinsky scheme.

The hydrodynamics of Magnetic Island, situated within the central area of the Great Barrier Reef, are significantly influenced by large-scale forces such as the North Caledonian Jet, the East Australian Current (EAC), and the EAC lagoonal branch, originating from the South Equatorial Current (Kessler and Cravatte, 2013, Wolanski and Kingsford, 2024, Wolanski et al., 2024b). Furthermore, the shallow nature of the region renders it susceptible to local wind patterns that can induce currents on a smaller scale. Consequently, the model domain encompassed an area from the middle of the Queensland Plateau to the far south of Magnetic Island, extending from the Queensland

coast out into the Coral Sea (Figure S4.1), as per the domain utilised by Schlaefer et al. (2021). Open boundary forcings of wind and a combination of currents and tides over the entire domain were employed to simulate water movements.

Tide and current data, sourced from eReefs GBR 4 (Herzfeld, 2016), and wind data, source from the Australian Bureau of Meteorology's (BoM) Atmospheric high resolution Regional Reanalysis for Australia (BARRA) (Su et al., 2019), were utilised to simulate the hydrodynamic forces, with tides and currents at the open boundary and wind over the entire domain. An unstructured SLIM grid was created, with coarser elements in open water and finer elements near coasts and reefs, with emphasis on the area surrounding Magnetic Island (Figure S4.1). This grid was composed of 68,403 triangles with side length ranging from 25.90 m to 7.75 km (df = 205,209 by sea surface elevation, zonal and meridional current components). This design aimed to capture both regional-scale and small-scale currents near complex bathymetry. This allowed for fine-scale hydrodynamic forces within Horseshoe Bay to be simulated. The hydrodynamic model was run for four distinct time periods; two within the Australian box jellyfish season (October 2016 to January 2017 and October 2017 to January 2018) and two outside of the season (June to September 2017 and June to September 2018). These time periods were chosen due to the availability of tide, current, and wind data (Herzfeld, 2016, Su et al., 2019). The hydrodynamic fields were simulated every 3 min and saved every 30 min. This high temporal resolution was utilised to match the fine-scale resolution of the SLIM grid to ensure complex coastal hydrodynamic features were effectively simulated.

5.3.3.2 Hydrodynamic Model Validation

Validation of the hydrodynamic model demonstrated that it had a high level of accuracy. The model was validated through comparisons to measured tidal anomalies for the area from a local tidal gauge (Maritime Safety Queensland, Queensland Government) and to water current data collected in Horseshoe Bay at six sites from November 2017 to January 2018 through use of drag-tilt current meters (Figure S4.2). Measured and simulated tidal anomalies were firstly visualised together, and a linear regression was run to quantify the relatedness of the data (Figure S4.3, Table S4.1). This was additionally undertaken for measured and simulated water movements, for both zonal (west to east) and meridional (south to north) current components (Table S4.2). This was undertaken through use of ggplot2 (Wickham and Wickham, 2016) and the statsmodels (v 0.14.2) (Seabold and Perktold, 2010) python packages. Further detail can be found in Supplementary IV.1.1.

5.3.4 Simulating eDNA Particle Transport

5.3.4.1 Particle Releases

The transport of passive virtual eDNA particles was simulated by coupling a Lagrangian particle tracker model with the hydrodynamic model. This was created as per Schlaefer et al. (2022). Lagrangian particle tracker models have been extensively utilised for modelling passive particle dispersion (McDonald and Nelson, 2021, Robins et al., 2013, Schlaefer et al., 2022, Van Sebille et al., 2018). Initial particle release concentrations were determined from empirical field concentrations, following those reported by Morrissey et al. (2024a). During the Australian stinger season, initial concentrations were set at 200 copies L^{-1}, while outside of the stinger season, they were adjusted to 100 copies L^{-1}. These concentrations reflect the approximate maximum concentration of *C. fleckeri* eDNA captured during and outside of the Australian stinger season respectively, representing both life history stages (Morrissey et al., 2024b). This approach enhanced the ecological relevance of the simulations, ensuring that the modelled concentrations more accurately reflected real-world conditions and the sensitivity of utilised workflows (Morrissey et al., 2022). Particle releases during the Australian stinger season occurred from three locations (Figure 5.1). This was undertaken to mirror the presence and movements of *C. fleckeri* medusae along the shore of Horseshoe Bay where they were previously detected (Morrissey et al., 2024b). Conversely, only a single location was utilised outside of the Australian stinger season to reflect the locations where *C. fleckeri* polyps were putatively detected within Horseshoe Bay (Figure 5.1). A decay function, consistent with known *C. fleckeri* eDNA decay (Morrissey et al., 2022) was applied to the particles post-release. This function employed three differing decay rates to reflect the various observed stages of particle decay (Morrissey et al., 2022). Use of this decay function, instead of a first-order decay model which may oversimplify this process (Scriver et al., 2023), aids in recreating real-world eDNA “ecology” and provides a more accurate representation of eDNA persistence (Andruszkiewicz et al., 2019). To ensure robustness, the decay function was applied five replicate times upon each particle release, with randomly selected particles to be decayed, to capture any potential variation in particle transport. This approach allowed for a more accurate assessment of variability in dispersion patterns, ensuring that the results reflected a range of possible particle decay scenarios. This was undertaken for both a pulse release event condition, where particles were released once from each location, and for a multi-release event, where particles were released every 10 h. Particle release simulations were undertaken for a duration of 7 days.

5.3.4.2 Tide and Wind Conditions

The influence of two varying tidal and wind conditions on eDNA transport were examined. Regarding tidal conditions, spring and neap tidal conditions were chosen as they represent the two extremes of the tidal regime. For wind conditions, south-eastly (SE) and north-eastly (NE) conditions were selected. These wind conditions are the most common for the study area as per the Australian Bureau of Meteorology's wind data for the Townsville region (BoM, 2024). A combination of tidal and wind conditions resulted in four scenarios (Table 5.1), for both during and outside of the Australian stinger season. These scenarios were utilised to ensure a range of environmental conditions, potentially resulting in variable eDNA transport and dispersion, were comprehensively represented.

Table 5.1. Conditions for tidal and wind scenarios, including tidal stage, tidal range, average wind direction, average wind speed, timing in regard to the Australian stinger season, and start and end date of each scenario. Roses of wind direction and speed can be found in the supplementary materials and are representative of the entire dispersion period (Figure S4.4**)**.

Scenario	Tidal stage	Average wind direction	Average wind speed (km hr^{-1})	Australian stinger season	Start date	End date
A	Spring	123.3° (SE)	28.2	During	17/01/2018	23/01/2018
B	Spring	43.4° (NE)	19.6	During	26/12/2017	01/01/2018
C	Neap	126° (SE)	24.4	During	24/01/2018	30/01/2018
D	Neap	45° (NE)	16.9	During	29/12/2016	04/01/2017
E	Spring	142.7° (SE)	25.7	Outside	28/06/2018	04/07/2018
F	Spring	48.9° (NE)	12.3	Outside	20/09/2017	26/09/2017
G	Neap	145.9° (SE)	26.5	Outside	20/06/2018	26/06/2018
H	Neap	55° (NE)	18.7	Outside	28/09/2017	04/10/2017

5.3.4.3 Particle Transport Analysis

To explore the transport of the passive virtual eDNA particles a number of methods were utilised. Firstly, particle locations were visualised on map charts for every hour post-release utilising Matplotlib (v 3.8.4) (Hunter, 2007), Cartopy (v 0.23.0) (Office, 2010-2015), and cmocean (v 4.0.3) (Thyng et al., 2016) python packages. The map charts were further combined to create animations utilising the PIL (v 9.2.0) (Umesh, 2012) python package to effectively visualise temporal changes in particle movement patterns. Next, analysis of particle distribution within and outside of Horseshoe Bay through use of a defined geographical boundary box was undertaken. Boundaries were set as per Figure S4.6 and the relative abundance of particles over time within and outside of Horseshoe Bay were calculated (Pulse event only). This was undertaken through use of NumPy (v 1.24.0) (Harris et al., 2020), pandas (v 1.5.3) (McKinney et al., 2010), and PyProj (v 3.6.1) (Contributors, 2018) python packages. Additionally, particle distance travelled, in relation to the initial release location, was calculated for all particles at each hourly timestep (Pulse event only). This was undertaken through use of NumPy, pandas, geopandas (v 0.12.2) (Jordahl et al., 2021), Matplotlib, and PyProj python packages. Particle distance travelled during ebb and flood tides were also determined and were compared through use of a Mann-Whitney U test (McKnight and Najab, 2010) for each scenario at each release location (Pulse event only).

5.3.5 Comparison of Simulated eDNA Particle Transport to Field Measurements

To compare simulated eDNA particle transport with empirical field measurements, log-normalised relative concentration map plots were created from the multi-release events. These plots accumulated particle positions across the entire dispersion time, for all releases (n = 17), averaging the five decay scenario replicates, allowing for the visualisation of relative particle concentrations. These plots were created using a 2D histogram approach, in which the study area was divided into a grid of $1m^2$ cells. $1m^2$ cells were utilised to capture fine-scale dispersion patterns and to align the results with practical sampling areas where samples would be taken. Particle positions were aggregated over the entire simulation period, and the number of particles in each cell was recorded. A log-normalisation was applied to enhance the visibility of regions with lower particle concentrations. The log-normalised counts were subsequently scaled so that the maximum particle concentration corresponded to a value of 1. A threshold of <1 particle per grid cell was applied to exclude cells with negligible particle presence. A

custom colourmap was utilised to enhance visual interpretation, transitioning from blue for low-concentration areas (indicating particles below the detection limit), to yellow at the detection limit, and red for regions of high particle concentrations. A rapid transition at the detection limit was used to highlight areas of particular interest.

5.4 Results

5.4.1 Hydrodynamics of Horseshoe Bay

The hydrodynamic model of Horseshoe Bay revealed complex circulation patterns driven by tidal currents and further influenced by the bay's morphology **(**Animation S4.1 - 4.4). Tidal jets entered the bay on the western side during ebb tides and on the eastern side during flood tides, creating dynamic flows that bifurcated upon entering the bay. This formed gyre-like circulation patterns within the bay and shore-parallel currents. Slower alongshore currents were observed near the coastline. In contrast, faster water speeds occurred around the rocky headlands, where tidal jets were deflected and accelerated, leading to enhanced circulation and significant flushing in the central area of the bay. Eddies formed near the headlands and along the bay's periphery due to interactions between the faster tidal jets and slower moving coastal waters, hence free shear layers were present. The eastern section of the bay, sheltered by the headland, experienced reduced flow velocities and slower alongshore currents, resulting in a general area of 'sticky water' (Andutta et al., 2012), compared to other parts of the bay. These patterns highlighted the spatial variability of water movements throughout Horseshoe Bay. Animations of water movements for each scenario are available in the supplementary materials **(**Animation S4.1 - 4.4).

5.4.2 General Transport Patterns of eDNA within an Open Coastal Bay

The dispersion of eDNA displayed two main behaviours. Initially, particles remained in close proximity to the shore where they were transported by shore-parallel currents, and upon leaving the bay or moving away from the shore, they were quicky advected from the bay by reversing tidal flows where they were dispersed broadly (Figure 5.2, S4.14-4.21). Currents within and bordering the bay did also appear to cluster particles in certain areas, particularly along the sides and periphery of the bay. This was evident where particles were observed to be captured temporarily by eddy currents

creating transient aggregations of particles (Figure 5.2). Further, the effect of tidal flow was observed where particles showed oscillating movement both within and outside of the bay. Particles originating from each release location displayed similar behaviours, however, their trajectories varied. Those from the eastern side of the bay (release location C) generally travelled along the eastern headland while those from the central and western side of the bay (release locations B and A) travelled along the shore and western headland. These general patterns of eDNA transport held true both during and outside of the Australian stinger season. Animations of each scenario, for both pulse and multi-release event simulations are available in the supplementary materials (Animation S4.13 – 4.20).

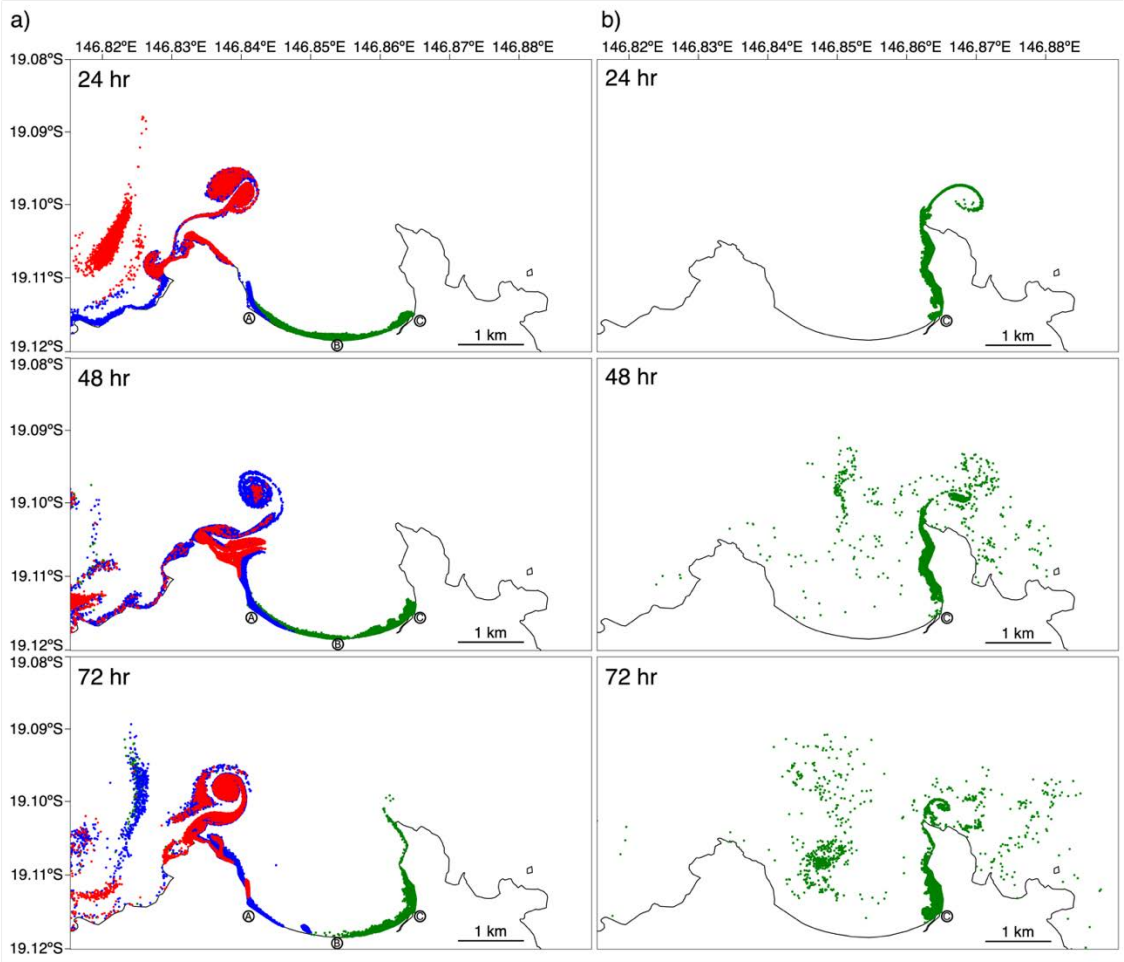

Figure 5.2. Maps of Horseshoe Bay displaying eDNA particle transport at 12 h, 24 h, and 72 h intervals a) during the Australian stinger season, during spring tide and SE wind conditions (scenario A), and b) outside of the Australian stinger season, during spring tide and NE wind conditions (scenario F). Red particles were released from location A, blue from location B, green from location C. Animations of multi-release eDNA particle dispersal can be found in Supplementary IV.3 for all particle releases.

5.4.3 Retention of eDNA within an Open Coastal Bay

Analysis of particle retention within the bay revealed the effects of both eDNA decay and environmental conditions on the above-described particle transport. In all scenarios, during the Australian stinger season, particle presence within the bay dropped below 1% at 20 - 23 h post-release. A maximum of 2.05 – 13.07% of released particles were found to actually exit the bay (from a single particle release), hence highlighting the influence of eDNA decay, and suggesting a rapid daily turnover of detectable signals within the bay. Retention varied by release location, for each scenario (Table 5.2). Particles originating from the eastern side of the bay consistently exhibited the longest retention times regardless of wind and tidal conditions (<1% at 26 h). These particles showed little advection outside of the bay (<1%), highlighting their persistence within this area. Those originating from the western side of the bay exhibited the shortest retention times (4 - 14 h), followed by those originating centrally (14 – 23 h), with environmental conditions having a clear influence. SE winds overall resulted in shorter particle retention times, with tidal state having a less pronounced effect. Outside of the Australian stinger season, similar patterns emerged, with particles again being retained within the eastern side of the bay (Table 5.2), dropping below 1% 23 - 26 h post-release. Clearly, there was spatial variation in particle retention across the bay where environmental conditions played a role.

Table 5.2. Time at which relative abundance of particles with Horseshoe Bay dropped below 1%, and the maximum relative abundance of particles that were transported outside of the bay during the simulation period. Results are shown for each location individually and for combined locations, among scenarios.

Scenario	Location	< 1% (h)	Maximum Outside (%)
A	A	4	48.50
	B	14	7.25
	C	26	0.19
	Combined	20	13.07
B	A	12	12.75
	B	23	1.05
	C	26	0.03
	Combined	23	3.44
C	A	12	14.45
	B	19	2.78
	C	26	0.42
	Combined	21	3.89
D	A	14	7.59
	B	23	2.23
	C	26	0.11
	Combined	23	2.05
E	C	26	0.11
F	C	23	0.83
G	C	26	0.08
H	C	26	0.01

5.4.4 Spatial Extent of Particle Transport across Seasonal and Environmental Conditions

Analysis of particle distance from associated release locations over time further highlighted the influence of environmental conditions upon eDNA. Regardless of season, wind direction had a greater effect than tide upon particle distance travelled, with SE winds (offshore winds) resulting in larger overall transport under both spring and neap tides during the Australian stinger season. On average, particles travelled 21.23 km (± 2.18) and 13.69 km (± 0.65), respectively, after 7 days. NE winds (onshore winds) resulted in shorter distances, 8.82 km (± 0.37) and 5.48 km (± 1.58), respectively. Ebbs tides generally extended particle travel, while flood tides shortened it, as reflected in the sine wave patterns of Figure 5.3**,** with this being significant under SE winds (Table S4.3). Outside the stinger season, SE winds still drove greater distances, however, neap tides resulted in larger transport distances than spring tides (8.62 ± 0.09 km and 7.84 ± 0.39 km, respectively), converse to what was found during the Australian stinger season. This further supported wind direction having a greater effect than tidal patterns on eDNA transport. Examination of particle transport distances when particles resided within the bay (12 h) revealed relatively similar distances under all scenarios for both during and outside of the Australian stinger season (0.99 – 1.45 km and 0.18 – 0.52 km, respectively). The influence of these environmental forcings was likely reduced in comparison to when particles were outside of the bay in open ocean, as indicated by the red arrows in Figure 5.3. Seeding location did influence particle distances, but overall did not result in significant changes to general patterns, with exception of those seeded from the eastern side of the bay during neap tidal and NE wind conditions (Figure 5.3).

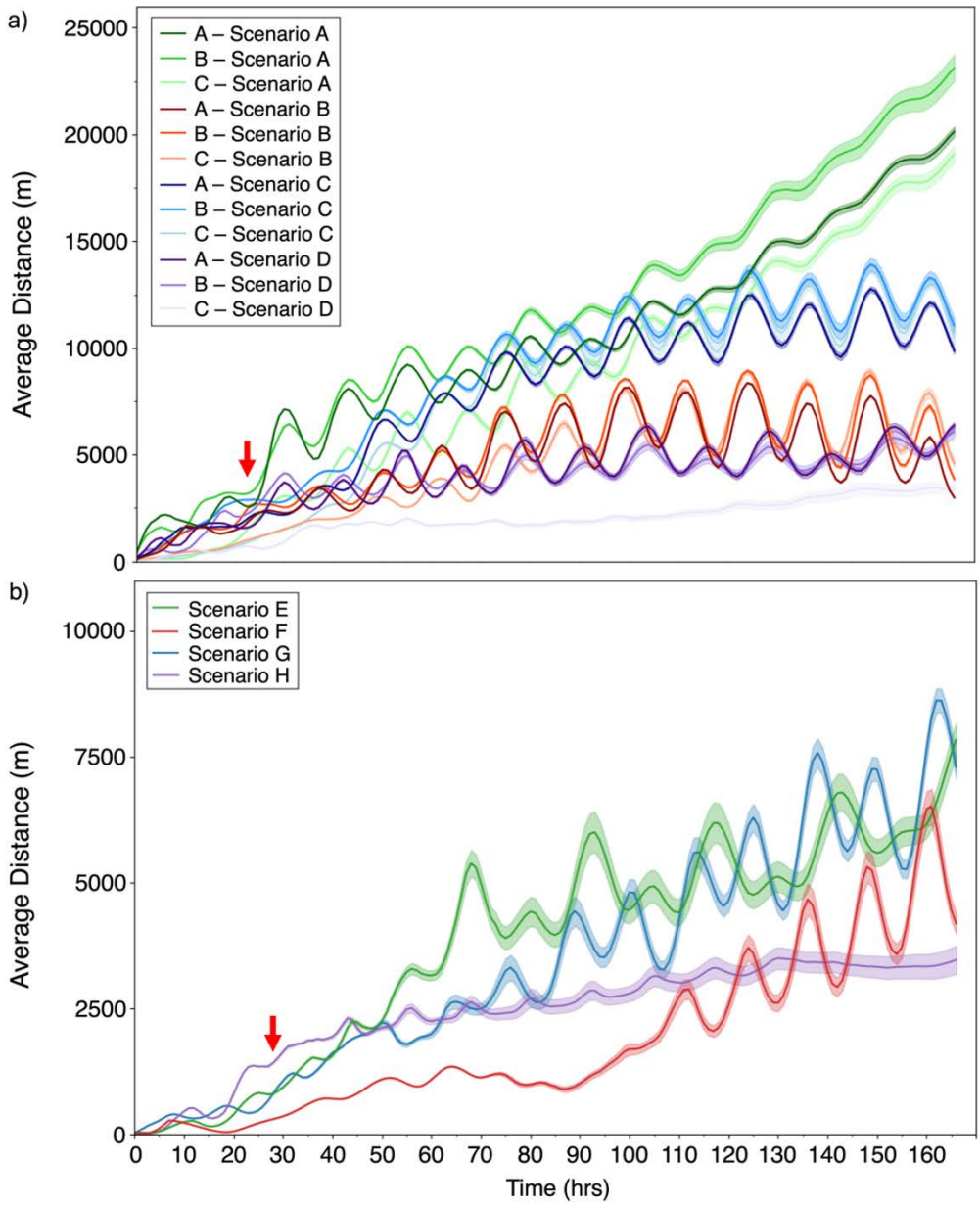

Figure 5.3. Average particle distance over time for a) wind and tidal scenarios during the Australian stinger season, with each release location displayed, and b) wind and tidal scenarios outside of the Australian stinger season. Green lines represent spring tide and SE wind conditions (scenario A and E), red lines represent spring tide and NE wind conditions (scenario B and F), blue lines represent neap tide and SE wind conditions (scenario C and G), and purple lines represent neap tide and NE wind conditions (scenario D and H). Lines represent average distances travelled of the five replicate particle releases from each release location (A - C). Shadows represent standard error. Red arrows approximately indicate the time at which relative abundance of particles within Horseshoe Bay dropped below 1%.

5.4.5 Spatiotemporal Distribution and Detectability of Simulated eDNA Particles

In all scenarios, the highest particle concentrations were consistently observed near the seeding locations, indicating an early phase of dispersion where particles remained concentrated. Additionally, higher particle concentrations were observed nearshore, particularly within the bay. There was a decrease in particle concentration with increasing distance from the seeding locations, reflecting the combined effects of particle dilution and decay. This decline spanned hundreds of meters to kilometres from each seeding location across all scenarios. Moving away from shore, this decline was more rapid highlighting the retention of particles nearshore. In most cases, particles that were outside of the bay were estimated to be below detection limits by in field testing.

Under SE winds (Figure 5.4a and 5.4c), the dispersion of detectable particles was tighter and more concentrated nearshore within the bay, while under NE winds (Figure 5.4b and 5.4d), particles displayed a broader nearshore spread. Among the seeding locations, for scenarios during the Australian stinger season, the eastern side of the bay (seeding location C) exhibited the broadest and most sustained particle concentration, again showing that particles persisted longer in this area compared to other locations. Additionally, eddy-like patterns were discernible in all plots (Figure 5.4 and 5.5), indicating that particles were temporarily trapped by this oceanographic phenomenon when exiting the bay. However, these particles were present at low concentrations, below the detection limit. An exception occurred for particles outside and to the west of the bay where they were estimated to be present at detectable levels in some areas. Similar particle dispersion patterns were seen within the eastern side of the bay outside of the Australian stinger season (Figure 5.5). Despite some variation in the spatial distribution of particles across scenarios, general trends remained consistent. These spatiotemporal distribution patterns aligned well with empirical field detections of *Chironex fleckeri*, where the species was only detected with eDNA at nearshore locations.

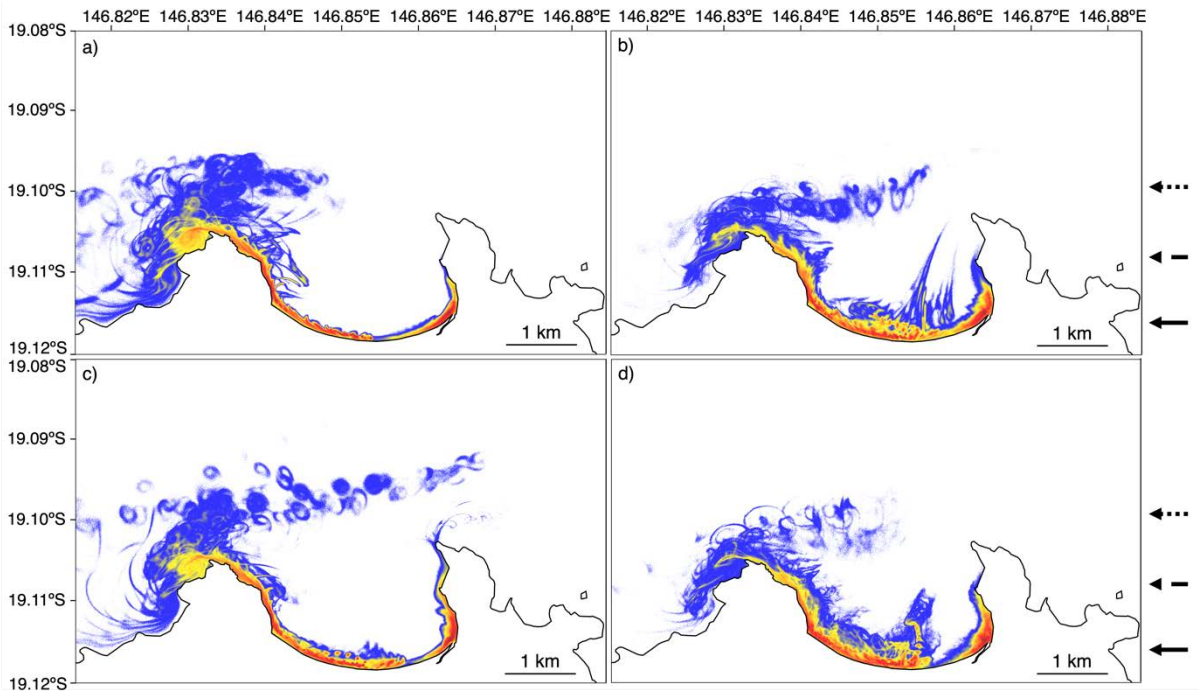

Figure 5.4. Log-normalised relative concentration of eDNA particles for a) spring tide and SE wind conditions (scenario A), b) spring tide and NE wind conditions (scenario B), c) neap tide and SE wind conditions (scenario C), and d) neap tide and NE wind conditions (scenario D). These plots represent the spatiotemporal distribution of particles from all particle releases over time, displaying the cumulative relative concentration of particles. Red to yellow colours represent areas where particles above the limit of detection while blue colours represent those below. Arrows indicate the latitudes of which field samples were taken, with dotted arrows representing offshore locations, dashed arrows representing mid-shore locations, and solid arrows representing nearshore locations.

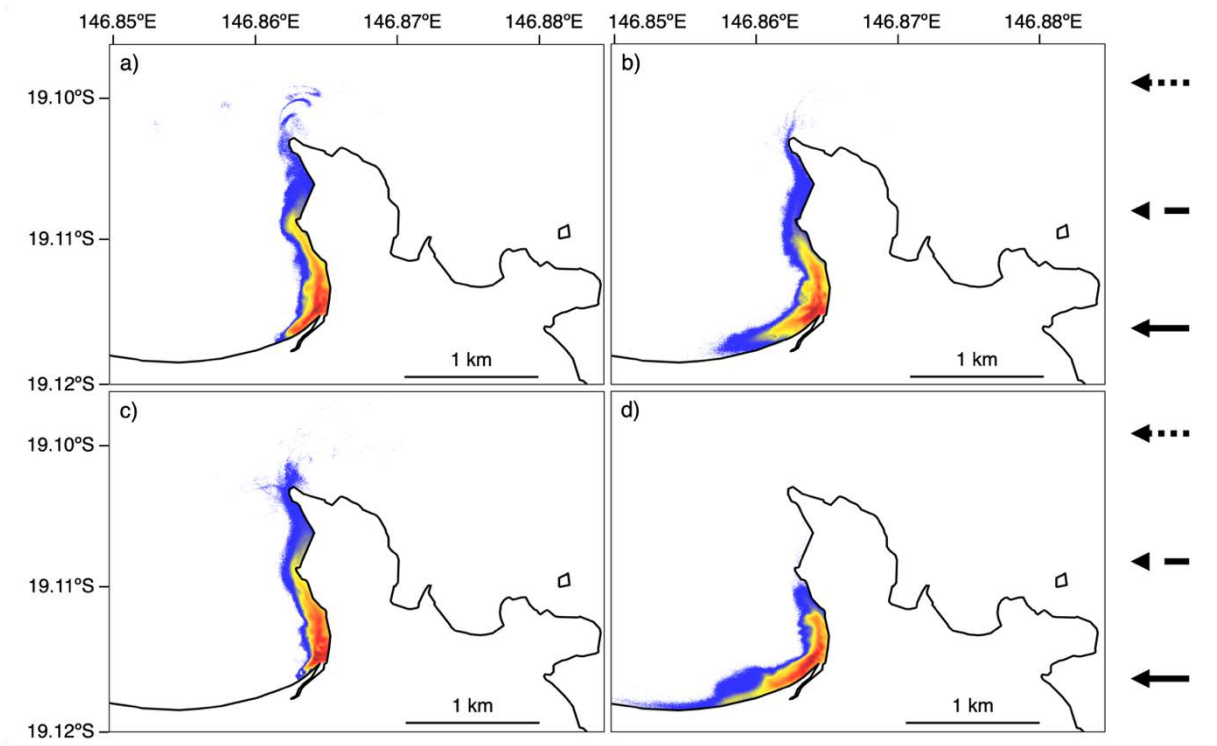

Figure 5.5. Log-normalised relative concentration of eDNA particles for a) spring tide and SE wind conditions (scenario E), b) spring tide and NE wind conditions (scenario F), c) neap tide and SE wind conditions (scenario G), and d) neap tide and NE wind conditions (scenario H). These plots represent the spatiotemporal distribution of particles from all particle releases over time, displaying the cumulative relative concentration of particles. Red to yellow colours represent areas where particles above the limit of detection while blue colours represent those below. Arrows indicate the latitudes of which field samples were taken, with dotted arrows representing offshore locations, dashed arrows representing mid-shore locations, and solid arrows representing nearshore locations.

5.5 Discussion

Through the incorporation of *Chironex fleckeri* specific eDNA parameters with hydrodynamic and Lagrangian particle tracker models, this study modelled eDNA transport mechanisms and spatiotemporal dispersion patterns within an open coastal bay. This enabled understanding of eDNA dispersal within this environmental setting and an understanding of the local hydrodynamic and environmental drivers influencing its transport and dispersal.

Local currents caused heterogeneous dispersal of particles across the bay, creating distinct distribution patterns. The dispersion and subsequent dilution of particles, combined with eDNA decay, limited the distances over which eDNA remained detectable, despite the transport of particles across distances ranging from kilometres to 10s of kilometres. Accordingly, spatially small eDNA 'detection shadows' were found. These modelled eDNA spatiotemporal patterns closely aligned with empirical findings (Morrissey et al., 2024b), where *in-situ* detections of *C. fleckeri* medusae and polyps occurred only in areas where modelled particles were shown to be detectable. Modelling, therefore, allowed for enhanced interpretation of *in-situ* detections and provided valuable insights for undertaking optimal sampling strategies for *C. fleckeri* monitoring.

5.5.1 Hydrodynamic and Environmental Drivers of eDNA Transport

This study highlights the necessity of environment-specific understandings of hydrodynamic and environmental drivers of eDNA transport and dispersal. Marine environments, whether distinctive, like semi-enclosed bays and open ocean areas, or similar, such as embayments of various sizes, likely demonstrate distinctive eDNA transport characteristics dictated by their unique hydrodynamic and environmental conditions. For instance, Ellis et al. (2022) showed, through biophysical models, that open coastline systems often exhibit greater alongshore eDNA transport than cross-shelf, however, this varies with each system's exposure to regional hydrodynamic forces. Similarly, Wolanski et al. (2024a) showed, again through biophysical models, that passive particles (representing coral larvae) exhibited differing degrees of retention within embayments due to variations in their morphology and exposure to prevailing hydrodynamic forces. This study builds on such findings, showing that the impact of hydrodynamic and environmental drivers of eDNA transport are inherently scale-dependent, with localised circulation patterns, wind influences, and geomorphological

features combining to create complex, context-specific transport pathways and distinct zones of eDNA movement and retention.

In Horseshoe Bay, simulations revealed that eDNA particles largely followed the shoreline and headlands, where relatively slow shore-parallel currents and free shear layers, which limit cross-boundary particle movements (Lazaro and Lasheras, 1989), drove this specific transport pathway. In contrast, particles in the bay's central areas were rapidly advected out by relatively fast, reversing tidal flows, effectively limiting eDNA presence within this area. Additionally, shear-induced eddy currents near the headlands temporarily accumulated particles, resulting in transient eDNA aggregations. These findings highlight how specific hydrodynamic characteristics within the bay produced varied transport pathways and spatially heterogeneous eDNA distributions. The bay's geomorphology further influenced these patterns. Particles released along the eastern shoreline exhibited high retention due to the eastern headlands sheltering effect to strong tidal flows. This created an area of 'sticky water', defined as a zone of reduced flow and circulation (Andutta et al., 2012), within this area of the bay allowing particles to be retained. In contrast, the central and western shorelines, exposed to stronger tidal flows due to the bay's open morphology, exhibited lower particle retention, contributing to distinct eDNA transport dynamics across the bay. Clearly, embayments with varying morphologies, open versus semi-enclosed, will exhibit differing eDNA retention characteristics (Wolanski et al., 2024a).

Wind direction modulated these transport and retention patterns, with SE winds promoting offshore currents and reduced retention, while NE winds enhanced retention by slowing offshore flows (Gill, 2016). Wind direction significantly influenced the transport potential of eDNA, where offshore winds resulted in eDNA travelling distances more than double that of particles experiencing onshore winds. Pastor Rollan et al. (2024) similarly observed that an introduced eDNA signal was largely transported by wind driven surface currents rather than prevailing flows. While tidal forces predominantly and broadly drove eDNA transport patterns within Horseshoe Bay, wind direction and bay geomorphology significantly influenced localised transport and retention patterns. This nuanced understanding of oceanographic and environmental influences on eDNA transport within this marine setting highlight the critical need for environment-specific models to estimate eDNA dispersal patterns accurately.

5.5.2 Spatiotemporal Dispersion and Detectability of eDNA

The simulations undertaken in this study demonstrated that some particles, despite rapid decay, travelled significant distances, over 10s of kilometres. However, these far-reaching particles are unlikely to be detected due to their low density. The combined effects of eDNA decay, particle dispersion, and subsequent dilution, reduced eDNA concentrations with increasing distance from source locations. Accordingly, understanding the interplay of these factors in relation to detection thresholds of the applied workflow is essential for understanding the spatiotemporal detectability, 'detection shadows' (Ellis et al., 2022), of eDNA. This understanding is crucial for interpreting eDNA detections and for understanding the relationship between these detections and the present location of source individuals.

Incorporation of the detection threshold of the currently developed *Chironex fleckeri* specific assay (Morrissey et al., 2022), with understanding of the transport dynamics of eDNA, described above, indicated that *C. fleckeri* eDNA remains detectable across distances ranging from hundreds of metres to kilometres from source locations. These spatially small 'detection shadows' were largely confined to the bay, shaped by eDNA decay, dilution and the previously discussed drivers of eDNA transport. Together, these factors resulted in rapid spatial and temporal turnover of *C. fleckeri* eDNA, with detectable signals persisting for only short periods, likely on a daily scale. Thus, eDNA-based detections of *C. fleckeri* likely reflect the species' close proximity and current presence (Morrissey et al., 2024b). This is highly beneficial for the genetic detection techniques application as an ecological survey and potential management tool for cubozoans. Similar results have been reported in previous studies. Ellis et al. (2022), who also utilised biophysical models to explore the spatial detection limits of *Undaria pinnatifida* (Wakame) and *Asterias amurensis* (North Pacific sea-star), found 'detection shadows' ranging between 1 to 3 km in size, with *in-situ* sampling providing additional support. Field studies have also demonstrated that eDNA detections are typically confined to distances of hundreds of metres to a few kilometres (Ely et al., 2021, Murakami et al., 2019, Baetscher et al., 2024, McCartin et al., 2024), or can distinguish communities separated by 10s of metres to kilometres apart (Port et al., 2016, Jeunen et al., 2019, Shea and Boehm, 2024, Kelly et al., 2018, Dugal et al., 2023, West et al., 2020). These findings, along with the present study, suggest a high site fidelity of eDNA to its source (Harrison et al., 2019). However, spatial inconsistencies across transects (detected at some distances and not others), as observed by Murakami et al. (2019) for caged juvenile *Psendoxaranx dentex* (Striped Jack), highlight the importance of

understanding eDNA transport dynamics when interpreting these patterns. Murakami et al. (2019) reported inconsistent detection along transects leading from the sea cage where occasional absences were found, even at nearby sampling distances. Accordingly, this understanding would be beneficial for determining the potential for false negative detections, particularly in scenarios where rapid eDNA decay or dilution may reduce detection probability.

This understanding has practical implications for detecting the presence of *C. fleckeri*. Given that the species is known to inhabit nearshore areas (Brown, 1973, Kingsford et al., 2012, Mooney and Kingsford, 2016a) and that eDNA signals in this bay were largely limited to nearshore regions, a targeted shoreline sampling strategy could enhance detection success. Additionally, as *C. fleckeri* is a generally low abundance species, eDNA-based methods may help distinguish its presence between neighbouring bays and sampling locations, aiding in assessing localised risk to water users. Further, this may allow for the assessment of medusae movements between locations, which would be beneficial to understandings of population connectivity (Kingsford et al., 2021). Finally, the rapid spatial and temporal turnover of *C. fleckeri* eDNA showcases the time-sensitive nature of detections, where signals likely diminish quickly once individuals leave an area. This reinforces the value of eDNA as a precise and cost-effective tool for ecological surveys and risk management.

5.5.3 Comparison of Modelled Transport with Empirical *Chironex fleckeri* Detection

The simulated spatiotemporal dispersion patterns of *Chironex fleckeri* eDNA particles showed strong alignment with empirical field detections of the jellyfish. Simulated particles were found in detectable concentrations along the shore of the bay, closely matching regions where both *C. fleckeri* medusae and polyps had been previously detected (Morrissey et al., 2024b) and are known to reside (Brown, 1973, Kingsford et al., 2012, Mooney and Kingsford, 2016a). Simulated particles were also present in some offshore and mid-shore areas where field sampling did not detect the species, however, these particles were present at theoretical concentrations below the detection threshold of the current workflow, making them unlikely to be detected. In some cases, eddies temporarily captured and aggregated particles, potentially increasing local concentrations. Such localised retention could create transient conditions where eDNA becomes detectable, depending on the timing and location of sampling. Given that field sampling was conducted across multiple times, under varying hydrodynamic conditions

(Table S4.4), eDNA would likely have been detected in these areas if it had been present in detectable concentrations. The alignment between simulated particle dispersal and empirical field detections supports the reliability of biophysical models in representing eDNA transport dynamics and provides additional support to these combined approaches for investigating this aspect of eDNA "ecology" (Ellis et al., 2022, Andruszkiewicz et al., 2019).

Specific field measurements further illustrate the utility of biophysical models for enhancing the interpretation of detections. For example, during the July 2020 field sampling, *C. fleckeri* detections were found at the nearshore and mid-shore sampling sites along the eastern headland (Morrissey et al., 2024b). During this time, SE winds and spring tides were in effect. Comparing these field detections to the simulated scenario for these same conditions revealed that eDNA released from the southeastern corner of the bay was transported along the headland and reached detectable levels at the mid-shore site. This detection, initially attributed to *C. fleckeri* polyps, could instead represent a transient eDNA signal, highlighting the enhanced interpretative power of biophysical models. Further, *C. fleckeri* medusae were detected within neighbouring bays later in the Australian jellyfish season. Simulated 'detection shadows' across all scenarios indicate that detectable levels of eDNA do not reach into neighbouring bays. This suggests that empirical detections within neighbouring bays arise from the presence of *C. fleckeri* individuals rather than transient eDNA signals. Together, these examples illustrate the potential value of biophysical models in resolving the complex spatiotemporal dynamics of eDNA transport and improving the interpretation of species detections.

5.5.4 Limitations, Considerations, and Future Directions

This study has demonstrated the considerable value of biophysical models combined with field sampling in revealing the transport dynamics and spatiotemporal detectability of *Chironex fleckeri* eDNA within a dynamic open coastal embayment. However, like any modelling approach, certain methodological considerations and limitations shape the interpretation of findings which should be addressed here. One key limitation is the challenge of representing eDNA behaviour in all its complexity. The model utilised in this study treated eDNA as passive particles, thereby neglecting processes such as aggregation, sinking, or interactions with suspended matter (Barnes et al., 2014, Harrison et al., 2019, Brandão-Dias et al., 2023). These factors may alter dispersal patterns, potentially leading to an overestimation of detectable eDNA signals

within this study (Harrison et al., 2019). Additionally, detectability is influenced not only by physical processes but also by the overall sensitivity of the eDNA survey, which can be shaped by sampling volume, processing workflow, and molecular assay performance (Furlan et al., 2016). These factors were considered in this study through the use of detection limits derived from the *C. fleckeri* assay and sampling workflow utilised by Morrissey et al. (2022). The sinking of particles was not considered in this study due to the well-mixed nature of the bay (Morrissey et al., 2024b). However, the use of three-dimensional hydrodynamic models, which offer more detailed insights into the vertical transport and retention, could provide a more comprehensive understanding of eDNA transport dynamics and detectability (Andruszkiewicz et al., 2019, McCartin et al., 2024).

The focus on eDNA detectability in this study also raises important considerations. While particles were shown to travel significant distances, dilution and decay processes constrained detectability to generally localised regions near the source. In some cases, detectability may be hindered by extensive spatial dilution rather than an absence of eDNA. As eDNA detection techniques become increasingly sensitive, such as with the adoption of digital PCR (dPCR) and CRISPR-based assays (Uthicke et al., 2018, Phelps, 2019, Williams et al., 2019), detectable signal ranges may expand. Without corresponding knowledge of eDNA transport dynamics, these advances may complicate the interpretation of the source of eDNA for ecological survey applications. A clear understanding of spatiotemporal eDNA patterns, tailored to the sampling workflow, is essential to ensure accurate ecological inferences. This understanding is especially important when undertaking sampling across small spatial scales. To further advance understanding of eDNA transport dynamics, future studies should include bays with varying geomorphologies and hydrodynamic exposures, as well as other environmental contexts.Such efforts would not only refine our understanding of the factors influencing eDNA dispersal, but also greatly improve sampling frameworks, allowing for more spatially comprehensive and effective ecological survey designs.

5.6 Conclusions

This study demonstrates the utility of biophysical models combined with field sampling in understanding eDNA transport and dispersion within a dynamic marine environment. Further, modelling reveals the critical role of hydrodynamics, geomorphology, and environmental conditions in shaping spatially constrained eDNA 'detection shadows'. By incorporating species-specific eDNA parameters, the model closely aligned with empirical detections of *Chironex fleckeri,* providing a robust framework for interpreting eDNA patterns and optimising sampling strategies. These findings highlight the importance of environment-specific understandings for eDNA sampling, particularly in systems influenced by complex hydrodynamic forces. While this study focused on an open coastal bay, future research should extend these methods to varied marine contexts and incorporate additional factors, such as more complex eDNA dynamics, to further refine this understanding. Overall, this study not only advances our understanding of eDNA transport dynamics but also demonstrates the broader potential of combining biophysical modelling with eDNA techniques to enhance interpretations of eDNA-based species detections, to improve sampling strategies, and to better understand the relationship between detections and the present location of target organisms.

Chapter 6.

General Discussion

The broad objective of research reported in thesis was to develop and advance the use of environmental DNA (eDNA) as a tool for detecting cubozoan jellyfish, specifically focusing on *Chironex fleckeri*, while also furthering our understanding of cubozoan ecology. Central to this objective was the development, optimisation, and validation of a highly specific and sensitive eDNA detection assay, along with a workflow ensured to result in accurate and reliable detections. Beyond assay development, the eDNA approach was deployed as an ecological sampling tool to investigate hypotheses surrounding the species' distribution, life history and population structure across two distinct, open and semi-enclosed, oceanographic environments. Sampling in these two environments enhanced the robustness of ecological conclusions and the broader applicability of the eDNA technique. Furthermore, biophysical modelling was utilised to simulate the transport dynamics of eDNA, shedding light on how the detection of eDNA with distance correlates with the physical presence of the species. This approach contributed to a deeper understanding of the use and interpretation of eDNA data.

This thesis comprises four data chapters. In Chapter Two, a TaqMan assay was meticulously designed and optimised for the sensitive detection of *C. fleckeri*. Additionally, this assay was multiplexed with an endogenous control assay to ensure robust quality control and reliable detection. This process involved rigorous testing and refinement to achieve high specificity and sensitivity in identifying *C. fleckeri* eDNA, laying a solid foundation for subsequent eDNA studies. This chapter additionally provides a framework for developing and applying this technique to other cubozoan species. Chapter Three applied the genetic detection technique to explore the distribution of the species within a semi-enclosed estuary. This allowed testing of the technique's use in the detection of the species' benthic polyp life history stage, which was successful. With a knowledge on both medusae and polyp distributions across the study area, it was concluded that the population stock proposed from a biophysical modelling approach (Schlaefer et al., 2018) aligned with the findings from eDNA. The sampling for Chapter Four was based in an open coastal system. Here, an anecdotal account of a spatially restricted population was also evaluated using eDNA. These two studies have contributed to increasing evidence that some cubozoan species may have spatially small population stocks. Finally, in Chapter Five the transport dynamics of

C. fleckeri eDNA were determined through the use of biophysical modelling techniques within an open coastal bay. This chapter examined how hydrodynamic and environmental conditions influenced the transport and dispersion of eDNA, and the findings of these models were compared to empirical field measurements. This allowed for an understanding of the detectability of *C. fleckeri* eDNA and for a more thorough interpretation of eDNA-based species detections. Overall, this thesis not only demonstrates the efficacy of utilising eDNA for the detection of *C. fleckeri* but also highlights its potential as a transformative tool for marine ecology, enabling advancement of our understanding of cubozoan ecology.

6.1 Detecting Cubozoan Jellyfish with Environmental DNA

Research in this thesis has demonstrated the efficacy of eDNA as a highly effective method for detecting cubozoan jellyfish, specifically the notorious Australian box jellyfish, *Chironex fleckeri*. Previous research has utilised various techniques to detect and locate cubozoan jellyfish. However, these taxa remain elusive due to a combination of factors. Their transparency, temporal and spatial dispersion, and residence, for some species, in coastal environments complicates detection efforts (Kingsford and Mooney, 2014, Tibballs et al., 2012). The development and optimisation of the *C. fleckeri* eDNA detection assay has, however, significantly enhanced our ability to detect the presence of this species. When multiplexed with an endogenous control assay, it additionally ensures accurate and reliable detection (Chapter 2). This progress in cubozoan detection is due to the technique removing the need for physical capture and morphological identification of individuals (Jerde et al., 2011, Sigsgaard et al., 2015, Barnes and Turner, 2016, Smart et al., 2016, Evans et al., 2017). Further, this thesis contributes to an increasing body of evidence that eDNA is effective in the detection of elusive and problematic taxa (Beng and Corlett, 2020, Duarte et al., 2023, Rishan et al., 2023, Larson et al., 2020).

The *C. fleckeri* assay was designed to be highly specific, capable of detecting very low traces of the species' eDNA (Modelled LoD of 0.45 copies/reaction, Chapter 2). This sensitivity is crucial when targeting organisms that are typically in low abundance and have great spatial variation in their occurrence. Additionally, the assay was designed to detect *C. fleckeri* across its biogeographic range in Australia, providing a tool that is useable across various environments. This versatility ensures the assay's effectiveness over broad spatial scales and in a diversity of habitats.

The research in this thesis contributes to the growing body of research exploring the use of eDNA-based techniques to assess and determine the presence of these problematic taxa (Bolte et al., 2021, Azama et al., 2023, Sathirapongsasuti et al., 2021, Osathanunkul, 2024, Ames et al., 2021, Son et al., 2023). Currently, species-specific assays have been developed for four other cubozoan species: *Copula sivickisi* (Bolte et al., 2021), *Chironex indrasaksajiae* (Sathirapongsasuti et al., 2021), *Chironex yamaguchii* (Azama et al., 2023), and *Chiropsoides buitendijki* (Osathanunkul, 2024). Additionally, metabarcoding approaches have been successful in detecting cubozoans (Ames et al., 2021). These studies utilised a range of approaches, including SYBR and TaqMan chemistries, and PCR, qPCR, and dPCR methods, each with its own benefits and limitations. The assay developed in the current research utilised TaqMan chemistry due to its increased specificity and sensitivity, quantification accuracy, and ability to be multiplexed. Additionally, qPCR methods were utilised for their cost-effectiveness. Although dPCR methods are suggested to offer higher sensitivity (Hunter et al., 2017), the LoD of the present assay shows a higher sensitivity compared to Osathanunkul (2024)'s dPCR assay, suggesting that assay design is of greater importance. It should be noted, however, that sensitivity alone is not sufficient for assessment of assay performance, consistency, inhibition control, and transparent justification of detection thresholds are equally critical, and these were addressed in this thesis (Chapter 2). Moreover, more sensitive methods provide little benefit without a more in-depth understanding on eDNA decay rates and dispersal for ecological survey applications (Chapter 5). Metabarcoding approaches have gained popularity recently due to their efficiency and throughput, however, there are 'blind spots' in these assays, resulting from factors such as template competition and primer bias, which can lead to false negative detections (Nester et al., 2020), especially for low abundance taxa (Wood et al., 2019, Dugal et al., 2024). For example, Dugal et al. (2024) successfully detected the presence of silverlip pearl oysters (*Pinctada maxima*) using an oyster-specific assay, but were unsuccessful when using a universal assay. Therefore, for efficient and cost-effective detection of cubozoan jellyfishes, species-specific eDNA workflows are recommended. Further, to ensure the reliability of these workflows, the inclusion of an endogenous control is essential. This control, which monitors for the presence of non-target eDNA in collected samples through use of a generic assay, ensures that both negative and positive results are not due to technical errors and hence reflect the true presence or absence of the target species (Furlan and Gleeson, 2016). By incorporating this control, the robustness and accuracy of the technique is enhanced, making it a critical component in the detection workflow, particularly in the context of monitoring potentially deadly jellyfish.

The literature has increasingly reported the success and efficacy of eDNA-based methods to detect cubozoan jellyfish. This thesis further demonstrates this success and highlights the technique's applicability for likely all cubozoan species, particularly the publicly feared 'Irukandji' species. The work presented within this thesis, goes beyond the currently published proof-of-concept studies and provides a robust method for accurate and reliable eDNA detection of cubozoans.

6.2 An Ecological Tool for Sampling

The application of eDNA represents a significant advancement in the detection of cubozoan jellyfish, offering an efficient and novel alternative to traditional methods. Traditional detection methods (i.e. visual surveys, netting, light attraction, and remote sensing) often suffer from inherent limitations (see Table 1.2), restricting the scope of ecological questions that can be effectively addressed (Kingsford et al., 2018). Research outlined in this thesis underscores the efficacy of eDNA in overcoming some of these challenges and highlights the benefits of eDNA (Chapter 3).

Current approaches have spatial and environmental constraints, such as limited access to remote areas, the need for clear water conditions, or the inability to sample in dangerous environments. Additionally, some of these methods sample passively, which can contribute to false negative detections (Chapter 1). In contrast, eDNA provides a tool which can be utilised across all environments and provides location-specific detections. This enables targeted and efficient monitoring of species presence across a diversity of habitats, and therefore contributes to a better understanding on the distribution patterns and population structures of cubozoan jellyfish, an area where traditional methods have some benefits, but often require more effort and can be spatially constrained (Kingsford et al., 2021, Kingsford and Mooney, 2014). The rapid decay of jellyfish eDNA (Chapter 2), coupled with its shedding by individuals as they move through their environment (hundreds of meters per hour; Gordon and Seymour (2009)), additionally increases the likelihood of detecting the presence of cubomedusae that may evade traditional sampling efforts. Beyond its accuracy of detection, eDNA offers logistical advantages. Its reduced labour requirements can facilitate larger sampling efforts, which are essential for in-depth ecological studies. This further results in a more cost-effective approach. Additionally, the non-invasive nature of eDNA sampling minimises risk associated with handling venomous taxa. Although eDNA appears inaccurate for estimating differences in abundances for *C. fleckeri* it emerges as a powerful tool for detecting and studying

cubozoans. The subsequent subsections delve deeper into the practical applications and implications of eDNA in this context.

6.2.1 Life History

Cubozoan jellyfish have a metagenetic life history, characterised by two major stages; medusae and polyps. Results reported in this thesis demonstrated that both stages of *Chironex fleckeri* can be detected using eDNA. Seasonality was a requirement in that medusae are only present for a few months of the year and, therefore, in the absence of medusae the detection of *C. fleckeri* eDNA can only be attributed to the polyps (as detailed in Chapters 2 & 3). It was demonstrated that eDNA analysis can detect polyps, a stage that was previously challenging to identify due to their small size and cryptic nature. Previously, researchers spent years intensively searching specific environments before finding specimens (Hartwick, 1991a). This utility of eDNA for detecting polyps is supported by a proof-of-concept study undertaken by Bolte et al. (2021), where *Copula sivickisi* polyps were putatively detected near the benthos, outside of the species medusae season. eDNA hence can be utilised to determine the source locations of the threatening medusae stage, which has numerous benefits for enhancing our ability to manage the threats posed by these taxa through early detection, targeted monitoring, and improved risk assessment in vulnerable coastal regions where other preventative measures (i.e. stinger nets) are not suitable. This capability of eDNA further allows for efficient detection and the investigation of ecological hypotheses surrounding this life history stage.

The inaccessibility of polyps has resulted in considerable knowledge gaps surrounding their ecology, particularly concerning their habitat, the environmental conditions in which they reside, as well as the source of medusae. Consequently, inferences on polyp habitat have largely been limited to broad environmental categories, such as estuaries (Hartwick, 1991a, Cutress and Studebaker, 1973, Mooney and Kingsford, 2012, Mooney and Kingsford, 2017). This thesis provided the first direct insights into the specific habitats of *C. fleckeri* polyps, through examining their presence within numerous habitats, revealing their occurrence in both estuarine and marine conditions (Chapters 3 & 4). This finding refutes a longstanding paradigm, that cubozoan polyps are exclusively found in estuarine habitats, and supports Mooney and Kingsford (2012)'s findings from age-structured statolith chemistry profiles that they occur in both of these physically contrasting environments. Notably, polyps in this thesis were predominately detected in habitats with rocky substrata and shallow carbonate reefs,

consistent with Hartwick (1991a)'s observation of polyps on stone undersurfaces. Additionally, polyps were not detected in mangrove habitats, again aligning with Hartwick (1991a)'s observation of the absence of polyps on mangrove structures. These findings were consistent across two oceanographically differing environments that were semi-enclosed (Chapter 3), or open (Chapter 4). These insights not only refine our understanding of cubozoan polyp habitats, but also prompt comparisons with other cnidarian species, specifically scyphozoan jellyfish. The reported habitat preference is similar to that of scyphozoan polyps, which are typically found in concealed environments that offer protection and favourable conditions for growth and development (Brewer, 1976, Cargo and Schultz, 1966, Svane and Dolmer, 1995, Kikinger, 1992, Pitt, 2000). The detection of polyps with eDNA at these sites suggests that cubozoan polyps, like scyphozoan polyps, may seek out habitat that provide shelter from predators and sedimentation, highlighting the ecological parallels between these two cnidarian groups. Overall, it is likely that polyp presence is determined by the availability of suitable substrate and proximity to medusae 'hotspots', where medusae undertaking external spawning may lead to higher concentrations of settling polyps (Chapter 3).

eDNA not only facilitates the detection of polyps but also holds potential for aiding in their physical locating. Further, recent advancements in environmental RNA (eRNA) analyses, as demonstrated by Parsley and Goldberg (2023) on detecting larval and adult stages of American bullfrogs (*Lithobates catesbeianus*) and tiger salamanders (*Ambystoma mavortium*), offer an exciting avenue for more precise detection, and confirmation, of specific life history stages. As eRNA decays more rapidly than eDNA, it can provide finer temporal and spatial resolution, enabling ecologists to more accurately pinpoint 'polyp beds'. This ability to physically locate cubozoan polyps would allow for targeted collection and study, essential for understanding their environmental conditions, improving cubozoan husbandry, and elucidating their role in medusae abundances and distributions. This knowledge would be invaluable for understanding cubozoan ecology. Additionally, due to the ubiquitous nature of these environmental nucleic detection techniques, they can be applied to determine the presence of scyphozoan jellyfish benthic and larval stages, as demonstrated by Gaynor et al. (2017).

Overall, the use of eDNA to efficiently detect the benthic polyp stage of *C. fleckeri* represents a major advancement in cubozoan ecology. This approach not only aids in the filling of knowledge gaps, but, also opens new possibilities for research. The ability to detect polyps lays the groundwork for future studies aimed at investigating cubozoan population dynamics and understanding the ecological roles of these taxa, which will

significantly aid in managing the threats posed by these taxa to human health and commercial enterprise.

6.2.2 Population Structure

Understanding on the spatial structure of cubozoan populations is currently limited (Kingsford and Mooney, 2014, Kingsford et al., 2021). This knowledge is fundamental to furthering understanding of cubozoan ecology. While metapopulations of cubozoans are known, significant gaps exist in our understanding of the substructure within these broad population units (Kingsford and Mooney, 2014). This thesis demonstrates the use of eDNA to efficiently monitor and determine the real-time distributions of cubomedusae (Chapter 3 & 4). Additionally, as benthic polyp stages can be putatively detected, eDNA analysis enables incorporation of the distribution of this life history stage into this understanding, which is critically important for a comprehensive understanding of cubozoan population structures (Kingsford et al., 2021). Polyps, being the source of medusae, likely play a key role in the distribution of medusae, as seen with scyphozoan jellyfish (Colin and Kremer, 2002, Toyokawa et al., 2011, Shahrestani and Bi, 2018). This thesis has subsequently utilised distributions of medusae and, for the first time, polyps from eDNA detections to infer and test hypothesised population stocks of *Chironex fleckeri*. This use of eDNA analysis is highly supported by the literature, where the distributions of numerous species, including scyphozoan jellyfish, have been determined (Jo et al., 2019, Minamoto, 2022, Rees et al., 2014, Gaynor et al., 2017, Minamoto et al., 2017), enabling critical insights into the distributions and population structures of elusive taxa (Rees et al., 2014, Beng and Corlett, 2020).

A number of studies have been undertaken prior to the current body of work to explore the population structures of cubozoans (Mooney and Kingsford, 2017, Mooney and Kingsford, 2012, Schlaefer et al., 2018, Schlaefer et al., 2021, Mooney and Kingsford, 2016a). These studies utilised indirect techniques due to the challenges of detecting cubozoans. Mooney and Kingsford (2012, 2017, 2016a) utilised statolith morphometrics (Mooney and Kingsford, 2017, Mooney and Kingsford, 2016a) and elemental chemistry (Mooney and Kingsford, 2012), common tools for population stock discrimination (Campana and Casselman, 1993, Campana et al., 2000), to undertake this for *C. fleckeri*. They found significant differences in statolith shape and elemental chemistry across North Queensland, Australia, with differences between locations separated by 10s of kilometres, suggesting the presence of population stocks. Schlaefer et al. (2018), with the use of biophysical modelling of cubomedusae swimming behaviour

and dispersal, concluded a population restricted to a semi-enclosed bay (Port Musgrave). Similarly, *Copula sivickisi*, with their diurnal behaviour, were found to maintain their position within bays (Schlaefer et al., 2020, Schlaefer et al., 2021), indicating local populations and an island-based population stock. These studies collectively indicated that some cubozoan populations may be more complex and localised than previously thought, challenging the traditional view of them as broadly dispersing and highly connected taxa.

The research within this thesis builds on these findings by directly assessing the population structure of *C. fleckeri* with the use of eDNA. Within Chapter 3 of this thesis, the hypothesised *C. fleckeri* population stock, proposed by Schlaefer et al. (2018), was examined using eDNA where supporting evidence was found. Medusae, while prominently detected within the estuarine system, were also detected outside of it, whereas polyps were exclusively detected within the port. This pattern suggested a localised origin of medusae, with polyps serving as the source within the estuary. These findings added considerable support to *C. fleckeri* having spatially small population stocks. Additionally, this direct assessment validated the use of biophysical models to inform cubozoan population structure. The robustness of spatially small population stocks of *C. fleckeri* was additionally examined in a contrasting oceanographic environment, an open coastal bay (Chapter 4). Again, supporting evidence was found suggesting a potentially isolated population stock of the species encompassing the northern side of Magnetic Island, Australia. Medusae within this area were detected only nearshore, displaying the species' known nearshore distribution, with polyps consistently detected within the coastal bay of study. The findings, from both Chapter 3 and 4, reinforce the notion that *C. fleckeri* population stocks may be more spatially constrained and exhibit more complex local structures than previously understood. This thesis highlights the utility of eDNA analysis, combined with biophysical modelling, in examining the population structures of cubozoans, significantly contributing to understanding of cubozoan ecology and enhancing management capabilities.

Given the spatio-temporal abundance and distribution data available for some cubozoan species (Kingsford et al., 2012, Bordehore et al., 2011, Rowley et al., 2022, Bordehore et al., 2023), it is plausible that similar patterns of localised population structures may exist for other cubozoan taxa. There will, however, be exceptions to this. Lawley et al. (2016) reported populations of *Alatina alata* across the Pacific to not be genetically distinct, therefore being highly connected. This connectivity of *A. alata* populations is not unexpected due to the species' movements to significant depths and regular mating cycles (Thomas et al., 2001, Chiaverano et al., 2013, Carrette et al., 2014,

Lewis et al., 2013, Morrissey et al., 2020b). This underscores the influence of cubozoan swimming abilities and behaviours upon their dispersal. As *C. fleckeri* medusae have been shown to swim at speeds up to 16.6 cm s^{-1} (Schlaefer et al., 2018, Schlaefer and Kingsford, 2024), undertake rheotaxis (Schlaefer et al., 2018), avoid obstacles (Hamner et al., 1995), and maintain nearshore positions (Kingsford et al., 2012, Brown, 1973, Hartwick, 1991a, Gordon and Seymour, 2009, Mooney and Kingsford, 2016a), demonstrating the high mobility and behavioural complexity of this taxon; this suggests that they can actively influence and maintain their distribution, providing further support to the findings of this thesis.

To further investigate the spatially restricted structure of *C. fleckeri* population stocks, assessment of the genetics of individuals across different geographic locations would be valuable. This population genetic analysis could provide deeper insights into the differentiation and connectivity between populations. This has been undertaken for scyphozoan and some cubozoan jellyfish (Kingsford et al., 2021). Further, eDNA analysis has been successfully utilised for this purpose (Sigsgaard et al., 2017, Sigsgaard et al., 2020a, Sigsgaard et al., 2020b, Adams et al., 2019, Parsons et al., 2018) and may provide a tool to concurrently detect genetic diversity and population structure.

6.2.3 Limitations

Despite the above discussed benefits, it is important to acknowledge the limitations of eDNA as an ecological survey tool. One significant limitation is eDNA's inability to provide information on species abundances, behavioural patterns, or size/age distributions, which traditional methods such as visual surveys and netting can offer. This thesis, in Chapter 3, explored the use of eDNA as a proxy of *C. fleckeri* medusae abundance, determining that it was unsuitable despite supportive findings in the literature for numerous species, including scyphozoan jellyfish (Takahara et al., 2012, Pilliod et al., 2013, Lacoursière-Roussel et al., 2016a, Wilcox et al., 2016, Yamamoto et al., 2016, Minamoto et al., 2017, Thomsen et al., 2012). The inaptness of using eDNA in this context likely results from greater variability in eDNA concentrations, from this spatially disperse and low abundance taxa, resultant from both biotic and abiotic influences upon eDNA presence and persistence. Therefore, to fill these limitations of eDNA analysis, a multifaceted approach that integrates eDNA with traditional methods is recommended for the comprehensive study of cubozoan ecology. By combining these methods, ecologists can leverage the strengths of each method to overcome their respective

limitations. This approach will enable assessments of population dynamics and environmental response, essential for furthering understanding of cubozoan ecology. Furthermore, ongoing advancements in eDNA's application, such as standardisation of protocols and understanding on eDNA dynamics and behaviours will further enhance its integration into broader ecological research frameworks (De Brauwer et al., 2023, Harrison et al., 2019).

6.3 eDNA Dynamics

The dynamic nature of eDNA highlights the need for a thorough understanding of its "ecology" across different environmental contexts (Harrison et al., 2019). Specifically, understanding the temporal and spatial dynamics of eDNA is critical to its effective use and reliable interpretation (Harrison et al., 2019). Foremost is knowledge of the technique's temporal resolution, particularly the decay of eDNA within the environment and abiotic factors influencing this. Exploration of the persistence time of *C. fleckeri* eDNA within this thesis revealed a rapid decay rate (0.060 h^{-1}, Chapter 2), where the large majority of eDNA decayed within the first 24 hours. This rate falls within the 10-50 hour half-life range found for the majority of marine organism eDNA (Collins et al., 2018) and was similar to those reported for a number of jellyfish species (Minamoto et al., 2017, Bolte et al., 2021, Azama et al., 2023). These findings suggest that *C. fleckeri* eDNA detections reflect the species current or recent presence.

Additionally, this thesis found small-scale temperature changes did not affect eDNA degradation rates, indicating that the technique can be used across the species' biogeographic range without concern of differing temperature influences. This consistency is supported by water temperatures being similar (within 5°C; AIMS (2017)) across this range at any one time. This understanding is primarily for marine conditions, and further research is needed to explore those reflective of estuarine systems, i.e. lower salinity, high turbidity, and lower oxygen levels. These abiotic influences are known to have varying effects upon the decay of eDNA (Okabe and Shimazu, 2007, Schulz and Childers, 2011, Stoeckle et al., 2017, Weltz et al., 2017). However, due to the low-abundance and spatially disperse nature of *C. fleckeri*, is it likely that these influences would have minimal effect upon the species' detection. This understanding may, however, provide further insight into the applicability of eDNA analysis as a proxy of *C. fleckeri* abundance.

Regarding the spatial dynamics of eDNA, this is an area of research widely recognised as a priority by eDNA researchers and practitioners globally (Harrison et al., 2019, Blackman et al., 2024). Understanding eDNA's spatial dynamics is essential for improving the accuracy and interpretation of eDNA-based species detections. In this thesis, the spatial dynamics of *C. fleckeri* eDNA were investigated through a biophysical modelling approach within an open coastal bay revealing that a combination of factors influenced its dispersion (Chapter 5). These included local hydrodynamics, environmental conditions, and coastal geomorphology. These factors caused heterogeneous dispersal of simulated eDNA, resulting in distinct distribution patterns. Consequently, marine environments, whether distinctive or similar, likely exhibit unique eDNA transport characteristics dictated by their associated hydrodynamic and environmental conditions (Ellis et al., 2022, Wolanski et al., 2024a). Therefore, this understanding is key for accurately interpreting eDNA-based species detections and for determining the potential of false negative detections.

Combining temporal and spatial insights provides a comprehensive framework for interpreting eDNA detections. In this thesis, this knowledge for *C. fleckeri* eDNA was combined to explore the likelihood of detection and its relation to empirical field detections. Detection 'shadows' of *C. fleckeri*'s eDNA signal were found to be spatially restricted. This spatial restriction was a result of the rapid decay and dilution of the eDNA signal which limits the detection range. These finding suggests that detections reflect the current or very recent presence of the species in the area, and this is supported by the close alignment of these 'detection shadows' with empirical field detections (Chapter 4). This adds to growing evidence suggesting a high site fidelity of eDNA to its source within marine environments (Ely et al., 2021, Murakami et al., 2019, Baetscher et al., 2024, McCartin et al., 2024, Port et al., 2016, Jeunen et al., 2019, Shea and Boehm, 2024, Kelly et al., 2018, Dugal et al., 2023, West et al., 2020, Ellis et al., 2022, Harrison et al., 2019). These spatially restricted detection 'shadows' have implications for sampling efforts, such as that samples should be taken in a spatially close manner (100s of meters apart). This also highlights the accuracy of the technique in detecting cubozoan jellyfish, and other low-abundance and elusive taxa.

Understanding the temporal and spatial dynamics of eDNA is essential for its effective application in ecological studies. The biophysical model utilised in this thesis, however, only accounts for a select number of parameters influencing the detectability of eDNA. Incorporating eDNA production rates, eDNA aggregation, sinking, and interactions with suspended matter (Barnes et al., 2014, Harrison et al., 2019, Brandão-Dias et al., 2023) could refine this understanding by accounting for these factors which

influence eDNA concentrations. Additionally, applying 3D biophysical models that include vertical stratification, mixing, and subsurface currents and eddies could provide more detailed insights into this understanding (Andruszkiewicz et al., 2019, McCartin et al., 2024). Overall, this use of biophysical models, combined with field sampling, provides a more comprehensive understanding of eDNA dynamics, which enhances the interpretation of eDNA-based detections of *C. fleckeri*.

6.4 Conclusions

Research reported in this thesis aimed to develop and advance the use of environmental DNA (eDNA) as a reliable tool for detecting cubozoan jellyfish, specifically *Chironex fleckeri*, and to enhance our understanding of their ecology. Through meticulous development, optimisation, and validation of a highly specific and sensitive eDNA detection assay, this research was successful and has significantly advanced cubozoan jellyfish detection. The findings underscore the potential of eDNA to expand on traditional detection methods, offering a spatially comprehensive, accurate, and efficient alternative for detecting and studying these elusive marine stingers. The application of eDNA as an ecological sampling tool demonstrated its effectiveness in investigating ecological hypothesises surrounding the taxa, specifically regarding life history stages and population structures of *C. fleckeri*. The detection of both medusae and polyps provided insights into their distribution, challenging longstanding paradigms on the dispersion of medusae and the habitat preferences of both medusae and polyps. Furthermore, the research reported in this thesis provided additional evidence of spatially small population stock structures through direct assessments. The capability of eDNA to putatively detect the benthic polyp stage is particularly noteworthy, as it enables crucial insights into this highly elusive stage needed for advancing an understanding of population sources, and subsequently management, of this potentially deadly taxon.

The integration of biophysical modelling in this thesis provided a deeper understanding of how hydrodynamic and environmental factors govern the transport and detectability of eDNA. This approach revealed the critical influence of a combination of extrinsic factors on eDNA dispersal patterns and detectability, highlighting the importance of considering local environmental factors in the interpretation of eDNA data. These findings suggested that *C. fleckeri* eDNA detections are indicative of the recent presence of *C. fleckeri*, showcasing eDNA as a powerful tool for the real-time monitoring of cubozoan jellyfish. Despite the demonstrated benefits, this thesis also acknowledges the limitations of eDNA as an ecological tool for sampling, particularly its inability to

provide detailed information on species abundances. A multifaceted approach, integrating eDNA with traditional methods, is recommended for comprehensive ecological studies. Future research into cubozoan ecology will benefit from the integration of this genetic detection technique into ecological frameworks, enhancing our ability to study and manage these organisms.

Finally, this thesis has advanced the application of eDNA analysis for the detection of cubozoan jellyfish, moving beyond proof-of-concept studies to providing a robust framework for ecological research and potential management efforts. The ability to accurately detect, study, and monitor *C. fleckeri* and potentially other cubozoan species has significant implications for understanding of their ecology and mitigation of their threats to both human health and commercial enterprise. The continued refinement and application of eDNA techniques will undoubtedly continue to play a crucial role in the understanding of the ecology and management of cubozoan jellyfish.

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Appendix I

Chapter 2 Supplement

Table S1.1. Primers used to isolate 584 bp length of jellyfish mt 16S rRNA gene.

Assay	Label	Sequence (5'-3')	Target Sequence Length (bp)
Universal Jellyfish Assay	16SL_F Aa_H16S_15141H	GACTGTTTACCAAAAACATA AGATTTTAATGGTCGAACAGAC	584

Table S1.2. List of marine fish species NCBI accession numbers and country of origin for all existing mt 16S rRNA/complete genome sequences used for *in silico* testing of the endogenous control assay.

Species	Accession Number	Country of Origin	*In silico* Amplification
Lates calcarifer	KR349919	Australia	Yes
Platycephalus fuscus	KT862661	Australia	Yes
Acanthopagrus butcheri	KX234643	Australia	Yes
Acanthopagrus australis	JN688792	China	Yes
Acanthopagrus pacificus	MK919144	Vietnam	Yes
Rhabdosargus sarba	KM272585	China	Yes
Scartelaos histophorus	NC_017888	China	Yes
Eleutheronema tetradactylum	KT593869	China	Yes
Lutjanus johnii	NC_024572	Australia	Yes
Sillaginidae punctatus	MF572031	China	Yes
Triacanthus blaculeatus	AP009174	Japan	Yes
Monodactylus argonteus	NC_009858	Japan	Yes
Hephaestus fuliginosus	MH606192	China	Yes
Gnathanodon speciosus	NC_054367	China	Yes

Table S1.3. Primer selection characteristics.

Selection Characteristics	Sense Primer	Anti-Sense Primer	MGB Probe
bp length	24	22	20
T_m (Nearest Neighbour)	64.98°C	65.23°C	N/A
GC%	45.8	54.5	45
Hairpins	34.4 (weak)	None	None
GC Clamps	2	3	N/A
Self-Dimer	None	None	None
Hetero-Dimer	None	None	None

Table S1.4. Base pair (bp) differences between *Chironex fleckeri* identifiers and sympatric jellyfish species.

Species	Total bp Differences	Primer bp Differences (Sense & Anti-Sense)	Probe bp Differences
Alatina alata	17	6 & 5	6
Carukia barnesi	28	8 & 8	12
Carybdea xaymacana	32	11 & 10	11
Copula sivickisi	22	8 & 5	9
Morbakka fenneri	31	10 & 7	14
Tamoya ohboya	33	9 & 8	16

Table S1.5. Efficiencies of the *Chironex fleckeri* specific detection assay when independent and multiplexed with the endogenous control assay.

	Target Assay Only	Multiplexed Assay
Efficiency (%)	93.47	92.68
R^2	0.999	0.999
Slope	-3.48	-3.51

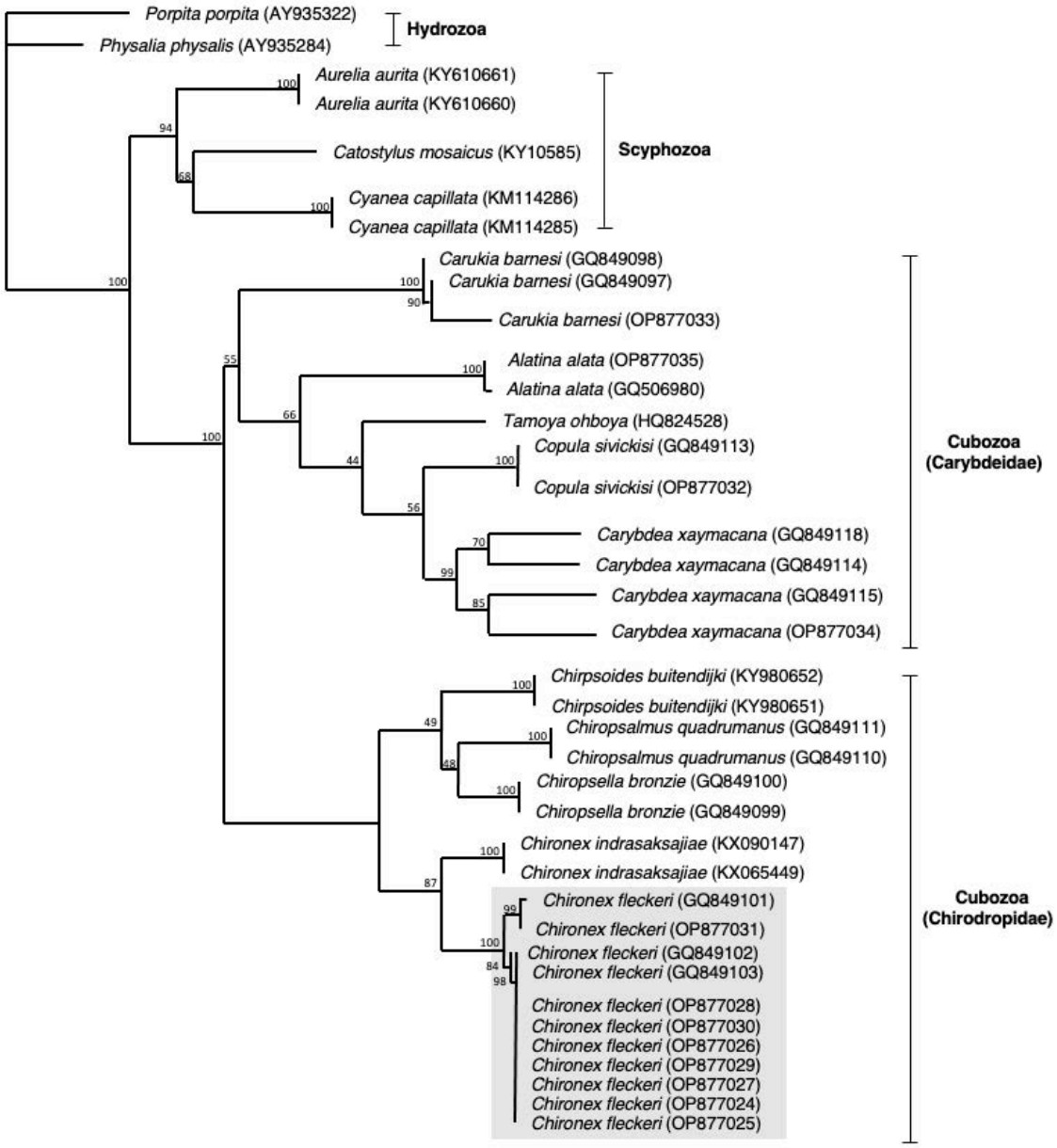

Figure S1.1. Intraspecific similarity of *Chironex fleckeri* sequences and interspecific divergence between *C. fleckeri* and sympatric species for the mt 16S rRNA gene. Bootstrap values greater than 50% are displayed above the branch nodes. The tree was maximum likelihood generated utilising the substitution models GTR+F+I+G4 with sequence regions ranging from ≤418 - ≥763 bp.

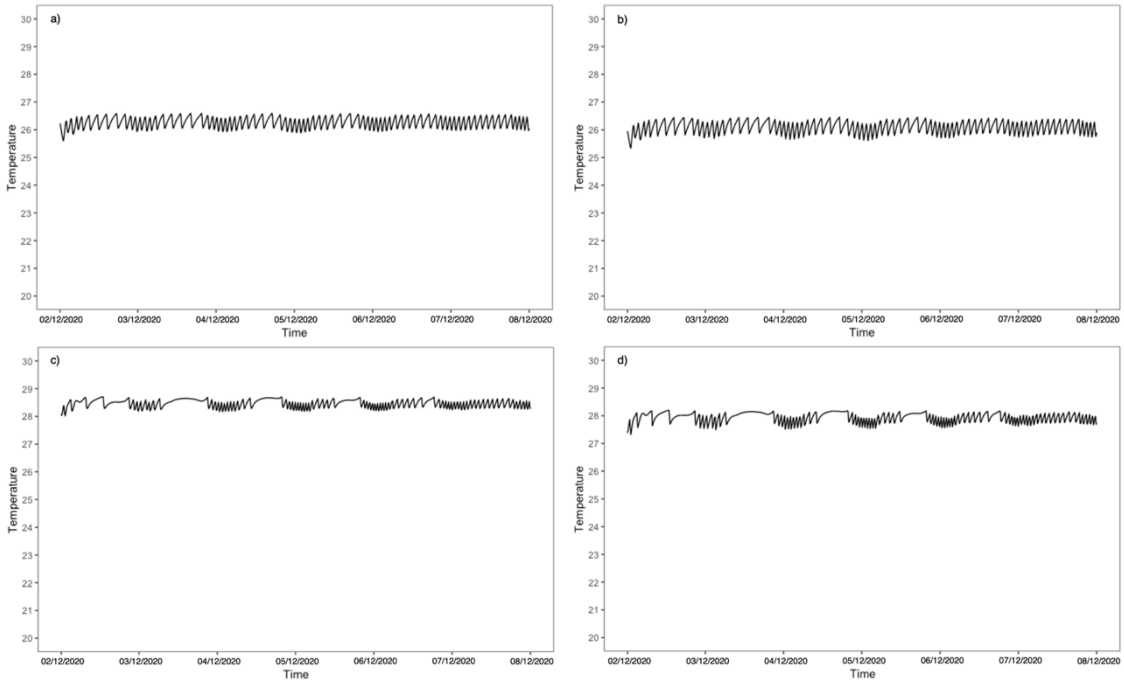

Figure S1.2. Tinytag TG-3100 data logger temperature data for duration of the *C. fleckeri* eDNA decay experiment. a) Logger 1 – 26 °C bath, b) Logger 2 – 26 °C bath, c) Logger 3 – 28 °C bath, d) Logger 4 – 28 °C bath.

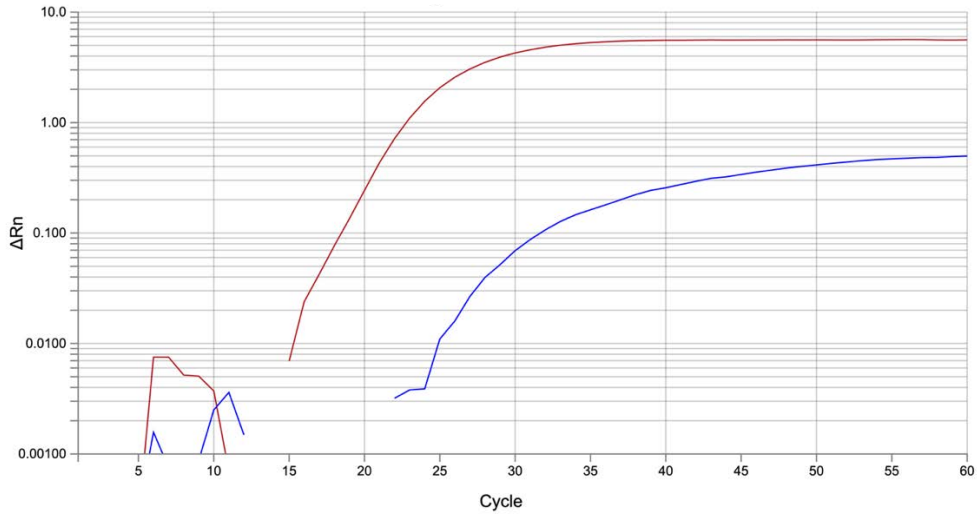

Figure S1.3. Amplification Plot of Multiplexed Assay showing amplification of FAM dye-labelled *Chironex*-specific assay (red) and VIC dye-labelled endogenous control assay (blue).

Appendix II

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Table S2.1. Multiplexed assay information for detection of *Chironex fleckeri* and endogenous control (Adapted from Morrissey et al. (2022)).

Assay	Label	Sequence	Reference
Chironex fleckeri	Chironex_16S_F	ATCTTCCACTGTCTCAGCTTTACC	Morrissey et al. (2022)
	Chironex_16S_R	CCTCAGTACTCGTGTCTCCCTA	
	Chironex_16S_P	(FAM)-CTCGTCCTTCCAAGTATAAG-(MGB)	
Endogenous Control – Generic Fish	Fish_16S_F	GACCTCGATGTTGGATCA	Furlan and Gleeson (2016)
	Fish_16S_R	CTCAGATCACGTAGGACTTTA	
	Fish_16S_ probe	(VIC)-ACATCCTAWTGGTGC-(MGB)	

Table S2.2. Densities of *Chironex fleckeri* medusa obtained via seine net drags.

Site	No. Medusa Captured	Density (individuals/1000m^3)
4	1	0.89
5	2	1.76
6	2	1.54
7	1	0.73
13	1	0.92
14	17	20.96
15	9	8.84

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Appendix III

Chapter 4 Supplement

Table S3.1. eDNA sample collection sites with depth, depth integrated temperature and salinity, number of positive technical replicates and eDNA concentrations (copies L^{-1}) during the December 2020 and February 2021 sampling periods.

Sampling Time	Site no.	Depth (m)	Temperature (°C)	Salinity (ppt)	No. of positive detections / 12	Copies per L
December 2020	5	2.5	29.5	36.2	1	32
	6	0.4	30.9	36.4	2	45.8
	16	2.2	29.5	36.2	5	97.9
	19	2.4	29.4	36.2	3	275.6
	22	1	31.1	36.2	0	0
	23	0.4	29	36.2	0	0
Average	–	–	29.9	36.2	–	–
February 2021	5	2.8	30.3	33.8	0	0
	6	2.1	29.9	32.4	2	63.4
	16	2.2	29.8	33.6	2	36.5
	19	3.1	30.3	33.8	2	92.4
	22	0.8	30.2	33.6	2	17.8
	23	0.7	30	33.4	1	26.8
Average	–	–	30.1	33.4	–	–

Table S3.2. eDNA sample collection sites with depth (m), depth integrated temperature and salinity, number of positive technical replicates and eDNA concentrations (copies L^{-1}) during the March and December 2021 sampling periods. * indicates sites where data was not collected.

Sampling Time	Site no.	Depth (m)	Temperature (°C)	Salinity (ppt)	No. of positive detections / 12	Copies per L
March 2021	1	8.1	28.1	32.8	0	0
	3	5.1	28.8	32.9	0	0
	5	0.5	29.5	32.8	0	0
	6	0.8	27.2	31.4	4	134.8
	14	10.7	28.4	32.9	0	0
	15	8.2	28.5	32.9	0	0
	16	1.4	29.6	31.4	3	58.2
	17	11.4	28.3	32.9	0	0
	18	8.9	28.6	32.9	0	0
	19	1.7	*	*	0	0
	20	11.2	28.3	32.9	0	0
	21	7.8	28.3	32.9	0	0
	22	1.2	27.9	32.8	0	0
	23	1.2	28.2	31.9	1	18.8
Average	–	–	28.4	32.6	–	–
December 2021	1	11.7	30	35.3	0	0
	3	8.8	30.1	35.2	0	0
	5	0.5	31.2	35.2	0	0
	6	0.4	30.2	35.5	1	33.5
	14	12.3	30.1	35.3	0	0
	15	8.9	30.1	35.2	0	0
	16	3	30.4	35.3	0	0
	17	11.8	30	35.2	0	0
	18	8.7	30.3	35.2	0	0
	19	4.2	30.6	35.3	0	0
	20	13	30.1	35.3	0	0
	21	8.1	30.2	35.3	0	0
	22	0.9	30.7	35.3	0	0
	23	0.4	30.6	35.3	1	22.5
Average	–	–	30.3	35.3	–	–

Table S3.3. eDNA sample collection sites with depth (m), depth integrated temperature and salinity, number of positive technical replicates and eDNA concentrations (copies L^{-1}) during the July 2020 sampling period. Note, temperatures and salinities were not measured during this sampling period.

Sampling Time	Site no.	No. of positive detections / 12	Copies per L
July 2020	1	0	0
	3	1	62.5
	5	0	0
	6	1	63.3
	14	0	0
	15	0	0
	16	0	0
	17	0	0
	18	0	0
	19	0	0
	20	0	0
	21	0	0
	22	0	0
	23	0	0

Table S3.4. eDNA sample collection sites with depth (m), depth integrated temperature and salinity, number of positive technical replicates and eDNA concentrations (copies L^{-1}) during the July 2022 sampling period. * indicates sites where data was not collected.

Sampling Time	Site no.	Depth (m)	Temperature (°C)	Salinity (ppt)	No. of positive detections / 12	Copies per L
July 2022	2	0.5	20.3	34.3	0	0
	3	0.7	20.2	34.2	0	0
	4	0.3	21.2	34.1	0	0
	6	0.8	20.2	34.1	0	0
	7	0.4	21	34.3	0	0
	8	0.8	22.7	7.6	0	0
	10	0.8	20.5	2.5	0	0
	9	0.8	20.8	11.3	1	73.6
	11	0.8	20.5	2.5	0	0
	12	0.7	20.5	34.4	0	0
	13	1	*	*	0	0
	16	0.6	20.7	34.3	0	0
	19	0.6	21	34.2	0	0
	22	0.1	*	*	0	0
	23	0.4	20.2	34.2	2	82.3
	14	10.1	20	31.5	0	0
Average	–	–	20.7	25.96	–	–

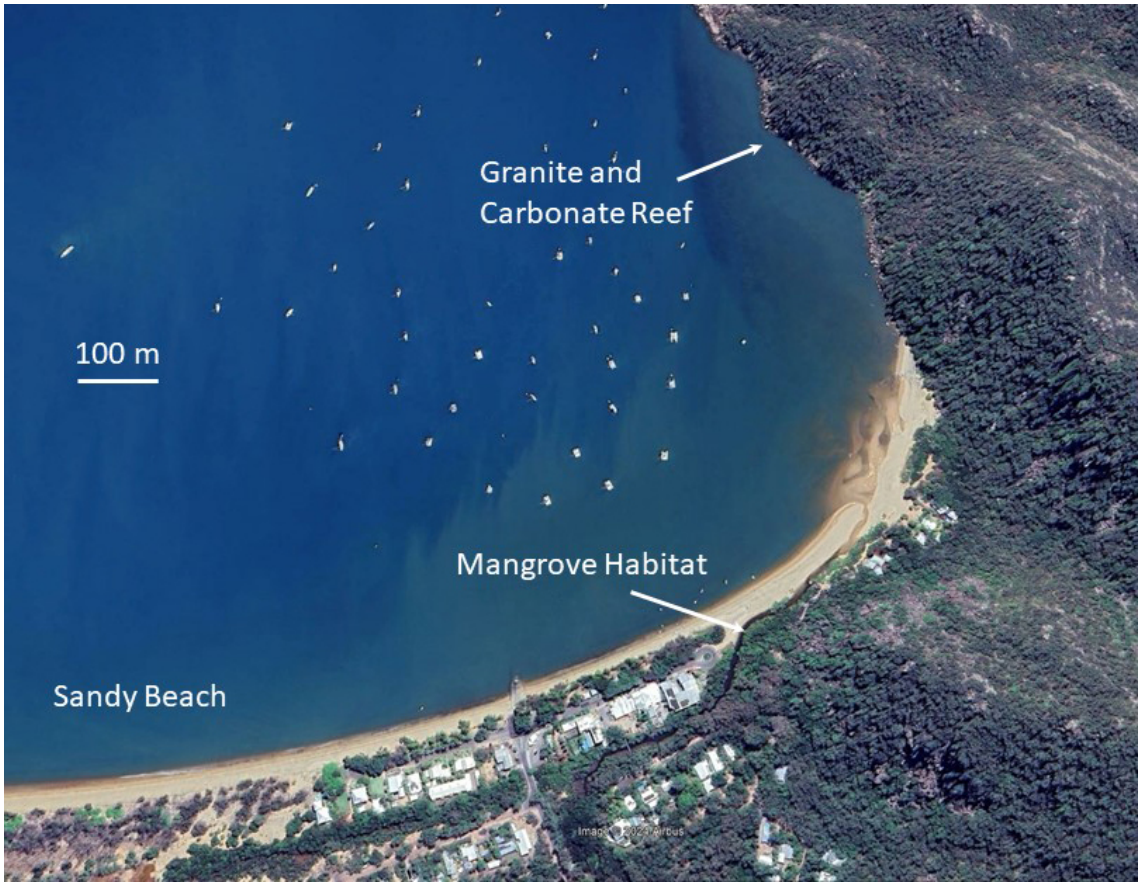

Figure S3.1. Labelled satellite image displaying the flora and landform of Horseshoe Bay, Magnetic Island.

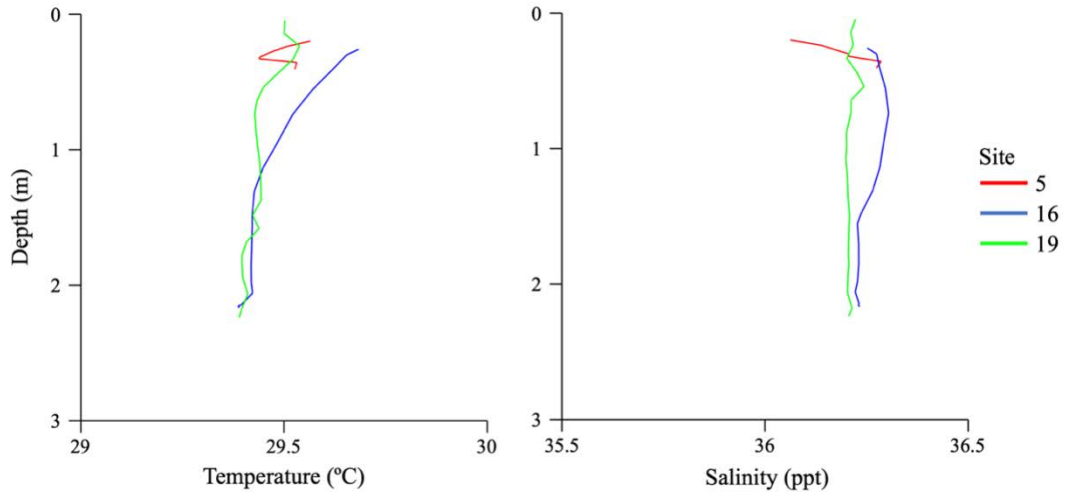

Figure S3.2. Depth profiles of temperature and salinity at nearshore sample sites for the December 2020 sampling time.

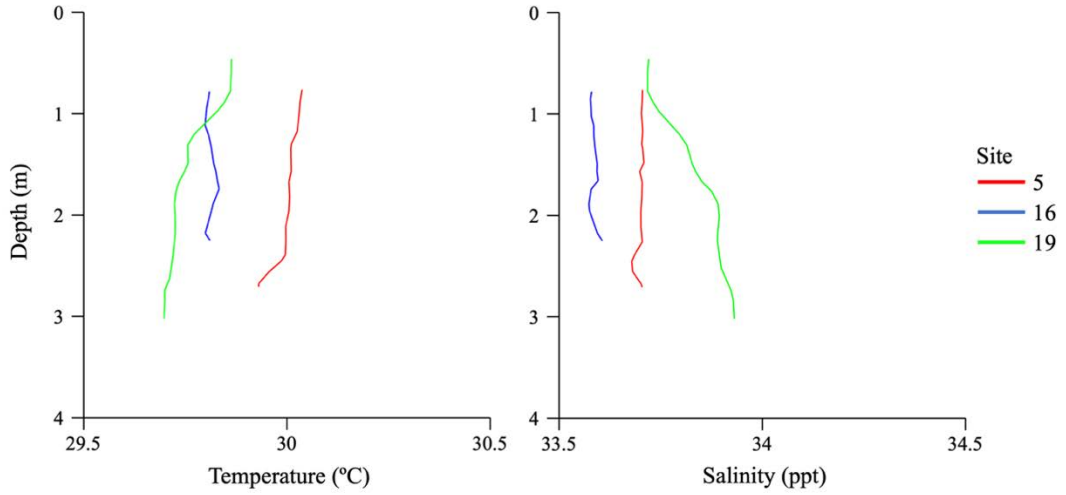

Figure S3.3. Depth profiles of temperature and salinity at nearshore sample sites for the February 2020 sampling time.

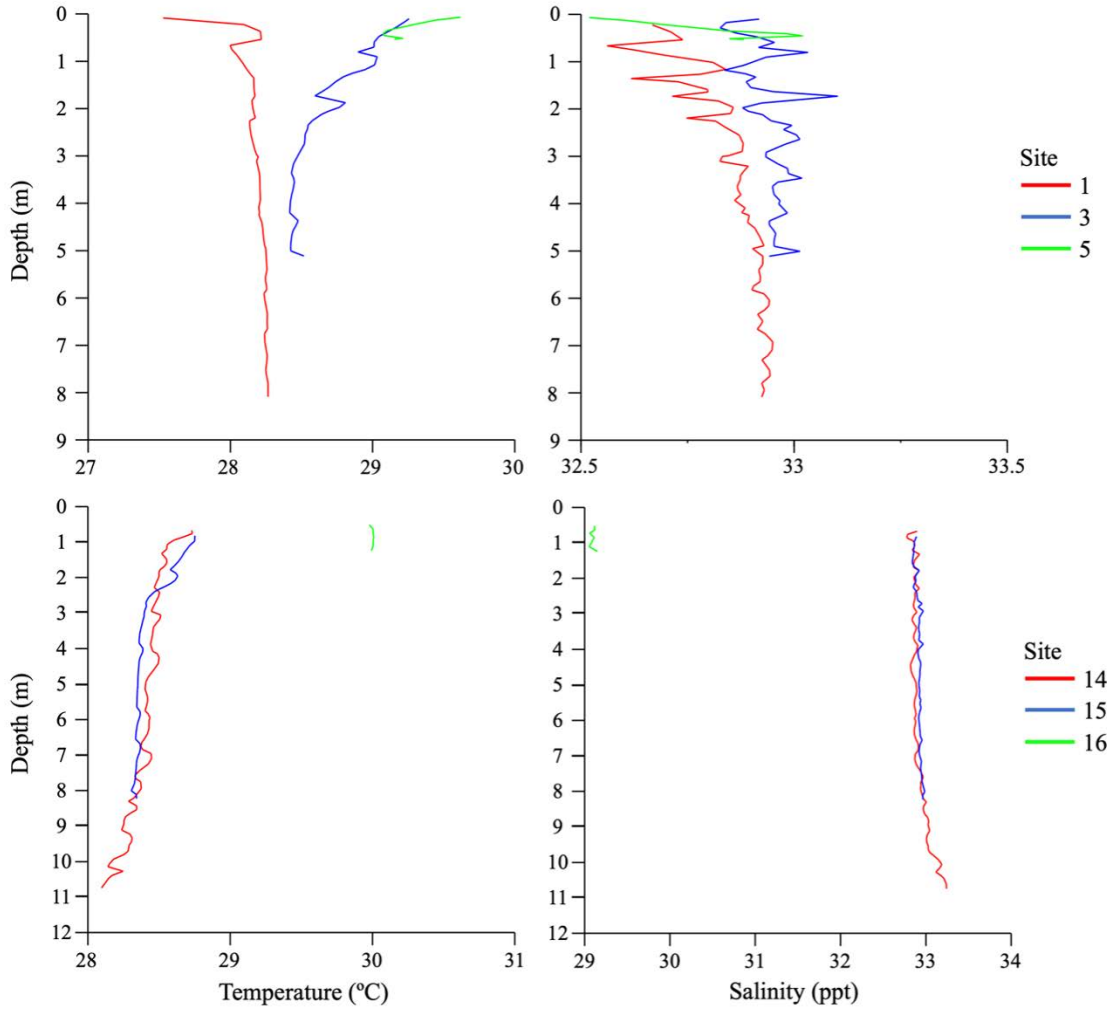

Figure S3.4. Depth profiles of temperature and salinity at offshore (sites 1 and 14), mid-shore (sites 3 and 15) and nearshore (sites 5 and 16) sample sites for the March 2021 sampling time.

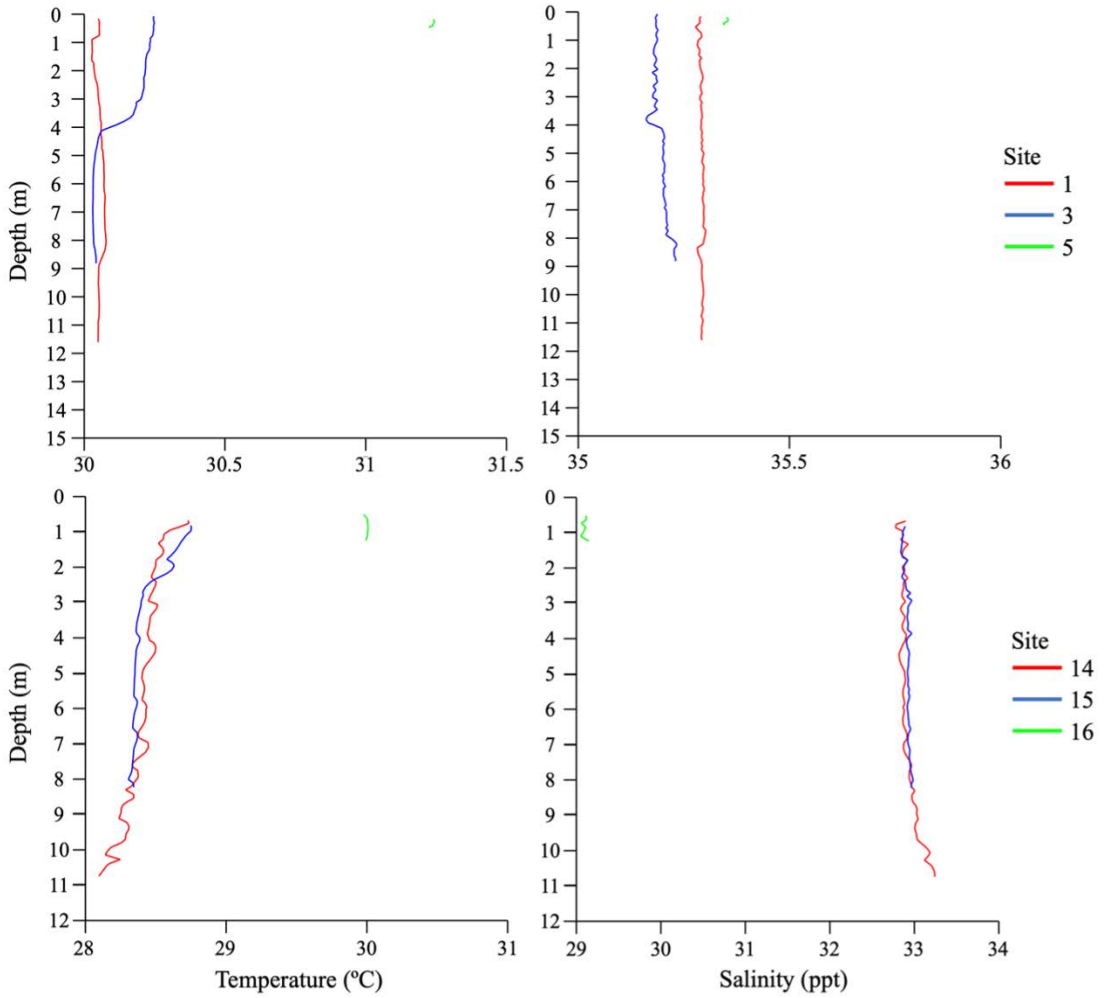

Figure S3.5. Depth profiles of temperature and salinity at offshore (sites 1 and 14), mid-shore (sites 3 and 15) and nearshore (sites 5 and 16) sample sites for the December 2021 sampling time.

Appendix IV

Chapter 5 Supplement

IV.1 Biophysical Model

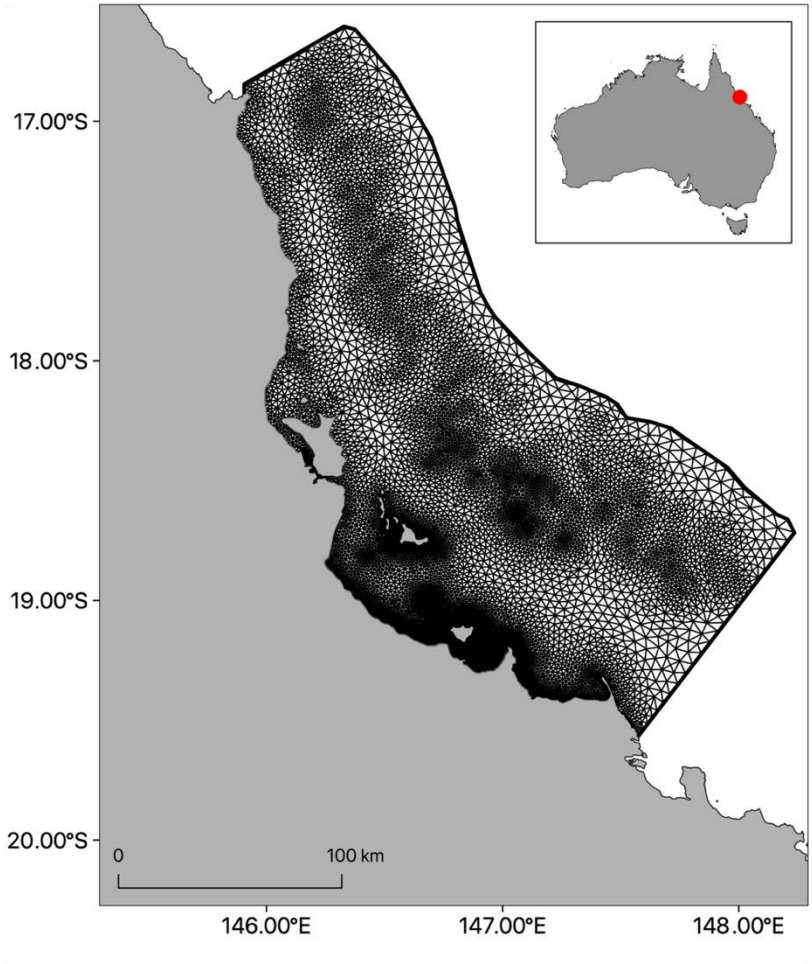

Figure S4.1. Map displaying the model domain (black border lines) and elements (triangles) of the hydrodynamic model, with a map of Australia showing the relative location of the area (red dot).

IV.1.1 Hydrodynamic Model Validation

The tides simulated in SLIM closely matched the tidal data measured at the tidal datum in Townsville (Figures S2 & S3). The simulation accurately tracked the rise and fall of the tides across all four time periods, despite some discrepancies. Notably, the simulated tide's fall is slightly overestimated. When SLIM's tidal anomalies were

compared to measured anomalies through linear regression, the resulting lines had intercepts near zero and slopes close to one, with normalised root mean square errors approaching zero (Figure S4.3). Consequently, these regression lines nearly coincided with the identity lines, indicating a strong fit.

The water current data simulated in Horseshoe Bay shows discrepancies to that of the measured data. The trends of the simulated currents were however well captured within the model (NRMSE range: 0.10 to 0.35). The discrepancies likely result from the influence of the coastline in the model, which may not accurately reflect the complex interactions of currents as the bay contains varying coastal features such as a tidal estuary, reefs, and rocky headlands. These coastal features can create localised eddies and divergences in water movement that are challenging to model precisely. These, specifically eddies, can been observed during particle releases. Future improvements in the model could involve higher resolution coastal mapping, enhanced parameterisation of coastal dynamics, and integration of more detailed observational data to better capture these complex interactions.

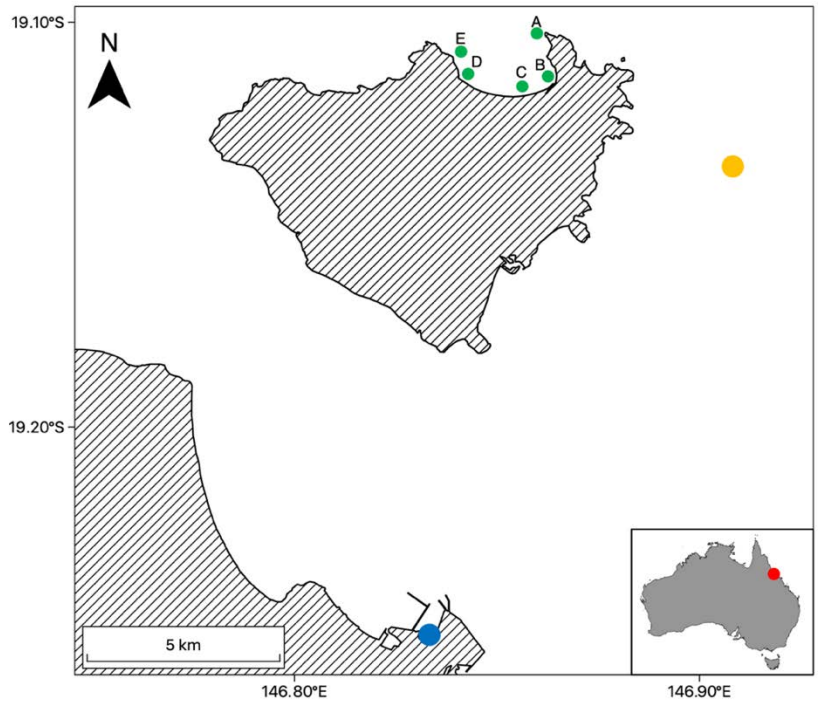

Figure S4.2. Map displaying the location of drag-tilt current meters (green dots), tide datum (blue dot), Australian Institute of Marine Science weather station (yellow dot), with map of Australia showing the relative location of the area (red dot).

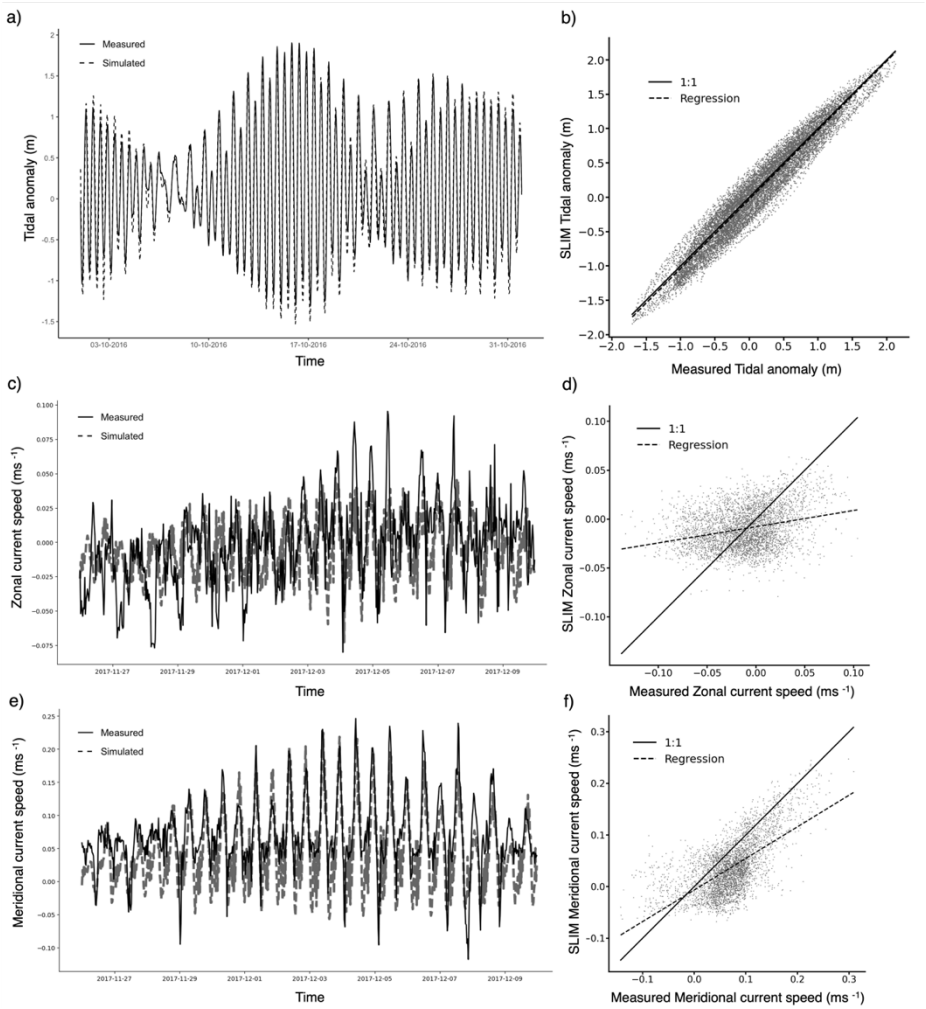

Figure S4.3. Hydrodynamic model validation plots showing time series and x-y plot comparing the measured and simulated tidal anomalies and zonal (U) and meridional (V) water current components. a) and b) display measured and simulated tidal anomalies for October 2016 to January 2017, c) and d) display measured and simulated zonal (U) water current for drag-tilt current metre location C for November 2017 to January 2018, and e) and f) display measured and simulated meridional (V) water current for the same location and time period. The measured data are displayed with a solid black line and the SLIM data are displayed with a dashed grey line for time series plots. In each x-y plot the regression line for the SLIM data (y) modelled as a function of the measured data (x) is displayed as a black dashed lined and compared to a solid black identity line (y = x).

Table S4.1. Hydrodynamic model validation of tidal anomalies through use of a regression analysis for all four time periods. The slope (m), y-intercept (c), normalised root mean square error (NRMSE), and the number of measurements compared between measured and simulated data are shown.

Model	m	c	NRMSE	n
October 2016 – January 2017	1.005	-0.03	0.04	13011
June 2017 – September 2017	0.992	-0.04	0.05	17202
October 2017 – January 2018	0.998	-0.08	0.05	12915
June 2018 – September 2018	0.997	-0.06	0.05	17445

Table S4.2. Hydrodynamic model validation of zonal (U) and meridional (V) water current components through use of a regression analysis at five locations within Horseshoe Bay for the period of November 2017 to January 2018. The slope (m), y-intercept (c), normalised root mean square error (NRMSE), and the number of measurements compared between measured and simulated data are shown.

	Location	m	c	NRMSE	n
U	A	0.17	-0.01	0.14	4007
	B	0.07	0.01	0.16	4007
	C	0.33	-0.01	0.13	3985
	D	0.01	-0.01	0.19	4007
	E	0.44	-0.05	0.15	4007
V	A	0.61	-0.01	0.12	4007
	B	0.15	0.01	0.10	4007
	C	0.21	-0.01	0.11	3985
	D	0.13	0.02	0.17	4007
	E	0.75	0.06	0.35	4007

IV.2 Scenario Conditions

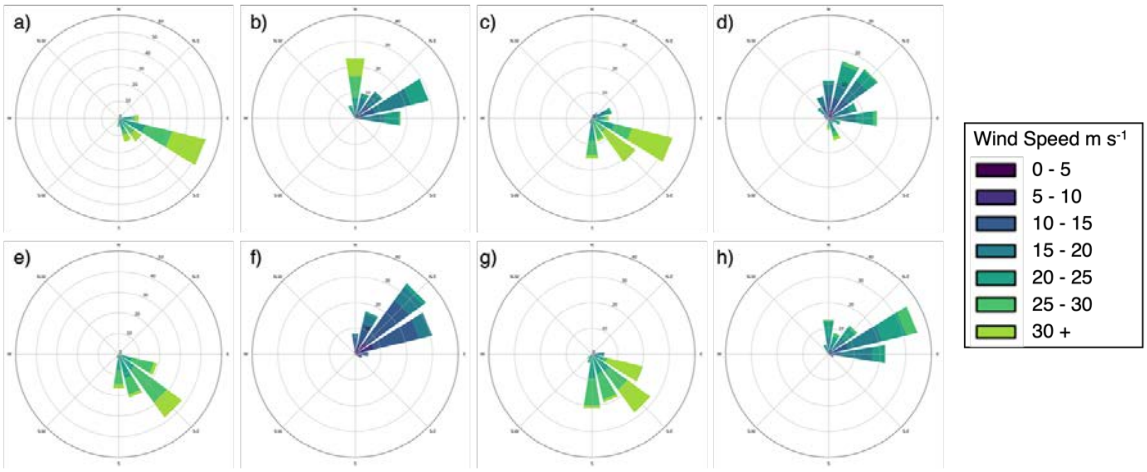

Figure S4.4. Wind roses showing wind speed and direction for, a) scenario A, b) scenario B, c) scenario C, d) scenario D, e) scenario E, f) scenario F, g) scenario G, h) scenario H.

IV.3 Particle dispersion

Figure S4.5. Map of Horseshoe Bay displaying the boundary box (black lines) utilised to calculate the relative abundance of remaining particles (post decay) within and outside of Horseshoe Bay.

Animation S4.1. Simulated currents in and around Horseshoe Bay across a 24 h period during a spring tidal cycle under south-easterly wind conditions.

Animation S4.2. Simulated currents in and around Horseshoe Bay across a 24 h period during a spring tidal cycle under north-easterly wind conditions.

Animation S4.3. Simulated currents in and around Horseshoe Bay across a 24 h period during a neap tidal cycle under south-easterly wind conditions.

Animation S4.4. Simulated currents in and around Horseshoe Bay across a 24 h period during a neap tidal cycle under north-easterly wind conditions.

Animations are available on Open Science Framework (DOI 10.17605/OSF.IO/B5ECS)

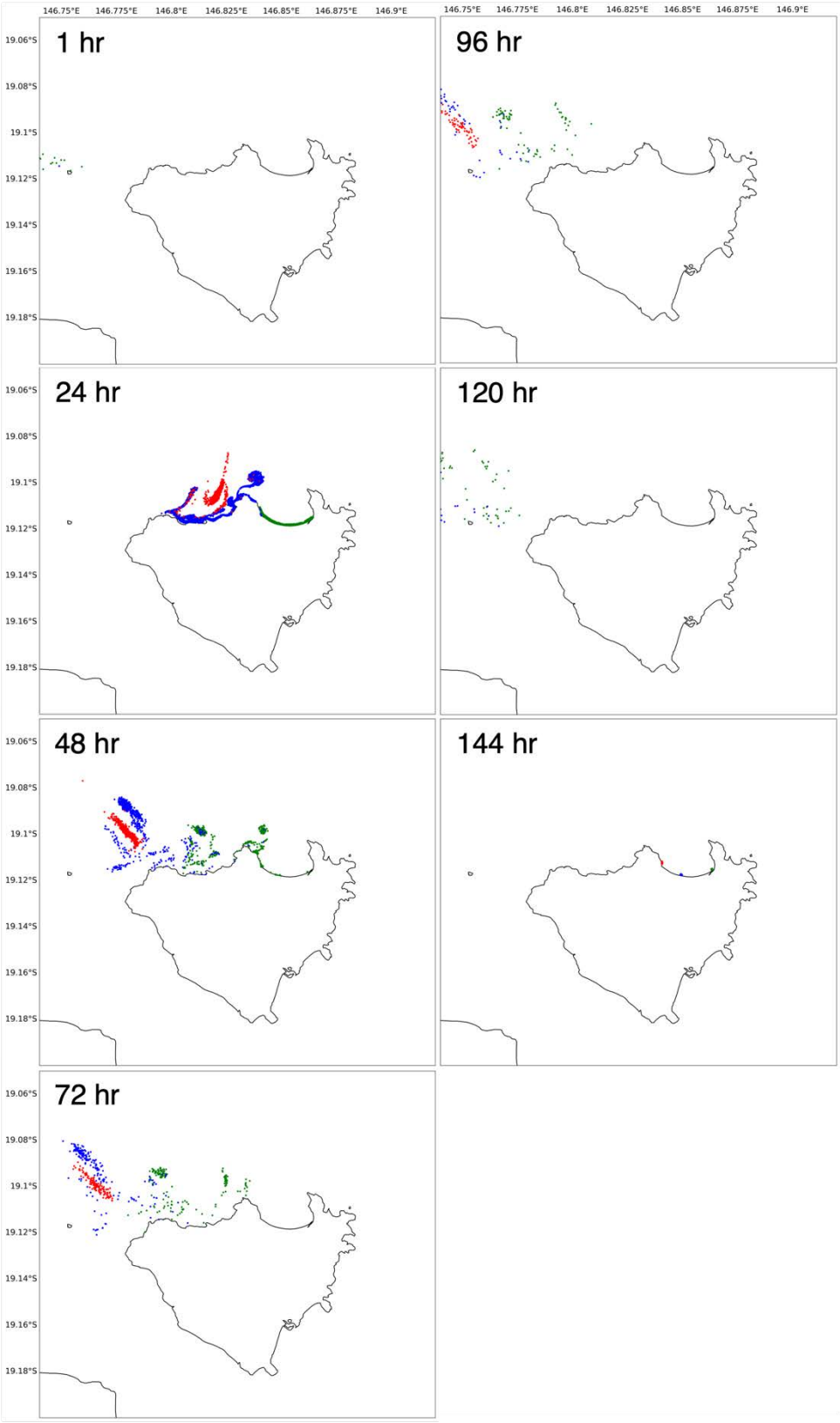

Figure S4.6. Maps of Magnetic Island displaying eDNA particle dispersion at 1 h, 24 h, 48 h, 72 h, 96 h, 120 h, and 144 h intervals under scenario A. Red particles were seeded from location A, blue from location B, and green from location C.

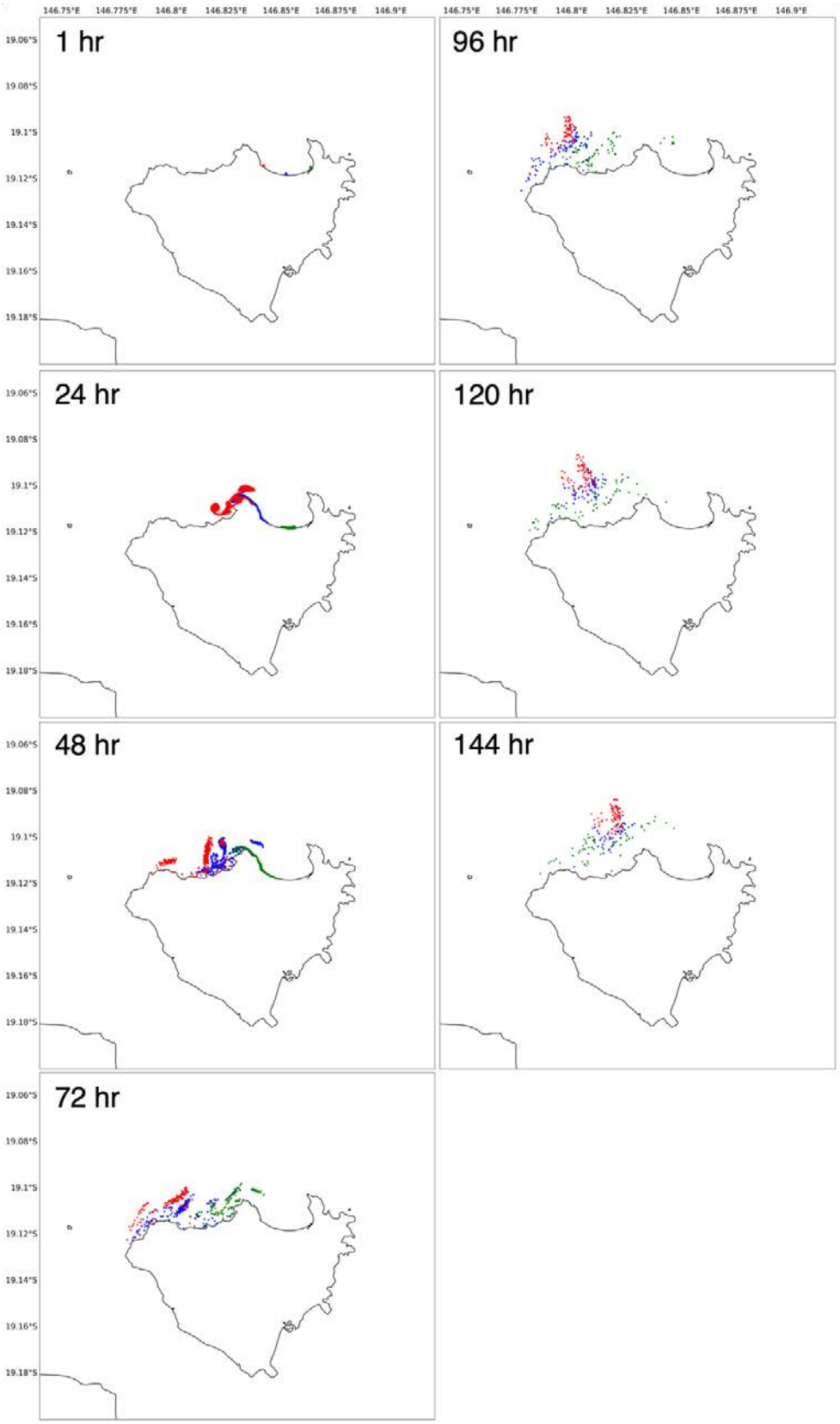

Figure S4.7. Maps of Magnetic Island displaying eDNA particle dispersion at 1 h, 24 h, 48 h, 72 h, 96 h, 120 h, and 144 h intervals under scenario B. Red particles were seeded from location A, blue from location B, and green from location C.

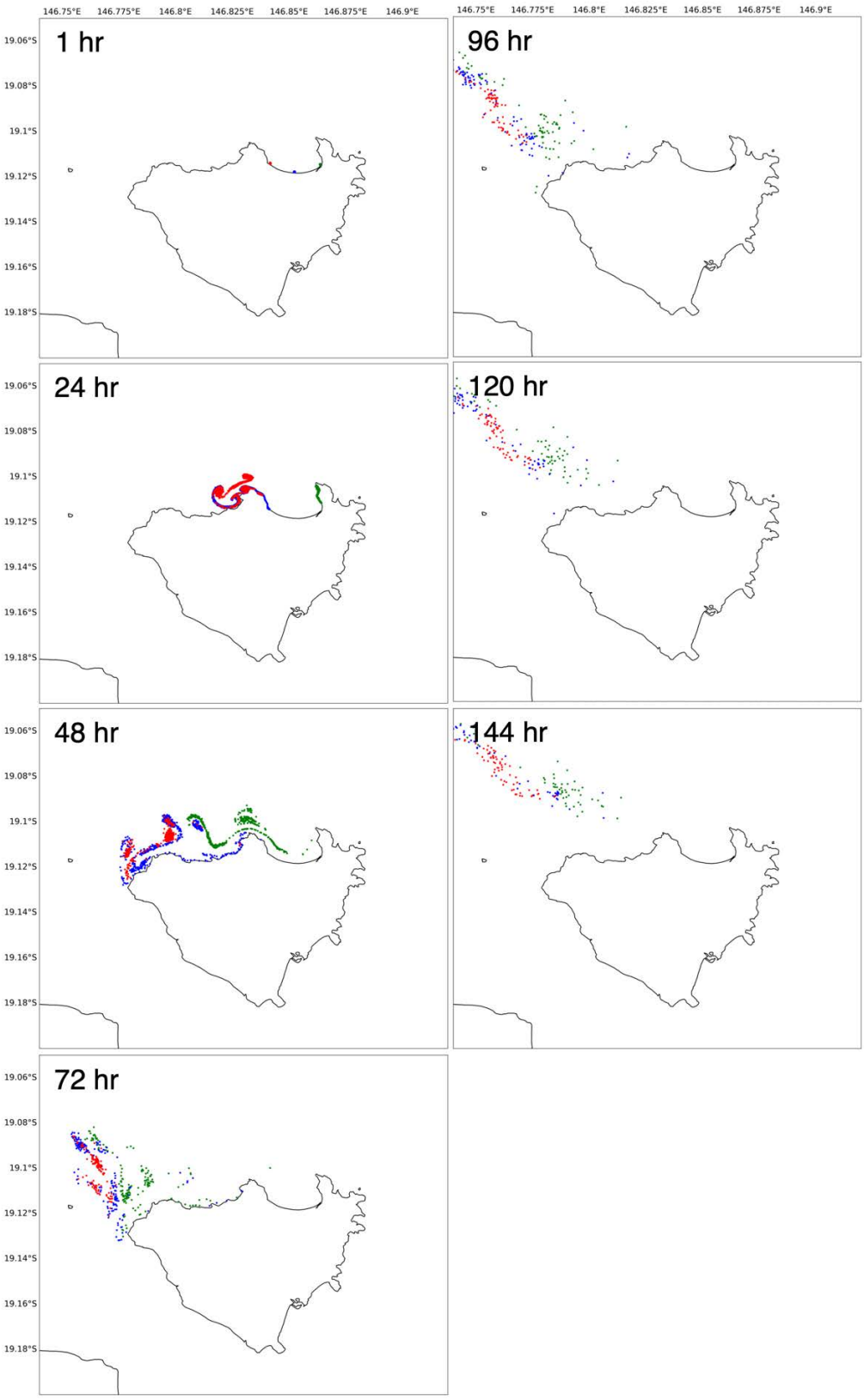

Figure S4.8. Maps of Magnetic Island displaying eDNA particle dispersion at 1 h, 24 h, 48 h, 72 h, 96 h, 120 h, and 144 h intervals under scenario C. Red particles were seeded from location A, blue from location B, and green from location C.

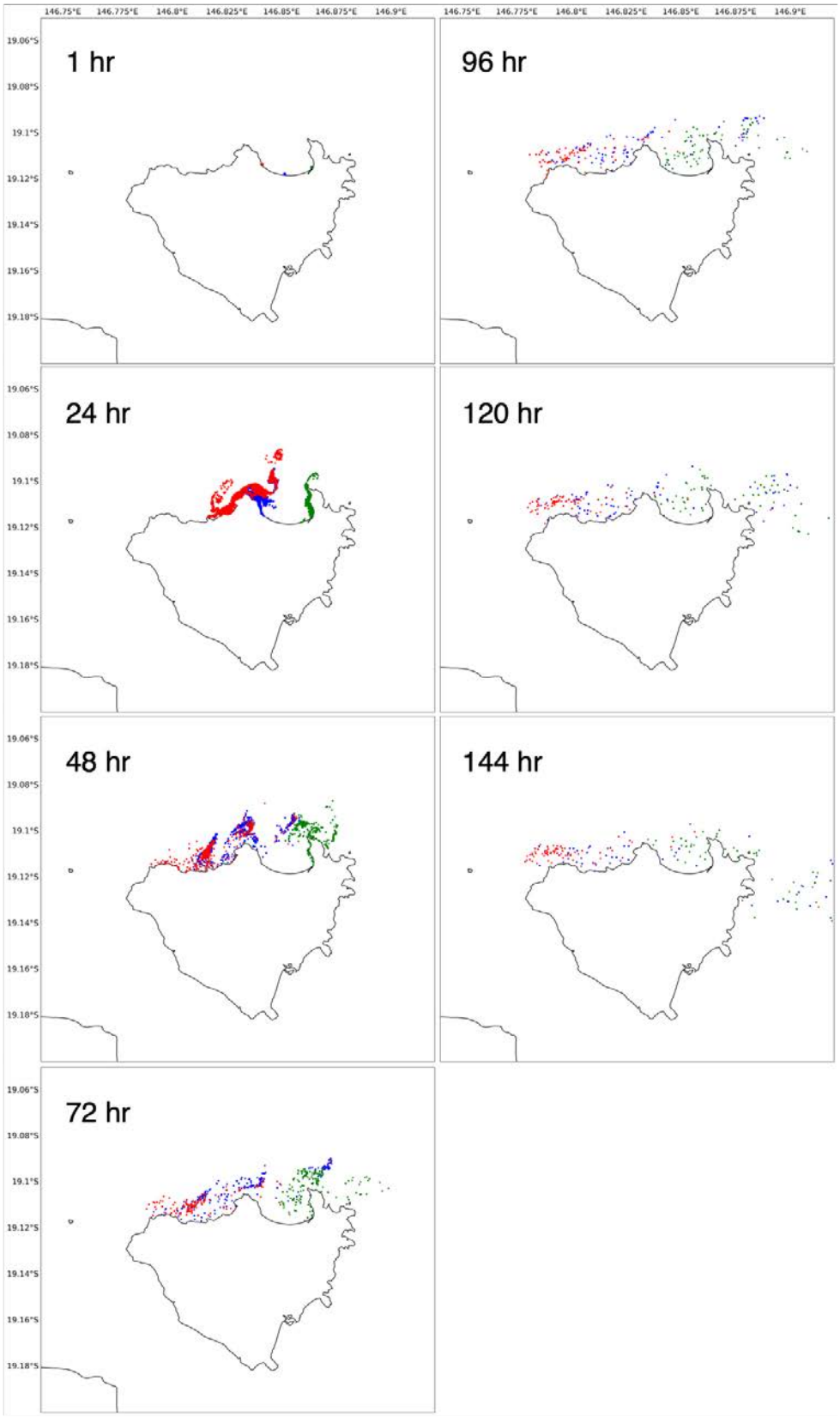

Figure S4.9. Maps of Magnetic Island displaying eDNA particle dispersion at 1 h, 24 h, 48 h, 72 h, 96 h, 120 h, and 144 h intervals under scenario D. Red particles were seeded from location A, blue from location B, and green from location C.

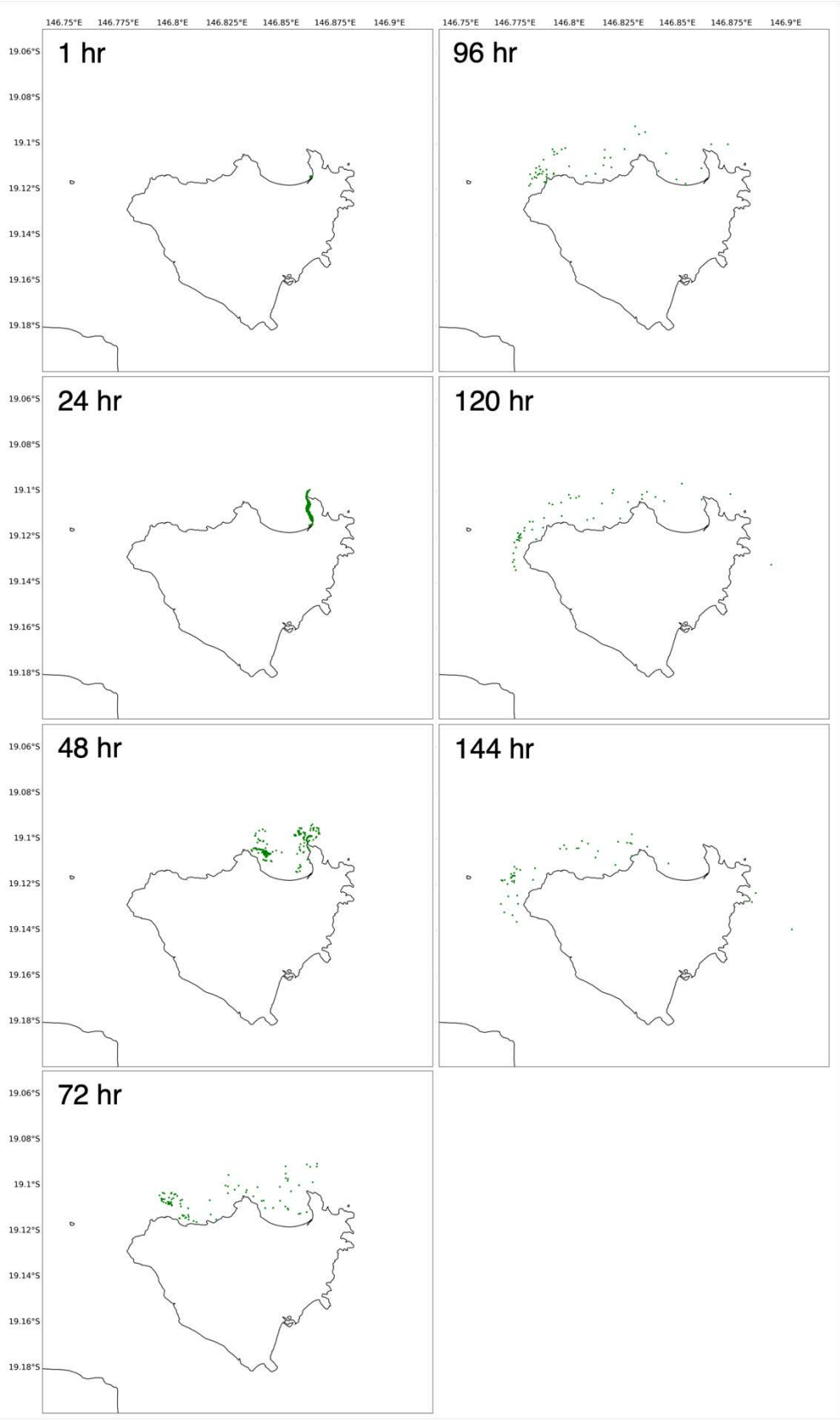

Figure S4.10. Maps of Magnetic Island displaying eDNA particle dispersion at 1 h, 24 h, 48 h, 72 h, 96 h, 120 h, and 144 h intervals under scenario E. Red particles were seeded from location A, blue from location B, and green from location C.

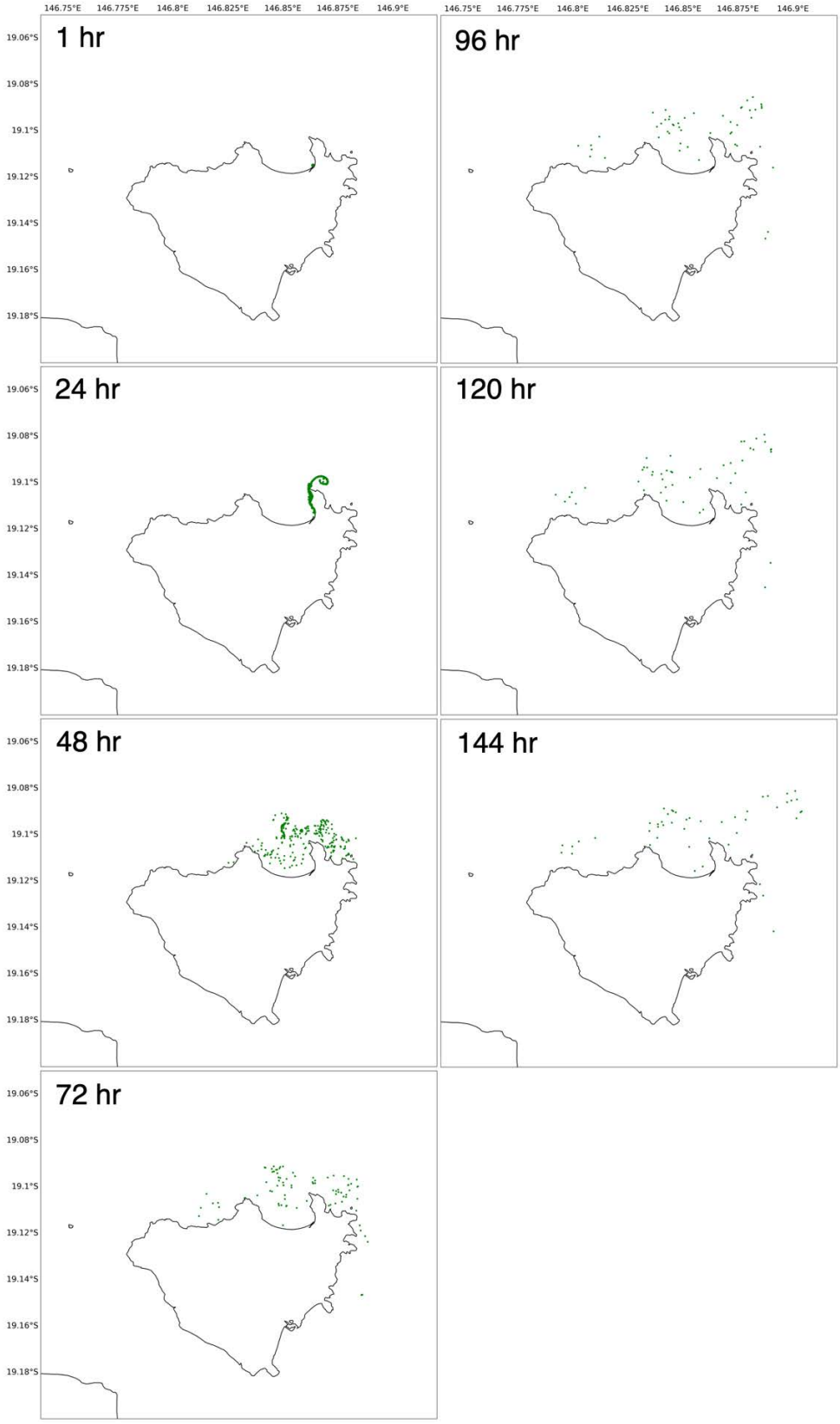

Figure S4.11. Maps of Magnetic Island displaying eDNA particle dispersion at 1 h, 24 h, 48 h, 72 h, 96 h, 120 h, and 144 h intervals under scenario F. Red particles were seeded from location A, blue from location B, and green from location C.

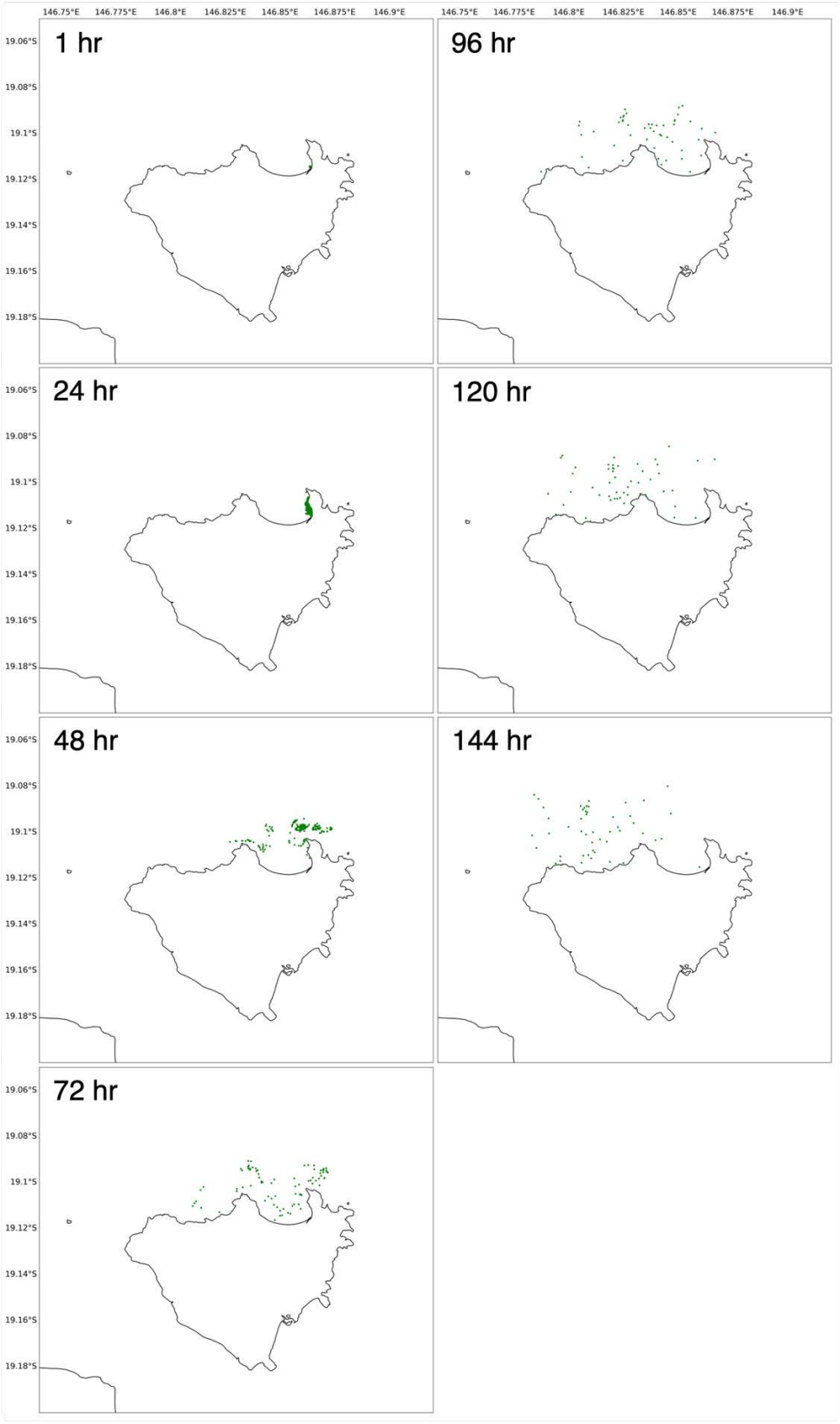

Figure S4.12. Maps of Magnetic Island displaying eDNA particle dispersion at 1 h, 24 h, 48 h, 72 h, 96 h, 120 h, and 144 h intervals under scenario G. Red particles were seeded from location A, blue from location B, and green from location C.

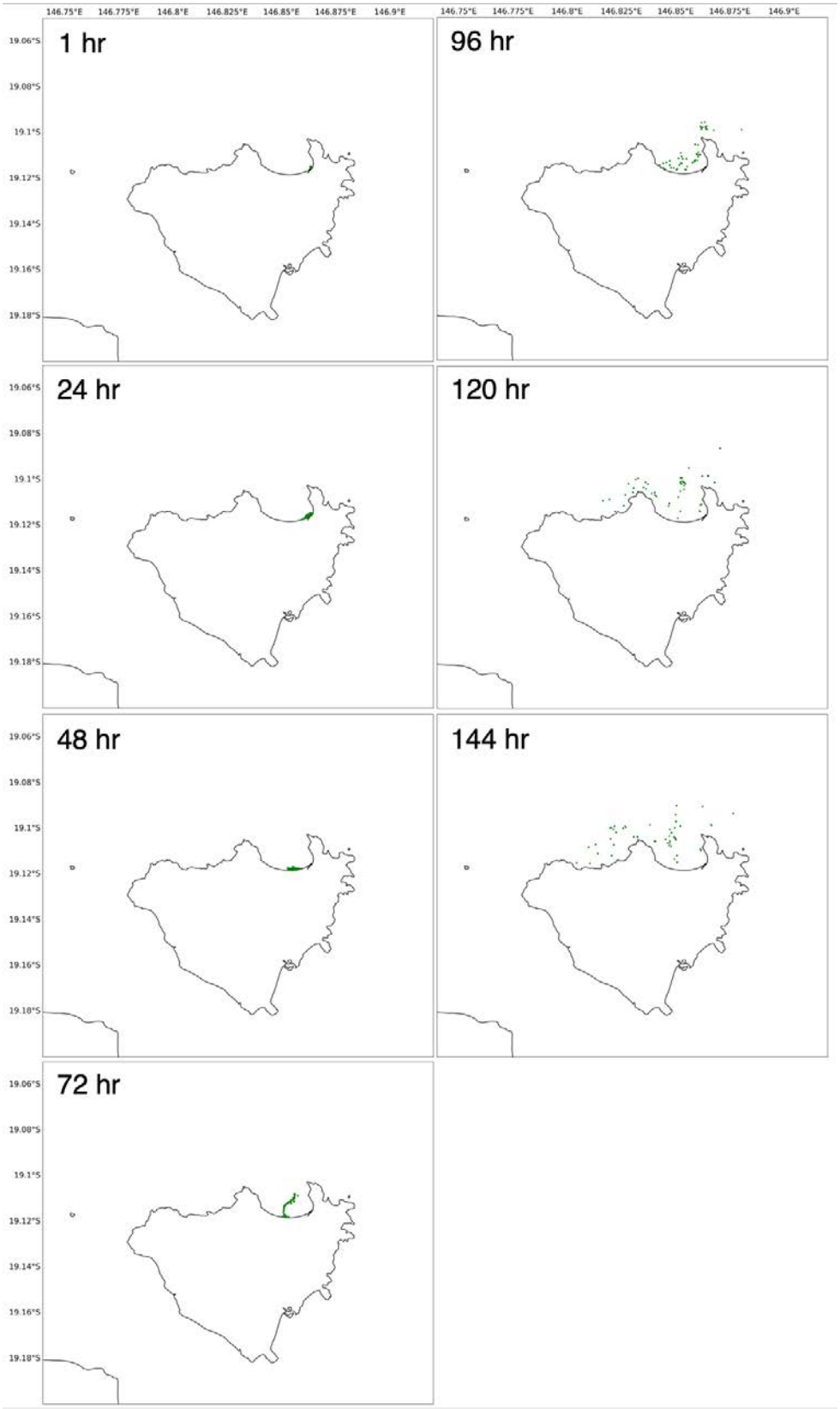

Figure S4.13. Maps of Magnetic Island displaying eDNA particle dispersion at 1 h, 24 h, 48 h, 72 h, 96 h, 120 h, and 144 h intervals under scenario H. Red particles were seeded from location A, blue from location B, and green from location C.

Animation S4.5. Hourly eDNA particle dispersion under scenario A. Red particles were seeded from location A, blue from location B, and green from location C.

Animation S4.6. Hourly eDNA particle dispersion under scenario B. Red particles were seeded from location A, blue from location B, and green from location C.

Animation S4.7. Hourly eDNA particle dispersion under scenario C. Red particles were seeded from location A, blue from location B, and green from location C.

Animation S4.8. Hourly eDNA particle dispersion under scenario D. Red particles were seeded from location A, blue from location B, and green from location C.

Animation S4.9. Hourly eDNA particle dispersion under scenario E. Red particles were seeded from location A, blue from location B, and green from location C.

Animation S4.10. Hourly eDNA particle dispersion under scenario F. Red particles were seeded from location A, blue from location B, and green from location C.

Animation S4.11. Hourly eDNA particle dispersion under scenario G. Red particles were seeded from location A, blue from location B, and green from location C.

Animation S4.12. Hourly eDNA particle dispersion under scenario H. Red particles were seeded from location A, blue from location B, and green from location C.

Animations are available on Open Science Framework (DOI 10.17605/OSF.IO/B5ECS)

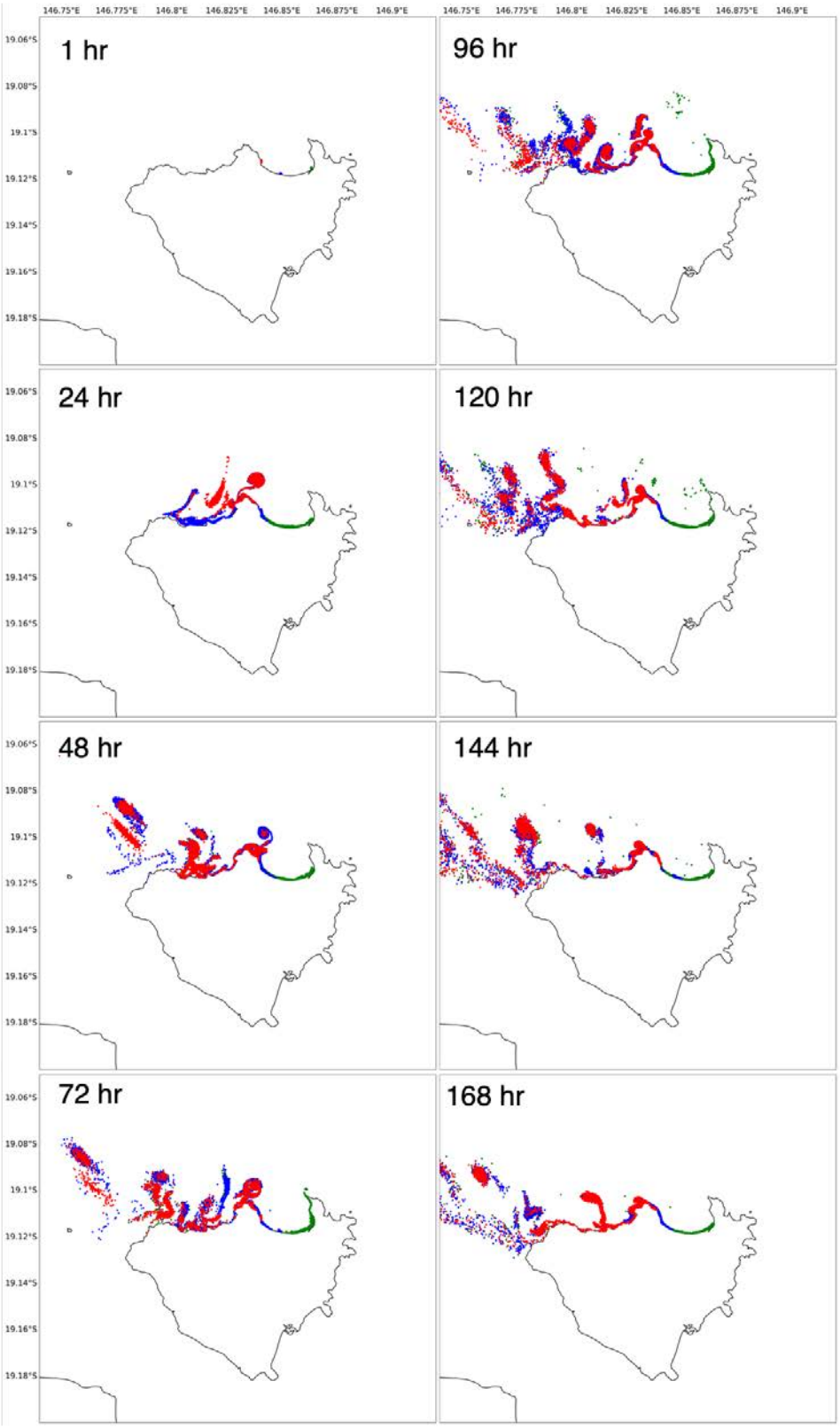

Figure S4.14. Maps of Magnetic Island displaying eDNA particle dispersion at 1 h, 24 h, 48 h, 72 h, 96 h, 120 h, 144 h, and 168 h intervals under scenario A with releases occurring every 10 hours. Red particles were seeded from location A, blue from location B, and green from location C.

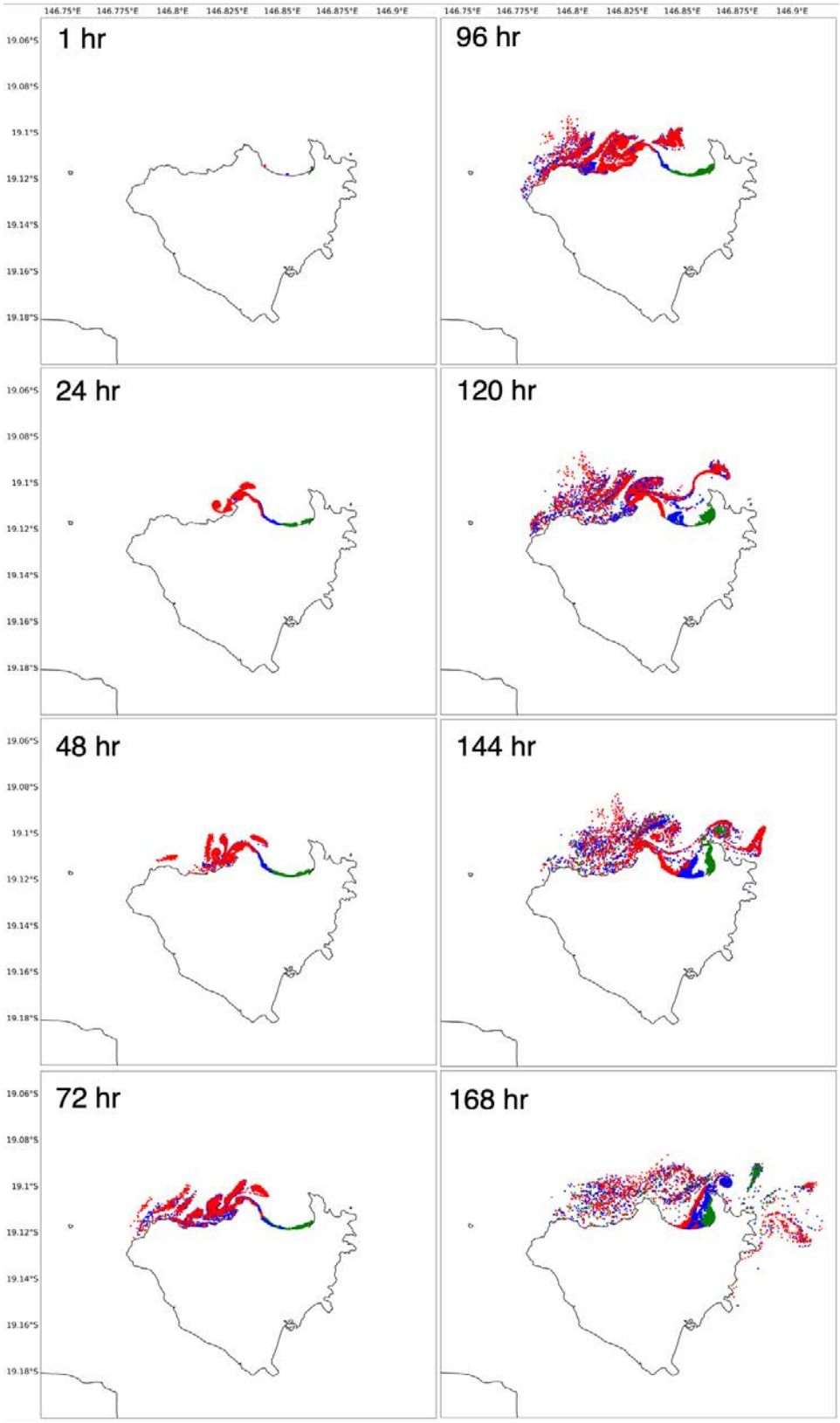

Figure S4.15. Maps of Magnetic Island displaying eDNA particle dispersion at 1 h, 24 h, 48 h, 72 h, 96 h, 120 h, 144 h, and 168 h intervals under scenario B with releases occurring every 10 hours. Red particles were seeded from location A, blue from location B, and green from location C.

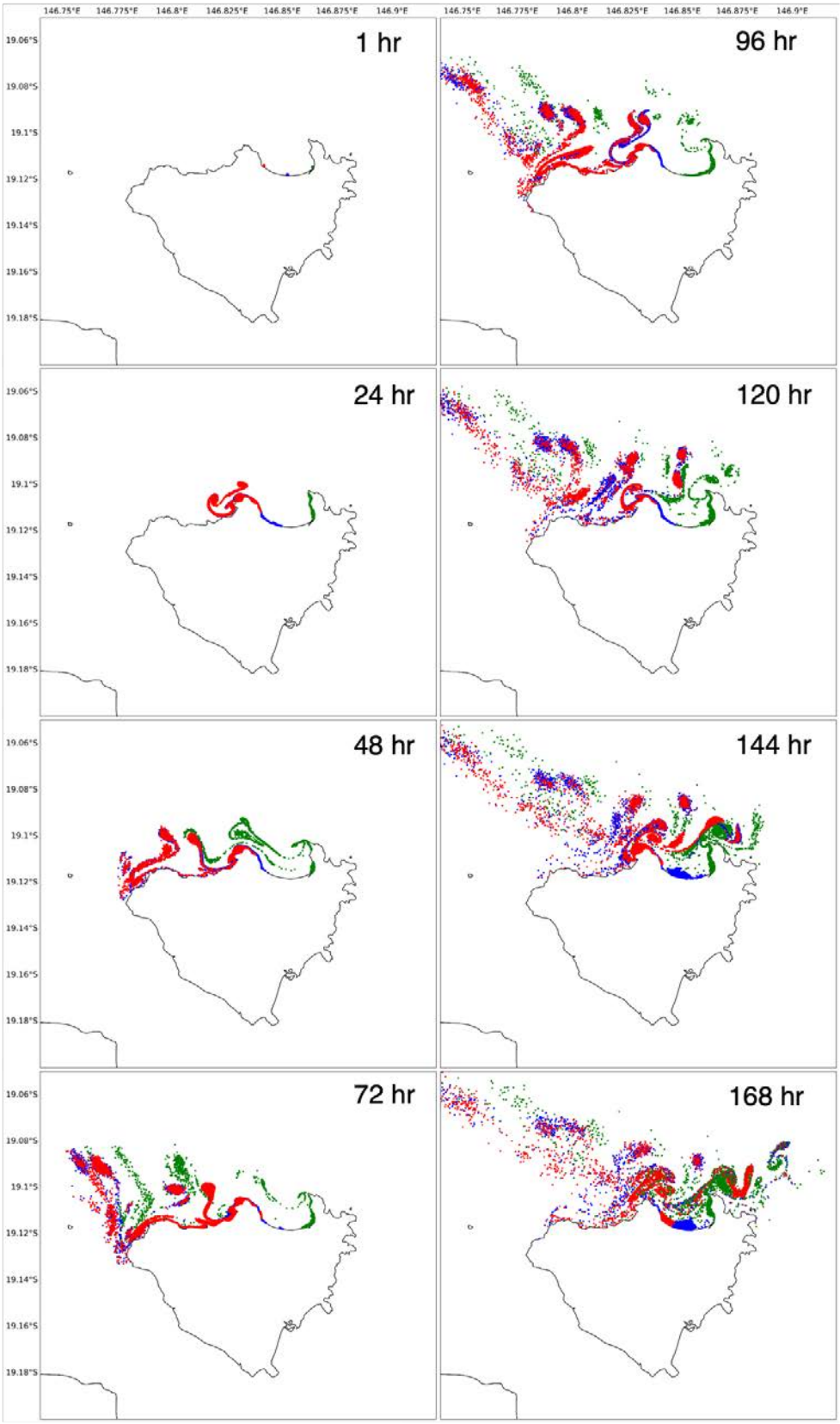

Figure S4.16. Maps of Magnetic Island displaying eDNA particle dispersion at 1 h, 24 h, 48 h, 72 h, 96 h, 120 h, 144 h, and 168 h intervals under scenario C with releases occurring every 10 hours. Red particles were seeded from location A, blue from location B, and green from location C.

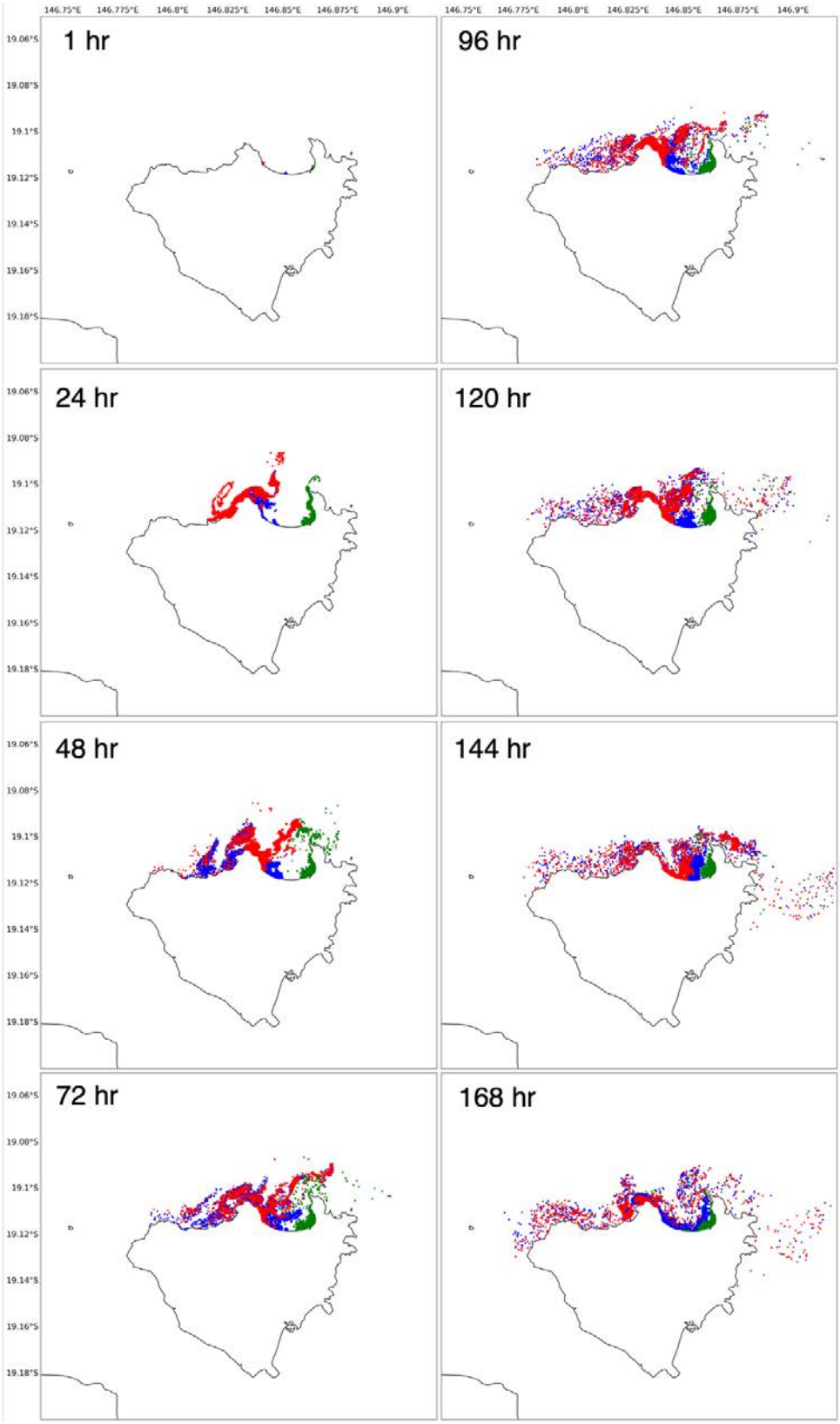

Figure S4.17. Maps of Magnetic Island displaying eDNA particle dispersion at 1 h, 24 h, 48 h, 72 h, 96 h, 120 h, 144 h, and 168 h intervals under scenario D with releases occurring every 10 hours. Red particles were seeded from location A, blue from location B, and green from location C.

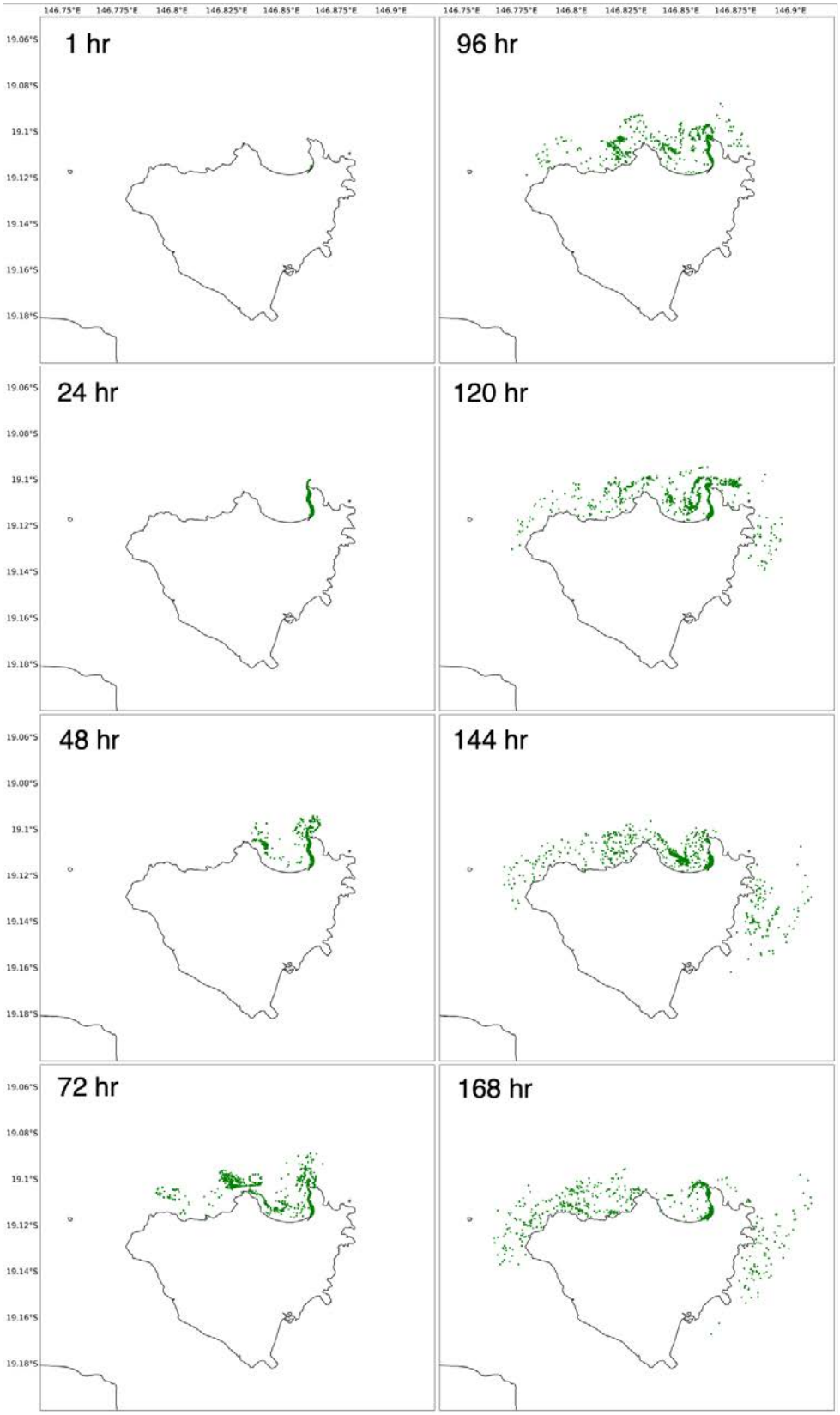

Figure S4.18. Maps of Magnetic Island displaying eDNA particle dispersion at 1 h, 24 h, 48 h, 72 h, 96 h, 120 h, 144 h, and 168 h intervals under scenario E with releases occurring every 10 hours. Red particles were seeded from location A, blue from location B, and green from location C.

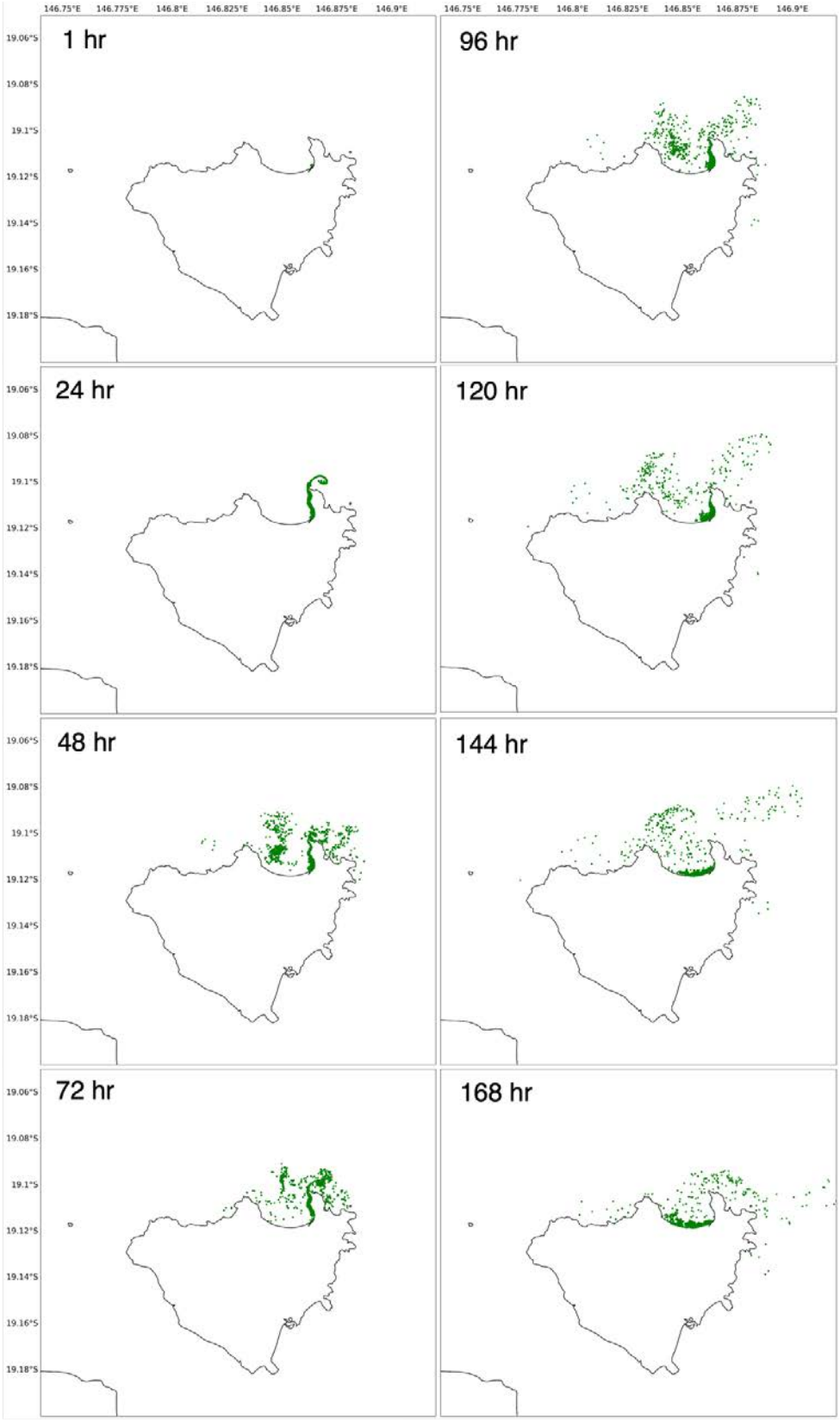

Figure S4.19. Maps of Magnetic Island displaying eDNA particle dispersion at 1 h, 24 h, 48 h, 72 h, 96 h, 120 h, 144 h, and 168 h intervals under scenario F with releases occurring every 10 hours. Red particles were seeded from location A, blue from location B, and green from location C.

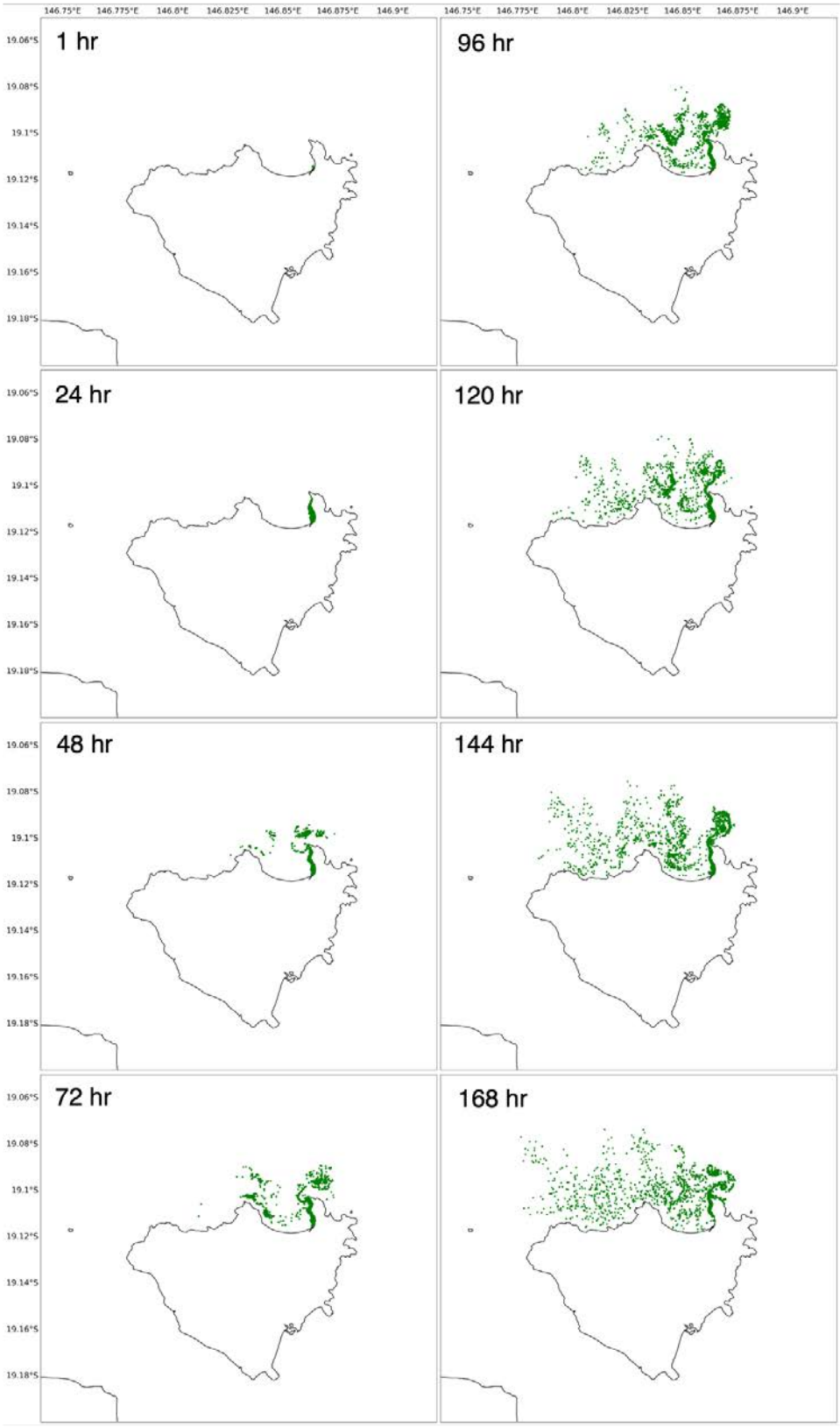

Figure S4.20. Maps of Magnetic Island displaying eDNA particle dispersion at 1 h, 24 h, 48 h, 72 h, 96 h, 120 h, 144 h, and 168 h intervals under scenario G with releases occurring every 10 hours. Red particles were seeded from location A, blue from location B, and green from location C.

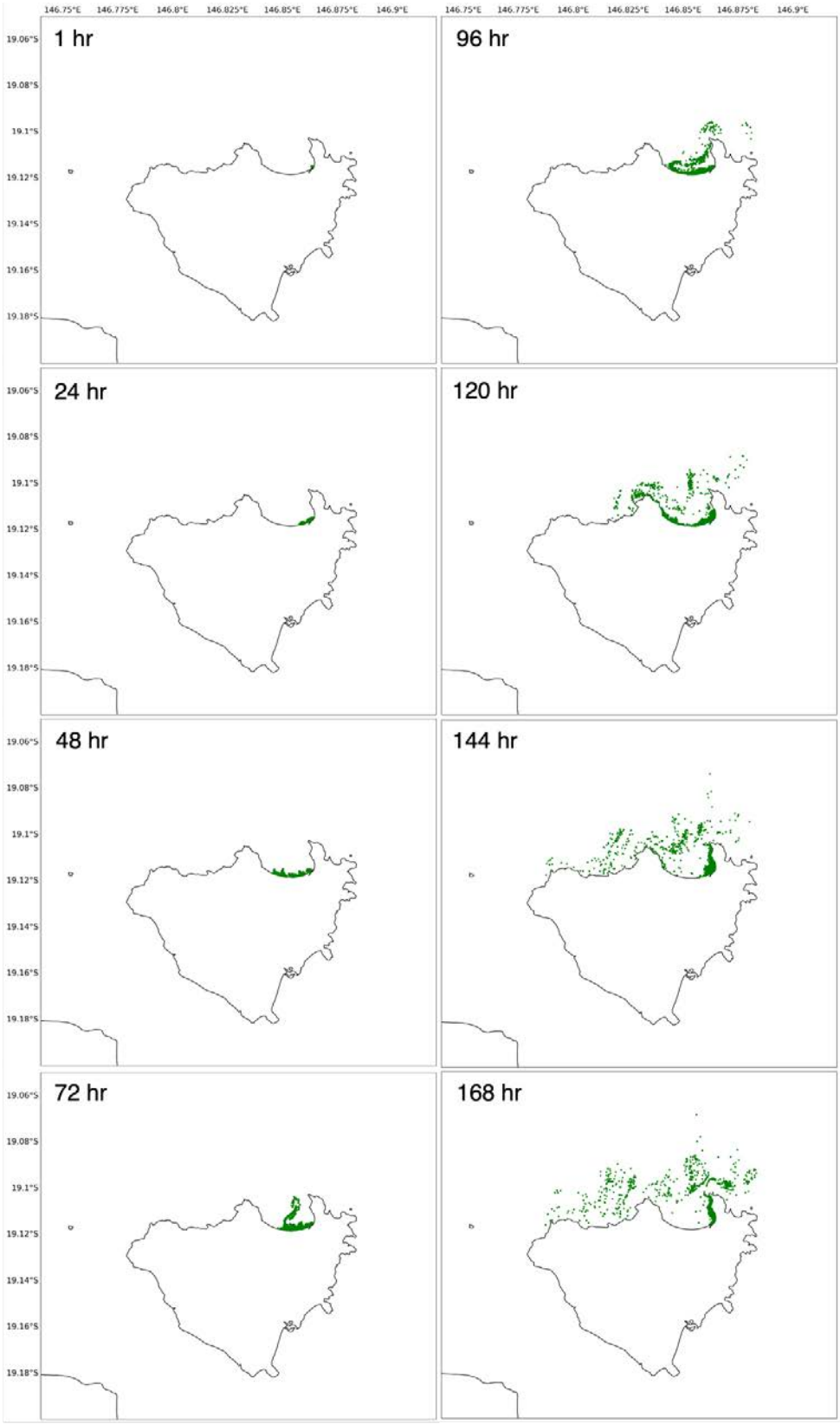

Figure S4.21. Maps of Magnetic Island displaying eDNA particle dispersion at 1 h, 24 h, 48 h, 72 h, 96 h, 120 h, 144 h, and 168 h intervals under scenario H with releases occurring every 10 hours. Red particles were seeded from location A, blue from location B, and green from location C.

Animation S4.13. Hourly eDNA particle dispersion under scenario A with releases occurring every 10 hours. Red particles were seeded from location A, blue from location B, and green from location C.

Animation S4.14. Hourly eDNA particle dispersion under scenario B with releases occurring every 10 hours. Red particles were seeded from location A, blue from location B, and green from location C.

Animation S4.15. Hourly eDNA particle dispersion under scenario C with releases occurring every 10 hours. Red particles were seeded from location A, blue from location B, and green from location C.

Animation S4.16. Hourly eDNA particle dispersion under scenario D with releases occurring every 10 hours. Red particles were seeded from location A, blue from location B, and green from location C.

Animation S4.17. Hourly eDNA particle dispersion under scenario E with releases occurring every 10 hours. Red particles were seeded from location A, blue from location B, and green from location C.

Animation S4.18. Hourly eDNA particle dispersion under scenario F with releases occurring every 10 hours. Red particles were seeded from location A, blue from location B, and green from location C.

Animation S4.19. Hourly eDNA particle dispersion under scenario G with releases occurring every 10 hours. Red particles were seeded from location A, blue from location B, and green from location C.

Animation S4.20. Hourly eDNA particle dispersion under scenario H with releases occurring every 10 hours. Red particles were seeded from location A, blue from location B, and green from location C.

Animations are available on Open Science Framework (DOI 10.17605/OSF.IO/B5ECS)

IV.4 Wind and tidal influence on eDNA transport

Table S4.3. Results of Mann-Whitney U test comparing ebb and flood tides for each scenario at each release location. * denotes $p < 0.05$, ** denotes $p < 0.005$, *** denotes $p < 0.0005$.

Scenario	Location	Ebb average	Flood average	U	p-value
A	A	1954.67	973.05	164	0.00817 *
	B	1842.59	809.89	163	0.00052 **
	C	1691.42	886.65	143	0.01245 *
B	A	2738.39	2049.43	123	0.12637
	B	2704.87	2002.13	122	0.13886
	C	2257.97	1737.55	118	0.19846
C	A	2206.82	1342.05	144	0.01084 *
	B	2125.29	1327.42	140	0.01859 *
	C	2356.26	1477.01	136	0.03081 *
D	A	1677.37	1387.98	112	0.31984
	B	1151.87	910.04	119	0.18205
	C	223.71	291.99	65	0.21593
E	C	1002.06	654.81	2755	0.03472 *
F	C	191.63	363.68	1201	0.0000023 ***
G	C	1281.04	1050.59	2372	0.67086
H	C	825.56	728.66	2766	0.18855

IV.5 Empirical *Chironex fleckeri* eDNA sampling time and conditions

Table S4.4. Tidal and wind conditions for empirical sampling times.

Australian stinger season	Time	Tidal stage	Wind speed range (km h^{-1})	Wind direction
Outside	July 2020	Spring	10 – 20	SE
Outside	July 2022	Between neap and spring	10 – 20	E/SE
During	December 2020	Spring	10 – 20	N
During	February 2021	Spring	15 – 30	E/NE
During	March 2021	Spring	10 – 20	E/SE
During	December 2021	Spring	15 – 30	SE

Appendix V

Population Structures and Levels of Connectivity for Scyphozoan and Cubozoan Jellyfish

Abstract

Understanding the hierarchy of populations from the scale of metapopulations to mesopopulations and member local populations is fundamental to understanding the population dynamics of any species. Jellyfish by definition are planktonic, and it would be assumed that connectivity would be high among local populations and that populations would vary little in both ecological and genetic clade-level differences over broad spatial scales (i.e., hundreds to thousands of km). Although data exists on the connectivity of scyphozoan jellyfish, there are few data on cubozoans. Cubozoans are capable swimmers and have more complex and sophisticated visual abilities than scyphozoans. We predict, therefore, that cubozoans have the potential to have finer spatial scale differences in population structure than their relatives the scyphozoans. Here we review the data available on the population structures of scyphozoans and what is known about cubozoans. The evidence from realized connectivity and estimates of potential connectivity for scyphozoans indicates the following. Some jellyfish taxa have a large metapopulation and very large stocks (> 1000s of km), while others have clade level differences on scales of tens of kilometers. Data on distributions, genetics of medusa and polyps, statolith shape, elemental chemistry of statoliths and biophysical modelling of connectivity suggest that some of the ~ 50 species of cubozoans have populations of surprisingly small spatial scales and low levels of connectivity. Despite their classification as plankton, therefore, some scyphozoans and cubozoans have stocks of small spatial scales. Causal factors that influence population structure in many taxa include, the distribution of polyps, behaviour of medusa, local geomorphology, and hydrodynamics. Finally, the resolution of patterns of connectivity and population structures will be greatest when multiple methods are used.

Introduction

Jellyfish are ecologically important as predators (Moller, 1984), prey (Strand and Mamner, 1988, Brodeur and Merati, 1993, Arai, 1997) and structures in the pelagic environment (Doyle et al., 2014, Kingsford, 1993). The abundance of jellyfish can vary greatly in space and time, and increases in abundance can have detrimental effects on ecosystems and human livelihoods. For example, predation on small planktonic food and specific groups (e.g., fish larvae) by jellyfish can result in important changes in marine food chains. These changes can in turn result in phase shifts that fundamentally alter trophic pathways, where in some cases baitfish maybe largely replaced by jellyfish (Lynam et al., 2006). Jellyfish blooms can also affect human infrastructure (Canepa et al., 2014) such as aquaculture facilities, power plants that utilise seawater and nets used to catch boney fishes (Uye, 2014). Jellyfish have also been introduced in ballast water so affecting native assemblages (Graham et al., 2003). Further, jellyfish are venomous and when humans are stung the affects can range from nuisance level irritations, to severe responses and even death, consequently, the presence of jellyfish affects tourist industries at multiple latitudes globally (Kingsford et al., 2018). In contrast, jellyfish are also an exploitable resource. They are targeted by fisheries (Kingsford et al., 2000) and annual reported catches are about 0.9 Mt (Brotz, 2016). The magnitude of species influences on ecosystem functions and human activities is inherently linked to their population dynamics, and this is particularly true for particular jellyfish species which have boom and bust abundance cycles. Accordingly, there are multiple reasons knowledge is required on the sources, sinks, connectivity and related population structure of jellyfish.

There are about 200 morpho-species of ‘true jellyfish’ (Class Scyphozoa (Daly et al., 2007)), with true (genetic) diversity likely being double (Dawson, 2004). In contrast only ~50 species of box jellyfish (Class Cubozoa) have been described (Collins and Jarms, 2018). Our knowledge of biology and population ecology by Class, based on number of publications, is as asymmetrical as species richness (Kingsford and Mooney, 2014). Accordingly, many general paradigms on jellyfish are based on scyphozoans. For this reason, we have combined the knowledge available on population structures of both classes of jellyfish to assess their commonality and differences.

Population structures have generally been described according to three levels. Metapopulations, mesopopulations and local populations (Waples and Gaggiotti, 2006). Metapopulations are made up of a number of mesopopulations, or in a fisheries context these are coined ‘stocks’. A metapopulation often corresponds with the

biogeographic range of a species. For example, the biogeographic range of the three described species of *Chironex* are likely to be composed of multiple stocks (Figure S5.1). In some cases, there may be anti-tropical distributions of a species where metapopulations are found in both hemispheres, but are quite separate (Hutchings and Kingsford, 2019). This scenario is likely for widespread taxa of jellyfish such as *Carybdea rastoni* and *Copula sivickisi* found in northern and southern hemispheres and multiple seas (Kingsford and Mooney, 2014). Separate metapopulations, or in some cases more than one, likely correspond to the Large Marine Ecosystems (LME) coined by Abboud et al. (2018).

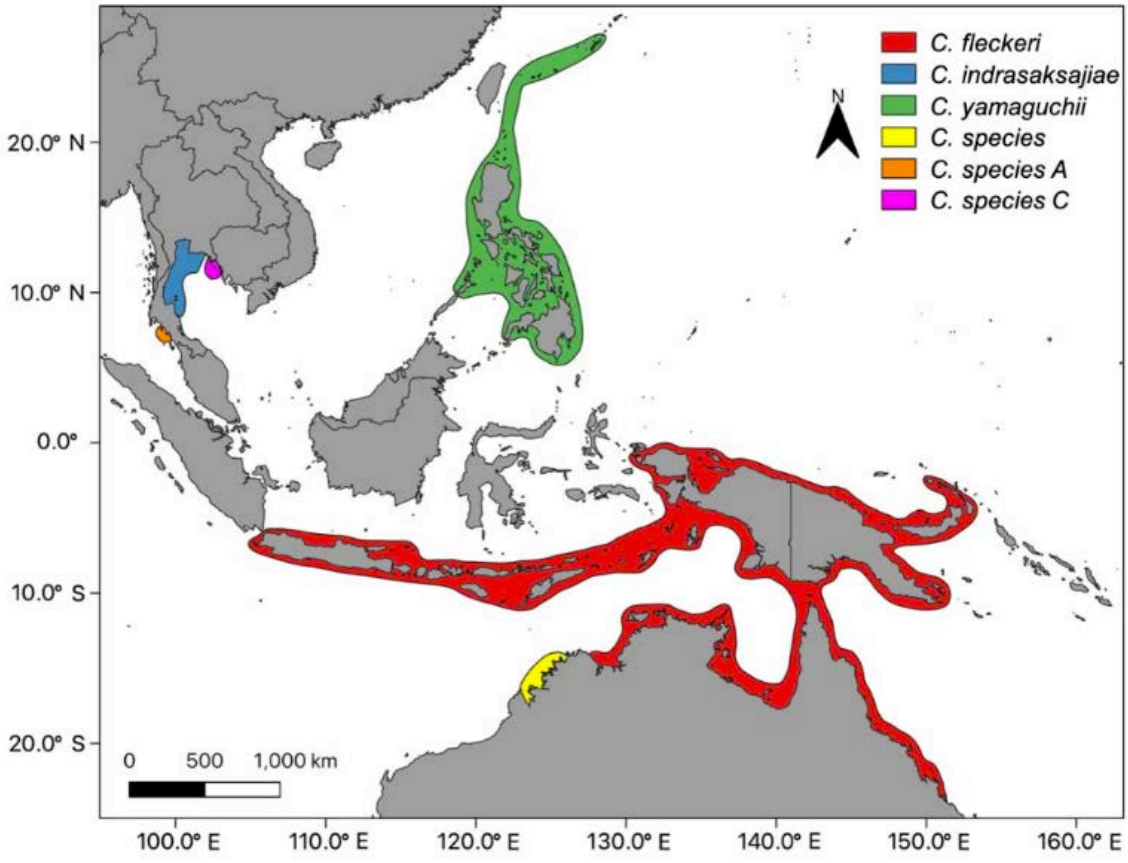

Figure S5.1. The geographic range of all known species of *Chironex*. *Chironex fleckeri* Southcott, 1956; *Chironex yamaguchii* (21); *Chironex indrasaksajiae* (22), and unnamed species in northern Australia (23) and Thailand (24); Taxonomy (25).

Population connectivity is the exchange of individuals among geographically separated population units (Pineda et al., 2007). Within metapopulations a robust biological stock should have little to no connectivity with other stocks. Accordingly, successful immigration and emigration, involving any life history stage, should be very low or zero (Begg et al., 1999). It is possible that an incipient stock is largely separate ecologically, but there is sufficient genetic exchange that they do not constitute separate clades. Where separation has been sufficient enough to result in significant clade-level genetic differences then there is the potential for incipient speciation. Within a stock, local populations can be identified that may be limited to a small geographic area such as a bay, estuary or island. Further, ecological interactions are likely among individuals from different local populations through the movement of adults or early life history stages, genetic connectivity therefore would be assumed. It is also possible that local populations with little or zero connectivity could contribute to a mosaic of genetic diversity within a stock and under the right circumstances could become a separate stock.

Plankton largely drifts or wanders by definition (McManus and Woodson, 2012). Jellyfish have historically been classified as plankton. Accordingly, it would be assumed that connectivity would be high among local jellyfish populations and that populations would vary little in both ecological and genetic clade-level differences over broad spatial scales (i.e., hundreds to thousands of km). However, findings on the behaviour and swim speeds of jellyfish suggest it may be more appropriate to classify them as actively swimming nekton. Nekton can swim to either maximize dispersal or minimize it by remaining close to natal areas and these strategies can spatially broaden or restrict species population structures, respectively.

Scyphozoans and cubozoans have a number of attributes that assist in restricting the dispersal of medusa from localized areas. Both classes are active swimmers. Scyphozoans cover a large size range, from just a couple of centimeters (e.g., *Linuche unguiculata,* the thimble jellyfish) to the lion's mane jellyfish (*Cyanea capillata*) which can grow to over 2 m bell diameter. Jellyfish in general are highly efficient swimmers (Gemmell et al., 2018) and small scyphozoans can reach surprising speeds. For example, Larson (1992) recorded *Linuche unguiculata* which ranged in size from 0.5 to 2.2 cm bell diameter swimming at average speeds ranging from 3.5 to 7.2 cm s-1. The larger scyphozoan taxa are even more mobile. *Stomolophus meleagris* which can grow to 25 cm bell diameter have been recorded swimming at speeds of 15 cm s-1 (Shanks and Graham, 1987).

Although cubozoans are generally small they are generally highly mobile. The fifty known species of cubozoans range in size from those with a bell diameter of a few cm (e.g., *Copula sivickisi*) to those of about 20-25 cm (e.g., *Chironex fleckeri*). All of the species that have been observed swim well. For example, relatively small *Copula sivickisi* (size range: 0.4 to 1.1 cm Inter Pedalial Distance) have been recorded swimming at maximum speeds of 12 cm s^{-1} in swim trials (Schlaefer et al., 2020). While the larger *Chironex fleckeri (*size range: 4 to 12 cm Inter Pedalial Distance) have been measured to swim at speeds of up to 16.6 cm s-1 in the field (Schlaefer et al., 2018).

Scyphozoans and cubozoans have excellent sensory abilities. The sensory systems of both classes include ocelli, specialized structures to sense light; however, cubozoans also possess image forming eyes similar in structure to the eyes of vertebrates and cephalopods (Nilsson et al., 2005). Jellyfish can migrate vertically and have some ability to move and orientate horizontally (scyphozoans, *Aurelia*, *Mastigias*, *Stomolophus*, *Pelagia* (Canepa et al., 2014, Hamner and Hauri, 1981, Hamner et al., 1994, Shanks and Graham, 1987)). More complex behaviours include responding to objects, conspecifics, currents, diel cycles, shadow and light, bioluminescent plankton, habitat type and small-scale geography (Table 1). Some cubozoans have even been observed to rest (*C. rastoni,* (Matsumoto, 1995)) or attach to the substratum (*Copula sivickisi* (Hartwick, 1991b)). Accordingly, predictions for population structures of jellyfish based on passive dispersal are likely to be highly inaccurate.

Table S5.1. Behaviours involving interactions with environmental factors documented in medusae from different scyphozoan and cubozoan species. The methods used to determine the behaviours are indicated. Mating behaviour is not included.

Behaviour	Species (Order)	Method	Study
Scyphozoa			
Sun-compass migration	*Aurelia aurita* (Semaeostomeae)	Field observation	Hamner et al. (1994)
	Mastigias (Rhizostomae)	Field observation	Hamner and Hauri (1981)
Wind and currents and bottom avoidance	*Stomolophus meleagris* (Rhizostomae)	Field observation	Shanks and Graham (1987)
Cubozoa			
Obstacle avoidance	*Carybdea rastonii* (Carybdeida)	Field observations (SCUBA) and laboratory experiments	Matsumoto (1995)
	Chironex fleckeri (Chirodropida)	Laboratory experiments	Hamner et al. (1995)
		Field observations	Schlaefer et al. (2018)
	Chiropsella bronzie (Chirodropida)	Laboratory experiments	Garm et al. (2007)
	Tripedalia cystophora (Carybdeida)	Laboratory experiments	Garm et al. (2007)
Maintain positions near the shore	*C. rastonii*	Field observations (SCUBA)	Matsumoto (1995)
	C. fleckeri	Opportunistic sampling (e.g., Surf Life Saver plankton tows and verbal records)	Kingsford et al., (2012)
		Electronic tagging	Gordon and Seymour (2009)
Rheotaxis		Field observations and biophysical modelling	Schlaefer et al. (2018)
	C. fleckeri	Field observations	Schlaefer et al. (2018)
	C. bronzie	Laboratory experiments	Garm et al. (2007)
	T. cystophora	Laboratory experiments	Garm et al. (2007)
	Copula sivickisi (Carybdeida)	Laboratory experiments	Buskey (2003)
		Laboratory experiments	Schlaefer et al. (2020)

Table S5.1. Continued.

Diel activity – nocturnal	*C. sivickisi*	Field observations (SCUBA)	Hartwick (1999b)
		Field sampling (plankton tows and SCUBA) and laboratory experiments	Garm et al., (2007), Schlaefer et al. (2020)
		Laboratory experiments	Schlaefer et al. (2020)
Attach to substrate	*C. sivickisi*	Laboratory observations	Hartwick (1999b)
		Laboratory Experiments	Garm et al., (2007), Schlaefer et al. (2020)
		Laboratory experiments and in-field video recording	Schlaefer et al. (2020), Hamner and Hauri (1981)
Rest on the bottom	*C. rastoni*	Field observations	Matsumoto (1995)
Swim toward bioluminescent plankton	*C. sivickisi*	Laboratory experiments	Garm et al., (2007), Schlaefer et al. (2020)
Exhibit habitat preference	*C. sivickisi*	Laboratory experiments	Garm et al., (2007), Schlaefer et al. (2020)
		Laboratory experiments	Schlaefer et al. (2020)
Diel activity - diurnal	*T. cystophora*	Field sampling (snorkel and SCUBA) and laboratory experiments	Garm et al., (2007), Schlaefer et al. (2020)
Maintain positions in light shafts	*T. cystophora*	Field observations (snorkel)	Stewart (1996)
		Laboratory experiments	Buskey (2003)
		Laboratory experiments and in-field video recordings	Garm and Bielecki (2008)
Orient via the mangrove canopy	*T. cystophora*	Eye orientation measurements, optical modelling and in-field experiments	Garm et al. (2011)

Further, the different life histories of scyphozoans and cubozoans could have great ramifications for species population structures. Jellyfish, including those in the classes Scyphozoa and Cubozoa, generally have bipartite lifecycles, the visible medusae which have been discussed produce free swimming larvae which settle and develop into sessile polyps. Scyphozoan polyps successively strobilate larval medusae called ephyrae, which are poor swimmers. In contrast, cubozoan polyps typically metamorphose into juvenile medusae that are nearly fully formed and have some swimming ability. The poor swimming ephyrae of scyphozoans, therefore, have greater potential to disperse from source locations compared to juvenile cubozoans. Notably, some scyphozoan jellyfishes lack a polyp phase and are holoplanktonic (e.g., *Pelagia noctiluca* and *Periphylla spp.*). Their entire lifecycle is completed in the plankton, where their planula larvae develop into ephyrae which develop into medusae. The lack of a polyp phase could also have a great effect on population structures. Indeed, the polypoid and podocyst phases of scyphozoans and cubozoans (Arai, 1997, Kingsford and Mooney, 2014) provides an additional mechanism to reduce dispersal and related emigration from a population unit. Where taxa already have a restricted distribution, as medusa, the benthic stages are likely to contribute to that pattern. The potential, therefore, for complex and highly differentiated population structures is great especially where the local geomorphology is convoluted and could facilitate retention; such as estuaries, bays and fjords.

Multiple methods have been used to determine levels of connectivity and to differentiate populations of marine organisms at different spatial scales, some of the methods demonstrate the potential for connectivity while for others it is realized connectivity (*sensu* (Olsen and McPherson, 1987)) as follows. Realized connectivity can be demonstrated though population genetics (Gerlach et al., 2016), tagging (Brill et al., 1999) and intergenerational tags (Almany et al., 2007), while methods that demonstrate the potential for connectivity include: morphometrics of body shape and body parts (e.g. (Haddon and Willis, 1995, Small, 1996)), elemental chemistry (Fowler et al., 2005) and biophysical modelling where predictions are made on physical oceanography and mobility of different life history stages (Wolanski and Kingsford, 2014). Where the information is spatially comprehensive in time and space, known patterns of distribution can also contribute to predictions on the potential for population connectivity (Pitt and Kingsford, 2000). Of course, the most compelling cases for the determination of stock structure comes from the use of multiple techniques and related corroborative data (Dawson, 2005a, Dawson, 2005b).

The objective of this study was to review the evidence for levels of connectivity and related differences in population structure for the Scyphozoa and Cubozoa. The specific aims of this review were to provide examples of: (1) metapopulations and spatially disjunct ecological patterns with evidence for allopatry; (2) finer scale stock and local population patterns, as informed by genetics data, statolith shape, elemental chemistry and biophysical modelling and; (3) the contribution of polyps to restricting distributions and dispersal. We conclude our synthesis by identifying multiple scyphozoan and cubozoan species that have stocks on scales of kilometers to tens of kilometers which has strong inferences for the nature of speciation.

Evidence from Scyphozoan and Cubozoan Populations

Metapopulations and Spatially Distinct Ecological Patterns

The biogeographic ranges of many jellyfish taxa have been determined from collections and in some cases from more detailed ecological studies. The data for some scyphozoans align with the traditional belief that the structure of zooplankton populations should be well mixed over broad spatial scales. For example, the scyphozoan *Aurelia aurita* is a cosmopolitan species that is found in the Pacific, Atlantic and the Red Sea (WoRMS, 2021). Although mostly found in the northern hemisphere, it is also found in the southern hemisphere around New Zealand. For this species, therefore, it would be expected that the range is made up of multiple metapopulations, most likely corresponding to parts of ocean basins. Within these metapopulations there is evidence of small-scale potential stocks (Table S5.2). The cubozoan, *Copula sivickisi,* also has a very broad distribution, where it is found over most of the Indo-east Pacific, has been recorded in the Indian and Atlantic Oceans and even extends to temperate areas of Japan and New Zealand (WoRMS, 2021).

Table S5.2. Examples of the spatial scales of populations of jellyfish by class. Methods used to detect differences among populations: museum collections and distribution patterns, genetics; elemental chemistry (LAICP-MS), ecological data on the timing of reproduction, recruitment, and movements; statolith morphology and biophysical modelling. Spatial scale is the scale at which differences were detected according to the method. Range is the maximum linear distance between detections, calculated as the distance along a coastline where applicable. WoRMS (WoRMS, 2021) has been used as a major source for the biogeography (range) of species. Evidence based on * realized connectivity. # potential for connectivity.

Species	Class	Methods	Spatial Scale	Range (Km)	Source
Aurelia aurita	Scyphozoa	Genetics *	100s of kms	>1000	Ben Faleh et al. (2009)
Aurelia spp.	Scyphozoa	Genetics *	10s to hundreds of kms	>1000	Dong et al. (2015)
Lychnorhiza lucerna	Scyphozoa	Genetics *	1000s of kms	>1000	De Angelis et al. (2017)
Catostylus mosaicus	Scyphozoa	Ecological #	10s of kms	>1000	Pitt and Kingsford (2000)
Catostylus mosaicus	Scyphozoa	Genetics *	10s of kms	>1000	Dawson (2005a)
Rhizostoma octopus	Scyphozoa	Genetics *	100s of kms	>1000	Lee et al. (2013)
Rhizostoma octopus	Scyphozoa	Genetics *	100s of kms	>1000	Glynn et al. (2015)
Pelagia spp.	Scyphozoa	Genetics *	1000s of kms	>1000	Miller et al. (2012)
Pelagia spp.	Scyphozoa	Genetics *	1000s of kms	>1000	Stopar et al. (2010)
Mastigias papua	Scyphozoa	Genetics *	10s of kms	>1000	Dawson and Hamner (2003)
Stomolophus spps.	Scyphozoa	Genetics *	100s of kms	>1000	Mamet et al. (2019)
Chrysaora melanaster	Scyphozoa	Genetics *	1000s of kms	>1000	Dawson et al. (2015)
Cyanea spp.	Scyphozoa	Genetics *	100s of kms	>1000	Abboud et al. (2018)
Alatina alata	Cubozoa	Genetics *	1000s of kms	>1000	Lawley et al. (2016)
Chironex fleckeri	Cubozoa	Biophysical #	Hundreds of meters to kms	>1000	Schlaefer et al. (2018)
Chironex fleckeri	Cubozoa	Statolith morpho. #	10s to hundreds of kms	>1000	Mooney and Kingsford (2017)

Table S5.2. Continued.

Chironex fleckeri	Cubozoa	Elemental chemistry [#]	Kms to 10s of kms	>1000	Mooney and Kingsford (2016)
Copula sivickisi	Cubozoa	Statolith morpho. [#]	10s to hundreds of kms	>1000	Mooney and Kingsford (2017)

Many taxa are not found in multiple oceans/seas, but their biogeographic ranges extend for over 1000 km (Table S5.2). For example, the four known taxa of *Chironex* are spatially discrete in tropical latitudes of the Indo-Pacific (Figure S5.3). *Chironex fleckeri* is only found along the east and northern coastlines of Australia, Papua New Guinea, and large parts of Indonesia. *Chironex yamaguchii* also has a broad range from tropical islands of Japan to the Philippines. Although *Chironex indrasaksajiae* and an unnamed species from Western Australia appear to have very restricted distributions, this may in part be due to their recent discovery and description. Further, a study using PCR primers has detected another two incipient species in the waters of Thailand (Nuankanya et al., 2021).

There are other examples of species where they are well known to have a very restricted distribution and therefore a small metapopulation. In an extreme case, incipient species of *Mastigias papua* have been identified in isolated marine lakes in Palau and the lakes are only separated by tens of kilometers (Dawson and Hamner, 2003, Dawson and Hamner, 2005). The chirodopid *Chiropsella bronzie* is recorded from the east coast of tropical Australia (Kingsford and Mooney, 2014), but is only found at mainland locations between Mission Beach (17°57.088 E, 146°05.799 S) and Cooktown (15°2.035, 145°15.051; J. Seymour pers. com.). The carybdeid, *Carybdea arborifera,* is only recorded in Hawaii (Kingsford and Mooney, 2014). Although some taxa such as *Aurelia aurita*, *Catostylus mosaicus*, *Chironex fleckeri* and *Copula sivickisi* have broad distributions, there is an increasing body of evidence for small stocks and local populations that are likely to facilitate incipient speciation (Table S5.2).

For example, the rhizostome *Catostylus mosaicus* is found in tropical to temperate latitudes around all of Australia, and this potentially forms one metapopulation. This species is generally found in estuaries, drowned river valleys, bays and coastal lakes. Detailed studies on the ecology of this species on the East coast of Australia have demonstrated that the species is highly mobile and is capable of maintaining position in coastal waters. Furthermore, patterns of recruitment and reproduction vary among locations that are only separated by tens to hundreds of kilometers (Pitt and Kingsford, 2000).

An added complexity to understanding patterns of distribution is introductions from shipping, be that in ballast water or via fouling. Perhaps one of the best documented cases is for *Phyllorhiza punctata*, which was historically only found in the western hemisphere, but was introduced to North America through the Panama Canal, and the same species has also been detected outside of its normal range in the Mediterranean (Galil et al., 1990). Although introductions are problematic, rapid progress in the sophistication of techniques in genetics will allow more rapid determination of source populations.

Population Genetics

Population genetics have been examined for scyphozoans, and to a lesser extent, cubozoans. This has allowed a fine-grain understanding of the presence of stocks within metapopulations. There is strong evidence for scyphozoans that at least some taxa have identifiable stocks at spatial scales of tens of kilometres to hundreds of kilometres (Table S5.2). However, even within the same genus, there are contrasting patterns. For example, Ben Faleh et al. (2009) concluded that there is high gene flow of *Aurelia* in the Mediterranean at scales of over 100 km. In contrast, there is evidence from China (Dong et al., 2015) that medium-scale genetic structuring of *Aurelia* is found at scales of hundreds of km.

The data on genetics for some scyphozoans align with the traditional belief that the structure of zooplankton populations should be well mixed over broad spatial scales. For example, the holoplanktonic semaeostome, *Pelagia,* has a life history with no scyphistoma stage, unlike many other scyphozoan taxa. *Pelagia noctiluca* was found to not have genetically or geographically distinct populations within the Mediterranean and East Atlantic despite two clades being identified (Stopar et al., 2010). Miller et al. (2012) also reported little variation among locations for *P. noctiluca* off Southern Africa. However, genetic differences were found to exist between populations in the northern and southern Atlantic, where at these spatial scales (thousands of kilometres) their status is likely to be at the level of metapopulations.

Rhizostoma octopus, which commonly occurs in large blooms in the north-eastern Atlantic Ocean, particularly within the Irish Sea, has “stocks.” Multiple studies have reported the species as having multiple distinct “stocks” at spatial scales of hundreds of kilometres (Lee et al., 2013, Glynn et al., 2015). Lee et al. (2013) reported three genetically distinct populations through examination of the mitochondrial gene cytochrome oxidase subunit 1 (*CO1*) and the nuclear calmodulin gene (*CaM*). The mitochondrial gene highlighted greater variation than that of the nuclear gene due to a

faster mutation rate (Brown et al., 1979), however both underlined the genetic variation between locations. Examination into the potential processes behind the identified genetic variation revealed physical oceanographic processes to be key. Locations with genetically distinct individuals were found to be relatively isolated by currents, hence explaining the genetic difference and supporting the separate “stocks,” while those locations with similarities were physically connected and so may allow migration/population connectivity between said locations. A comparable study by Glynn et al. (2015) reported similar findings, and both studies concluded that this jellyfish has genetically distinct, self-sustaining populations (stocks) at scales of as little as a few 100 kms.

Another rhizostome that has been found to contain a hierarchical population structure is *Catostylus mosaicus.* This jellyfish is characterized by local populations within a variety of coastal environments including estuaries, bays and coastal lakes. In line with ecological and behavioural evidence (Table S5.2), the species was found to have molecular variation at small spatial scales, ranging from tens of kilometres (Dawson, 2005a). Dawson (2005a, 2005b) examined the CO1 gene and internal transcribed spacer 1 (ITS1) of specimens collected along the east Australian coast and found there to be two distinct clades at spatial scales of hundreds of kilometres. Upon further examination of these clades, Dawson found there was significant genetic variation within and among collection sites within each clade, suggesting a finer-scale phylogeographic population structure. This genetic variation concurred with ecological differences reported by Pitt and Kingsford (2000), and thus populations within closely spaced individual estuaries and lakes should be considered as distinct, self-sustaining populations (Figure S5.2). These studies highlight the growing evidence of some scyphozoan taxa having distinct population units that inhabit relatively small, often geographically constrained areas.

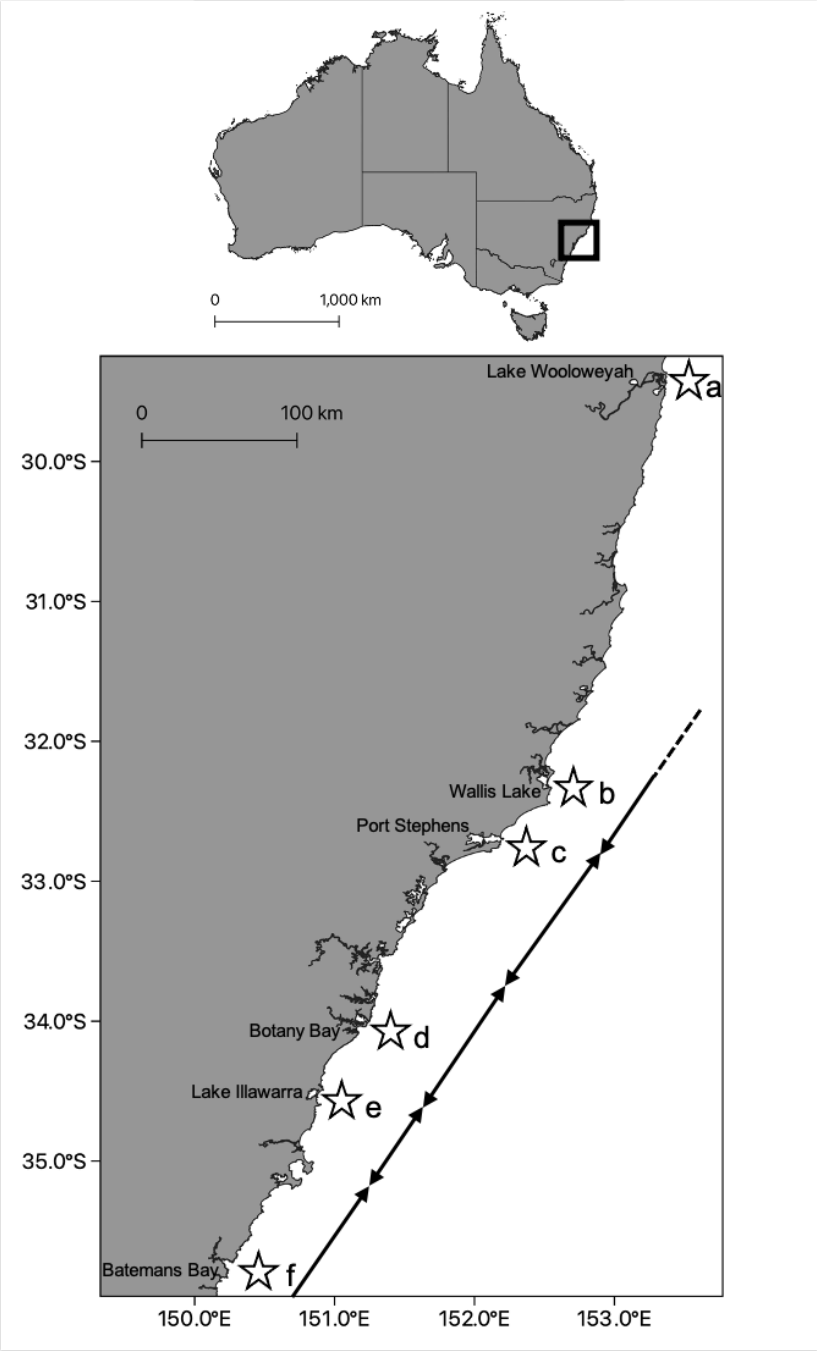

Figure S5.2. Incipient speciation of *Catostylus mosaicus* (Scyphozoa, Rhizostomeae, Catostylidae) (Dawson, 2005a). Populations of *C. mosaicus* were restricted to bays, coastal lakes and drowned river valley systems along the East coast of Australia in the state of New South Wales (Upper). (Lower) Ecological differences were found among sites (a–f) by Pitt and Kingsford (2000); these findings aligned with a high degree of genetic differentiation among sites (Dawson, 2005a). Where black arrows meet demarcates high genetic variation between sites. The dashed arrow indicates the direction of Dawson's next site in Southern Queensland, which differed from site a.

Abboud et al. (2018) focused on macromedusae of the hydrozoa and scyphozoa and asked two questions for what they considered Large Marine Ecosystems (LMEs); probably the equivalent of what we have termed metapopulations. (1) Do congeneric individuals found within the same LME constitute individual species? (2) Do congeneric individuals from different LMEs constitute different species? Overall, the scale of mismatch among LMEs and genetic structure was > 70%. LMEs did not match species boundaries for ~24% of comparisons. This may not be surprising given the spatial population structures of some taxa. However, over 19% of within LME comparisons detected cryptic species and 67% showed significant intraspecific phylogeographic structure.

Despite the obvious need, few studies have examined the genetics of cubozoan taxa. Of the references we could find, two were concerned with the development of microsatellites for *C. fleckeri* (Peplow et al., 2009) and *Carukia barnesi* (Coughlan et al., 2006) to facilitate the undertaking of studies on population genetics. The phylogeny of cubozoan species has also been investigated (Aungtonya et al., 2018, Lawley et al., 2016). The development of primers and probes allowed for the application of eDNA to detect cubozoans Bolte et al. (2021). Further, genetic sequencing used to develop species-specific PCR primers for *Chironex indrasaksajiae* revealed two incipient species in Thailand (Figure S5.1). Although there is a strong suggestion that these taxa may have small metapopulations, each species has only been detected at one or two sites (Nuankanya et al., 2021). This review highlights the need for population genetic studies to be undertaken on cubozoan taxa, as this would develop a more in-depth understanding of the ecology of these cryptic and elusive taxa, thereby assisting in the management of these potentially deadly organisms (Kingsford et al., 2018).

We predict that clade-level differences in the population structures of most cubozoans will be greater than that demonstrated for scyphozoans as they show greater complexity in behaviour, and they have greater sensory capabilities (Kingsford and Mooney, 2014). Furthermore, there is strong evidence of differences among stocks at small spatial scales (tens to hundreds of kilometres) from biophysical modelling, elemental chemistry and statolith morphometrics. However, there will be exceptions. For example, Lawley et al. (2016) suggested that populations of *Alatina alata* in the Pacific are not genetically distinct and must have connectivity over thousands of kilometres, or the less favoured argument of human vector-based introductions. It is also worthy to note that *Alatina alata* is generally found in oceanic waters rather than shallow coastal waters.

Contrasting genetic patterns within and among taxa are likely due to biological and behavioural attributes such as swimming ability and orientation, as well as coastal versus oceanic distributions and local differences in geography and physical oceanography. There are also potential methodological issues with the genetics methodology used. Techniques used to examine genetic variation within and between populations have varying sensitivities/resolutions. For example, with *Aurelia aurita,* allozyme electrophoresis was utilised by Ben Faleh et al. (2009) to investigate the genetic variation and population structure of the species. However, it is known that the technique has a low resolution as it only examines protein coding regions of DNA (Berta et al., 2015). In contrast, techniques such as microsatellites and mitochondrial markers (utilised by Dong et al. (2015)) have a higher sensitivity to reveal genetic variation, hence potentially resulting in the contrast of reported findings for *Aurelia*. Modern techniques will allow greater resolution of hierarchies of population structures and cryptic species Holland et al., 2004).

Morphometrics and Elemental Chemistry

Morphometrics of both soft tissue and carbonate statoliths have been used to differentiate populations and potential clades and to identify the likely connectivity. Differences in morphometrics and more recently elemental chemistry of the statoliths have been found among population units of jellyfish at scales that probably correspond to the separation of metapopulations (thousands of kilometers) to populations at stock or local population levels separated by hundreds of kilometers or less. For example, in addition to evidence from genetics, morphological differences in clades of the rhizostome *Catostylus mosaicus* have been found between locations separated by hundreds of kilometers on the East coast of Australia. The clades differed in color and the dimensions of their papillae, oral disk and bell depth. The differences were sufficiently great to be considered incipient species (Dawson, 2005a, Dawson, 2005b).

Bolton and Graham (2004) tested a hypothesis on the source of *Phyllorhiza punctata* that had invaded to the Gulf of Mexico by describing the morphology of specimens. They postulated that the invasion of the Gulf of Mexico by *P. punctata* represented a distribution shift from an invasive hub in the Caribbean. Further, that this could be detected through measurements of variation in morphological metrics that included the following: morphometrics of the bell, color and characteristics of different sections of anatomy, the shape of spots, and the presence of bumps and symbiotic zooxanthellae. Their model was that populations in the Gulf of Mexico and Caribbean

would be more morphological similar than specimens from more distant locations such as Australia and the East coast of the US. The model was rejected, as it turned out that populations in the Gulf of Mexico and Caribbean were morphologically dissimilar, implying separate invasions from distant sources such as Australia and the east Coast of North America. The sources, therefore, were separated by thousands of kilometers and likely represent separate metapopulations.

Elemental fingerprints of hard structures have been used to elucidate spatial patterns of fish stocks (Campana et al., 2000) and other taxa; recently the technique was applied to cubozoans. Mooney and Kingsford (2017) discovered that the morphometrics of the statoliths of cubozoans (hard structures made of a sulphate called “basanite” (Tiemann et al., 2006) that are components of medusae sensory systems) could be used to differentiate taxa. Furthermore, they also detected within-species variation. Discrimination among populations separated by hundreds of kilometers was detected for *Chironex fleckeri* and *Copula sivickisi.* At some locations, variation in shape was found at spatial scales of kilometres to tens of kilometres. For example, the elemental chemistry of *C. fleckeri* was different at Magnetic Island when compared to the mainland which was only ~20 km away (Mooney and Kingsford, 2016).

Biophysical Modelling

Advances in our knowledge of jellyfish behavior and improvements in the sophistication of oceanographic models has allowed biophysical models of jellyfish dispersion to be developed. The models can be used to estimate dispersal from polyp beds (Barz et al., 2006, Chen et al., 2014), potential connectivity between local populations and make predictions on likely stock boundaries (Schlaefer et al., 2018, Schlaefer et al., 2020). For example, in a biophysical modelling study, Fossette et al. (2015) programmed medusae of the scyphozoan *Rhizostoma octopus* to swim counter-current, and demonstrated that this behavior was integral to the maintenance of local blooms covering tens of kilometers over temporal scales of several months. Schlaefer et al. (2018) studied the behavior of the large cubozoan *Chironex fleckeri* in a semi-enclosed Bay in northern Australia (Port Musgrave, Figure S5.3). An oceanographic model that did not include jellyfish behavior indicated there was a high probability that jellyfish could remain inside the bay even if they behaved as passive particles. When active swimming, obstacle avoidance and directional behavior were included in the model, it demonstrated that not only was emigration out of the bay and to other bays unlikely, but that local populations within the bay were surprisingly robust (Figure S5.3).

Copula sivickisi is a small cubozoan that frequents tropical reefs (Kingsford et al., 2012). Schlaefer et al. (2020) focused on this species at the inner shelf location, Magnetic Island, on the Great Barrier Reef. The species is an active swimmer and is unique in that it can attach to the substratum, especially during the day. It prefers shallow algal beds of *Sargassum* and the abundance of medusae is highest in <5 m of water. Biophysical modelling determined that a high percentage of medusae were likely to stay within 2 km of the bay, in which they were released as virtual medusae. It was concluded that copulating medusae likely self-seed local populations (hundreds of meters to kilometers wide) and that connectivity with adjacent local populations was surprisingly low. Further, the chances of emigration from Magnetic Island (45 km in circumference) to other locations with suitable habitats was low and the island, therefore, likely corresponds to a stock unit.

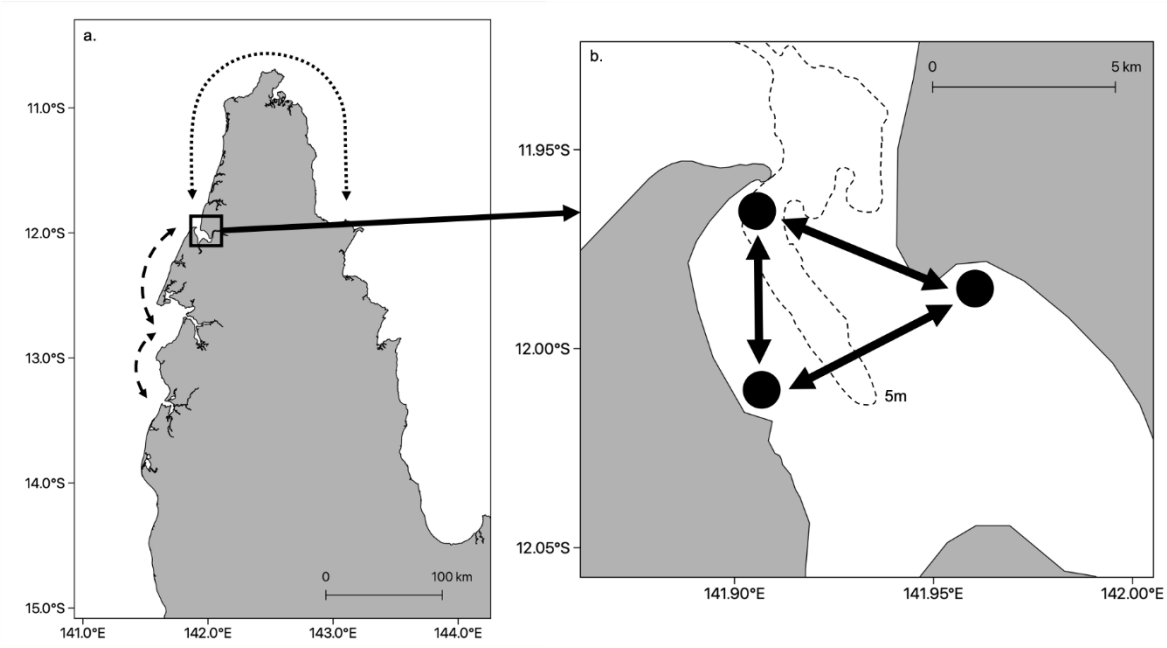

Figure S5.3. Level of connectivity among incipient stocks of *Chironex fleckeri* (Schlaefer et al., 2018). (a) Cape York in northern Australia showing low-to-zero connectivity between bays and estuaries at scales of tens of kilometres on the west coast (dashed line) and a potential rare event to the east coast (dotted line). The latter was only possible less than 5–7K years BP as land stretched from Cape York to Papua New Guinea during the last ice age (Hopley et al., 2007). (b) Local populations of *C. fleckeri* in Port Musgrave. Arrows indicate potential connectivity among local populations based on the current, but behavioural studies suggest this would be low. The dashed line indicates the 5 m isopleth.

The Contribution of Polyps to Restricting Distributions

Polyps are a key component of the life history of scyphozoan and cubozoan jellyfish and are the source of medusae (Arai, 1997, Kingsford and Mooney, 2014). We hypothesize, therefore, that the polypoid stages are important for a greater understanding of the point sources of dispersal, connectivity and the likelihood of retention in population. Given the abilities of polyps to prevail for long periods of time, generally longer than the medusoid phase, and their abilities to reproduce asexually, polyp beds can be a spatially robust source of medusae. Some field studies have examined the role of polyps in determining the abundance of medusa and, therefore, their role in the population dynamics of scyphozoan populations (e.g., (Shahrestani and Bi, 2018)). Distributional studies of polyps have also provided some evidence for insular populations, while others have not. For example, Marques et al. (2015) mapped the distribution and habitat preferences of *Aurelia* spp. polyps in the mostly enclosed Thau lagoon, north-western Mediterranean Sea (France). The polyps were primarily found on artificial structures which “raises the possibility of the potential isolation of this population from the other populations of the Mediterranean Sea.” An estuarine-bound population of *Cyanea* in the Niantic River, USA, had a strong relationship between the distribution of medusa and the planula and polypoid phases (Colin and Kremer, 2002). The estuary was only a few kilometers long and the polypoid phases were most abundant at the upper reaches of the estuary. Similar to the Thau Lagoon example, the estuary was largely enclosed. Toyokawa et al. (2011) carried out extensive surveys for polyps and also collected some data on the distribution of ephyra in Mikawa and Ise Bays. The bays are both tens of kilometers wide and/or deep, but they share a common boarder. They found that polyps were most abundant on artificial structures deep in the bays and that the two bays shared a population of medusae sourced from polyps in the two bays.

Recent findings on the genetics of *Aurelia* polyps have provided strong evidence for populations of small spatial scale. van Walraven et al. (2016) sampled *Aurelia* polyps in Southern North Sea and Gullmar Fjord (Sweden) and compared haplotypes using 18S mRNA and COI mDNA. Population differentiation in polyps was detected between the Dogger Bank in the English Channel and nearby coastal locations (marinas, wrecks and settlement plates separated by tens of kilometers), indicating extremely low connectivity. However, no differences were found among coastal locations.

The relationship between cubozoan polyps and the abundance of medusae has scarcely been addressed. Hartwick (1991a) did locate a wild population of *C. fleckeri* polyps and noted that young medusae occurred nearby; however, no data on the

relationship between polyp and medusae abundance were collected. The polyps of most jellyfish are difficult to find, but studies on connectivity may be refined through the detection of polyp beds using eDNA, as has been done for the cubozoan *Copula sivickisi* (Bolte et al., 2021).

Conclusions

It is clear that despite the classification of scyphozoan and cubozoan jellyfish as plankton, many of them are highly mobile and demonstrate orientation behavior that can result in restricted distributions. Moreover, the cubozoans in particular have good eyesight that can further assist in maintaining their position within the boundaries of a population. The biogeographic range of holoplanktonic and oceanic taxa can extend over thousands of kilometers and this, to some extent, has reinforced the view that jellyfish are plankton largely at the mercy of currents. However, evidence from distributional data and more recently information from population genetics, morphometrics/elemental chemistry and biophysical modelling has demonstrated that some taxa have a complex hierarchy of population units, from metapopulations to surprisingly small stocks and local populations, where the latter in some cases may turn out to be stocks. Discrete populations at scale of tens of kilometers or less are likely to be self-sustaining, where causal factors include behaviorally restricted distributions of medusae in space, and in some cases assistance from local geomorphology and currents. Further, sexual reproduction and the subsequent release of planulae by scyphozoans (Arai, 1997) and cubozoans (Kingsford and Mooney, 2014) will create localized polyp beds that are likely to make these stocks increasingly robust. The scales of jellyfish stocks are not only relevant to understanding their ecology but to demarcating boundaries for fisheries quotas (Kingsford et al., 2000), and for determining the risk of envenomation from dangerous jellyfish such as cubozoans (Kingsford et al., 2018). Using multiple methods to determine levels of connectivity and stock boundaries is recommended, as a combination of approaches (Table S5.2) provides greater resolution of the hierarchy of population units for each species.

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