Final Report -
Norfolk Marine Park
Invasive Marine Species (IMS) Survey

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Prepared for: Department of Infrastructure, Transport, Regional Development, Communications and the Arts and Department of Climate Change, Energy, the Environment and Water.




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EXECUTIVE SUMMARY

Biofouling Solutions Pty Ltd. (BFS) was commissioned by the Department of Infrastructure, Transport, Regional Development, Communications and the Arts (DITRDCA) with the support of Department of Climate Change, Energy, the Environment and Water (DCCEEW), to plan, design and implement a marine pest survey of the Norfolk Marine Park. In particular, the marine pest survey was required to include members of the Australian Priority Marine Pest List, Exotic Environmental Pest List and those species on the Ballast Water Risk Assessment Tables. This included a total of 27 different species of concern (hereafter referred to Invasive Marine Species or IMS). There is potential for a further two IMS (namely the New Zealand screwshell, *Maoricolpus roseus* and the invasive colonial ascidian, *Didemnum perlucidum*) to have been introduced to the Norfolk Marine Park because vessels visiting Norfolk Island also visit ports where these species are known to be present. As such, these were also included in the survey. A desktop likelihood assessment was conducted to assess the theoretical likelihood of the 29 different IMS being potentially introduced to and capable of establishment within shallow coastal waters of the Norfolk Marine Park.

Based on the nature, extent, general ports of call and anchorage locations of visiting vessels to Norfolk Island in recent times, the most likely locations and suitable habitats for IMS introductions include Cascade Bay, Ball Bay, Emily Bay and Slaughter Bay on Norfolk Island. Therefore, rather than devote valuable resources towards surveying other areas, existing data collected during the Reef Life Survey (RLS) visits were used to assess these regions for potential IMS. Of the 702 photographs of the benthic environment surrounding Norfolk, Nepean, and Phillip Islands collected during 2009, 2013 and 2021 surveys, none of the 29 targeted IMS were detected. This knowledge was used to primarily focus the surveys on four main locations, namely 1) Cascade Bay, 2) Ball Bay, 3) Emily Bay and 4) Slaughter Bay on Norfolk Island for potential IMS. In addition to these sites, some opportunistic surveys were conducted at five other locations around the Island including 5) Anson Bay, 6) Bumbora Beach, 7) Cemetery Bay, 8) Elephant Rock and 9) Duncombe Bay.

A three-person team consisting of Dr Ashley Coutts, Dr Joe Valentine and Antonia Cooper visited Norfolk Island and conducted the IMS survey between 24 April and 5 May 2022. The following sampling techniques were used: 1) Scuba diving and snorkelling 2) Benthic sediment cores 3) Crab traps 4) Intertidal shore searches 5) Plankton tows and 6) eDNA sampling (using metabarcoding, next generation sequencing and qPCR).

While no established IMS were detected during the survey, empty half-shell Pacific oysters, *Magallana gigas* were found on the seabed at the end of Cascade Pier and adjacent to Kingston Pier. In addition, empty New Zealand Greenshell mussel shells, *Perna canaliculus* were also found on the seabed adjacent to Kingston Pier. However, the specimens detected were all dead half-shells measuring 80-110 mm which matches the

commercial food consumption market size for these species. No other live or whole specimens were detected during the surveys; therefore, it is highly likely that these species were imported frozen for human consumption and discarded at these locations.

The lack of detection of any established IMS at Norfolk Island is not surprising. Given Norfolk Island lacks any substantial port infrastructure, this prohibits vessels from residing close and for prolonged periods, which significantly limits the ability for IMS to transfer and establish. For instance, while many different vessel types visit the Island (such as general cargo, oil and gas tankers, cruise ships, tug and barges, yachts, naval vessels, etc.) from mainland Australia, New Zealand and other international locations, all of these vessels either anchor or remain idle off Cascade Bay, Ball Bay or Kingston Pier and rely on smaller vessels for the interactions. Hence, the likelihood of any IMS associated with vessel biofouling departing their hulls and establishing at Norfolk Island is extremely low. It is also unlikely that IMS would be introduced to Norfolk Island via ballast water discharge because: a) the vast majority of vessels which visit the Island import commodities, hence tend take on ballast water rather than discharge, and/or b) all vessels visiting the Island are required to comply with Australia's Mandatory Ballast Water Management Requirements.

Despite the low likelihood of IMS arriving and establishing at Norfolk Island, it is vital that any proposed changes to maritime operations and/or infrastructure around Norfolk Island carefully consider the potential for IMS introductions. Specific activities could be risk assessed and if deemed to be high risk, there may be an opportunity to incorporate additional ballast water and biofouling management requirements into future tenders to ensure contracted vessels pose a low likelihood of introducing any IMS risks.

An effective biosecurity management system should also include post-border measures such as on-going surveillance for any newly established IMS. Fortunately, there is weekly surveillance occurring within Emily and Slaughter Bay by enthusiastic locals who regularly snorkel and are likely to notice any changes or newly established species. The most likely locations for biofouling vectored IMS to establish in the future is at Cascade and Kingston Piers. While it is acknowledged that these two locations are subject to heavy seas and are therefore difficult to access, it may be possible for Norfolk Island Diving to undertake the occasional dive (i.e., every 3-6 months) using a GoPro to record the nature and extent of the biofouling present. This footage could then be sent to an IMS specialist for review.

There may be an opportunity to incorporate Dr Katherine Dafforn's (Associate Professor and Environmental Scientist at Macquarie University) "Concrete Walls and Living Seawalls" concept. Dr Dafforn and her team have been designing and testing ecologically engineered surfaces for enhancing the establishment of native marine species which in turn increases the immunity to IMS recruitment. If some living seawall trials could

occur on both the Cascade and Kingston Piers, these would need to be monitored which could also include the surrounding areas. Furthermore, it would be worth incorporating this concept into any proposed extensions or new infrastructure developments around the Island.

Given the present lack of artificial structures at Ball Bay, such a location is less vulnerable to potential IMS recruitment. It is also the most difficult and expensive location to monitor considering it would require a boat, divers or a Remotely Operated Vehicle. Therefore, if there are budgetary constraints, routine monitoring should focus on Kingston Pier, Slaughter Bay, Emily Bay, and Cascade Bay (in order of priority). It is also recommended that a major survey similar to the one, as outlined in this report, occur every 2-3 years which would include Ball Bay and incorporate any other changes or developments.

1 INTRODUCTION

Biofouling Solutions Pty Ltd. (BFS) has been engaged by the Department of Infrastructure, Transport, Regional Development, Communications and the Arts with the support of the Department of Climate Change, Energy, the Environment and Water(Parks Australia), to plan, design and implement a marine pest survey of the Norfolk Marine Park.[1] DITRDCA is responsible for the provision of biosecurity functions on Norfolk Island that may typically be delivered by a state or territory government body. The Department of Agriculture, Fisheries and Forestry (DAFF) is responsible for First Port of Entry (Land & Sea) under the *Biosecurity Act 2015.* Parks Australia is responsible for the management of Australia's marine parks. This includes Norfolk Marine Park (Temperate East Network), surrounding Norfolk Island and covering 188,444 square kilometres.

As part of the marine pest survey, BFS was required to design a marine ecosystem survey capable of assessing the status of priority marine pest species surrounding Norfolk Island, Nepean Island and Phillip Island (with guidance provided in the Australian Marine Pest Monitoring Manual, the Australian Marine Pest Monitoring Guidelines, reports from Indian Ocean Territory Surveillance and the Monitoring Design Package). In particular, the marine pest survey was to include consideration of the members of the Australian Priority Marine Pest List, Exotic Environmental Pest List and those species on the Ballast Water Risk Assessment Tables.

2 METHODS

2.1 Establishing a Target Pest List

When the Australian Priority Marine Pest List, Exotic Environmental Pest List and those species on the Ballast Water Risk Assessment Tables are combined, there are a total of 27 different species of concern (hereafter referred to Invasive Marine Species or IMS). Although, there is potential for a further two IMS (namely the New Zealand screwshell, *Maoricolpus roseus* and the invasive colonial ascidian, *Didemnum perlucidum*), to have been introduced to the Norfolk Marine Park, because vessels visiting Norfolk Island also visit ports where these species are known to be present.

When the aforementioned list of species, including the two additional species, are combined there are a total of 29 IMS on the initial target list (see Table 1). There is a possibility, however, that due to their

[1] Given that the Norfolk Island Marine Park extends hundreds of nautical miles to the north and south, and Invasive Marine Species tend to be established within shallow coastal waters, the focus of this assessment is therefore within 50 m of water surrounding Norfolk, Nepean and Phillip Islands.

distribution, biotic and abiotic tolerances, and availability of transportation pathways, some IMS on this list may not have had the opportunity to be introduced to the coastal waters of the Norfolk Marine Park.

Table 1. Australian Priority Marine Pest List, Exotic Environmental Pest List and those species on the Ballast Water Risk Assessment Tables combined. Two additional species have also been added, *Maoricolpus roseus* and *Didemnum perlucidum.*

Phylum	Genus/Species	Common Name	Australian Priority Marine Pest List	Exotic Environmental Pest List	Ballast Water Risk Assessment Table List
Algae	Centric diatom	*Chaetoceros concavicornis*		✓	
	Toxic dinoflagellate	*Dinophysis norvegica*		✓	
	Japanese wireweed	*Sargassum muticum*		✓	
	Japanese seaweed	*Undaria pinnatifida*	✓		✓
Coelenterata	Comb jelly	*Mnemiopsis leidyi*		✓	✓
Annelida	Red-gilled mudworm	*Marenzelleria neglecta*		✓	
	Mediterranean fanworm	*Sabella spallanzanii*			✓
Mollusca	Asian date mussel	*Arcuatula senhousia*			✓
	Pacific oyster	*Magallana gigas*			✓
	New Zealand screwshell	*Maoricolpus roseus*			
	Soft shelled clam	*Mya arenaria*		✓	
	Black-striped false mussel	*Mytilopsis sallei*	✓	✓	
	New Zealand green-lipped mussel	*Perna canaliculus*	✓	✓	
	Brown mussel	*Perna perna*	✓	✓	
	Asian green mussel	*Perna viridis*	✓	✓	
	Asian brackish-water clam	*Potamocorbula amurensis*		✓	✓
	Rapa whelk	*Rapana venosa*		✓	
	European clam	*Varicorbula gibba*			✓
	Atlantic oyster drill	*Urosalpinx cinerea*		✓	
Echinodermata	Northern Pacific Seastar	*Asterias amurensis*	✓		✓
Crustacea	Japanese skeleton shrimp	*Caprella mutica*		✓	
	European green crab	*Carcius maenas*	✓		✓
	Lady crab / Asian paddle crab	*Charybdis japonica*		✓	
	Chinese mitten crab	*Eriocheir sinensis*	✓	✓	
	Japanese shore crab	*Hemigrapsus sanguineus*		✓	
	Brush-clawed shore crab	*Hemigrapsus takanoi*		✓	
	Harris' mud crab	*Rhithropanopeus harrisi*	✓	✓	
Chordata	Invasive sea squirt	*Didemnum perlucidum*			
	Carpet sea squirt	*Didemnum vexillum*		✓	
		Totals	**9**	**20**	**9**

Rather than devoting valuable resources searching for certain IMS which are highly unlikely to have had the opportunity to be introduced and establish in such an environment, a desktop likelihood assessment was conducted to assess the theoretical likelihood of the 29 different IMS being potentially introduced to and capable of establishment within shallow coastal waters of the Norfolk Marine Park (see Biofouling Solutions, 2021a for further background information). The likelihood assessment determined that IMS are most likely to have been introduced to the Norfolk Marine Park via vessel biofouling (i.e., attached to, associated with, entrained and/or via entanglement) (Biofouling Solutions, 2021a). Based on the nature, extent, general ports of call, and anchorage locations of vessels when visiting Norfolk Island in recent times, the IMS most likely

to have arrived and potentially established within the Norfolk Island Marine Park include the following six species: Japanese seaweed, *Undaria pinnatifida;* New Zealand screwshell, *Maoricolpus roseus;* Japanese skeleton shrimp, *Caprella mutica;* Lady crab/Asian paddle crab, *Charybdis japonica;* Invasive sea squirt, *Didemnum perlucidum;* Carpet sea squirt*, Didemnum vexillum* (**Figure 1**).

Of these six IMS, the most likely IMS to establish at Norfolk Island are those species with short propagule competency periods (i.e., settle within minutes up to 48 hours) which include *Undaria pinnatifida*; *Didemnum perlucidum* and *D. vexillum.* In addition, the two species of colonial ascidians (*D. perlucidum* and *D. vexillum*) are the most likely IMS to have arrived and become established given their ability to fragment and asexually reproduce (Biofouling Solutions, 2021a).

Japanese seaweed, *Undaria pinnatifida* *Photo credit: CSIRO*		New Zealand screwshell, *Maoricolpus roseus* *Photo credit: Biofouling Solutions Pty Ltd.*	
Japanese skeleton shrimp, *Caprella mutica* *Photo credit: NIWA*		Lady crab/Asian paddle crab, *Charybdis japonica* *Photo credit: Michio Otani*	
Invasive sea squirt, *Didemnum perlucidum* *Photo credit: Biofouling Solutions Pty Ltd.*		Carpet sea squirt*, Didemnum vexillum* *Photo credit: Cawthron Institute*	

Figure 1. Six of the most likely Invasive Marine Species (IMS) to have arrived and potentially established at Norfolk Island.

2.2 Refining Survey Locations

The DITRDCA and the DCCEEW requested that BFS plan, design and implement a marine pest survey to cover all of the Norfolk Marine Park (i.e., including Norfolk, Nepean and Phillip Islands). However, based on the nature, extent, general ports of call and anchorage locations of visiting vessels to Norfolk Island in recent times, the most likely locations and suitable habitats for IMS introductions include Cascade Bay, Ball Bay,

Emily Bay and Slaughter Bay on Norfolk Island (Biofouling Solutions, 2021a). Existing data collected during the Reef Life Survey (RLS)[2] visits were used to assess other regions of the Park for potential IMS. Of the 702 photographs of the benthic environment surrounding Norfolk, Nepean and Phillip Islands collected during 2009, 2013 and 2021 surveys, none of the 29 targeted IMS were detected (Biofouling Solutions, 2021b). This knowledge was used to primarily focus the surveys on four main locations, namely 1) Cascade Bay, 2) Ball Bay, 3) Emily Bay and 4) Slaughter Bay (**Figure 2**). In addition to these sites, some opportunistic surveys at five other locations around the Island including 5) Anson Bay, 6) Bumbora Beach, 7) Cemetery Bay, 8) Elephant Rock and 9) Duncombe Bay were undertaken (**Figure 2**).

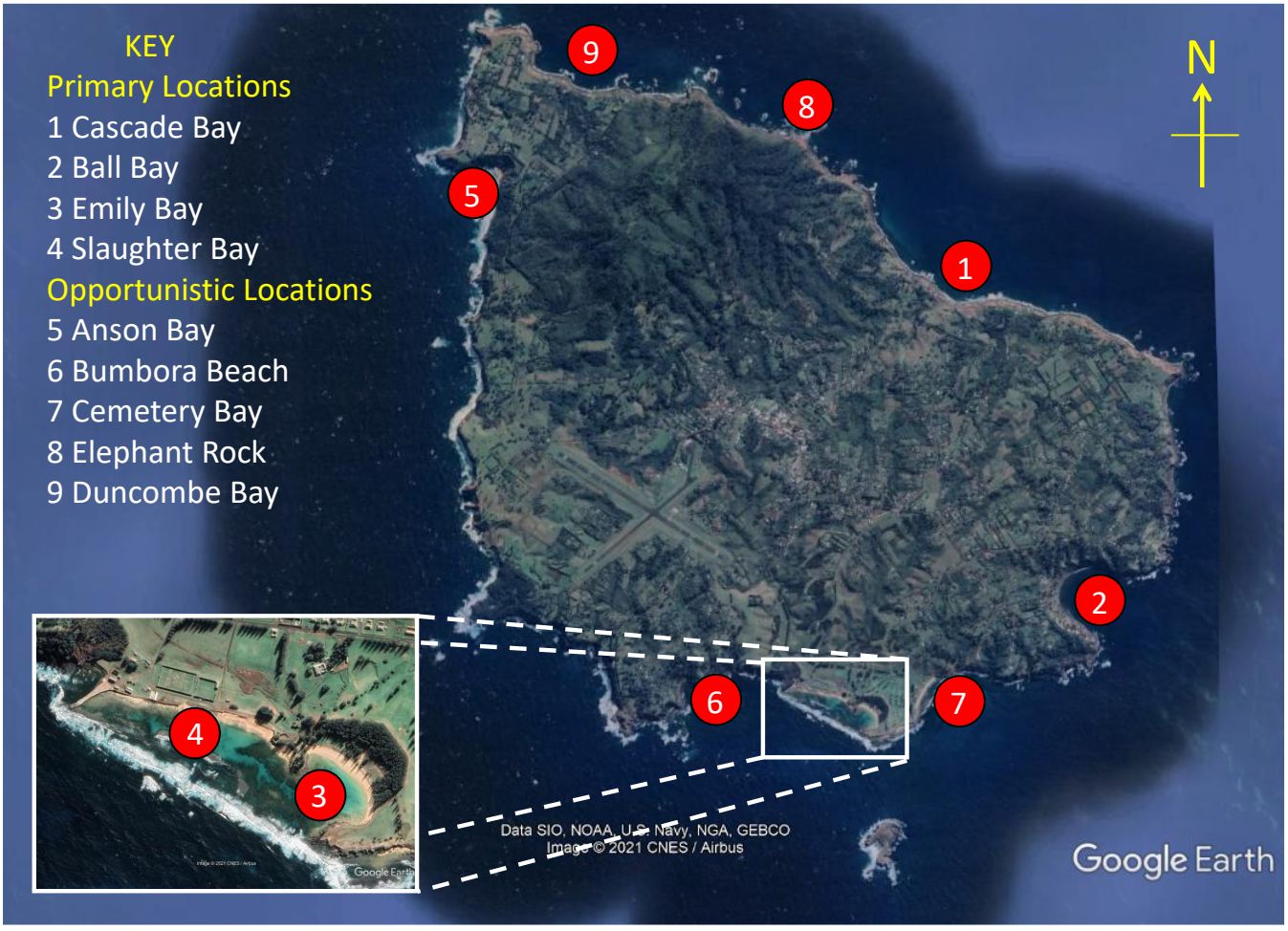

Figure 2. The location of the four primary and five opportunistic locations surveyed for detecting Invasive Marine Species (IMS) at Norfolk Island.

[2] *https://reeflifesurvey.com/*

2.3 Selection of Reliable, Proven and Cost-Effective Sampling Techniques

A wide variety of proven, cost-effective and practical sampling techniques exist for detecting different IMS in various habitats. The sampling techniques chosen for the survey at Norfolk Island are consistent with those identified in the Australian Marine Pest Monitoring Manual and have been widely applied in IMS surveys throughout ports/harbours around Australia and New Zealand. Sampling techniques outlined in the Australian Marine Pest Monitoring Manual were broadly based on protocols developed by the Commonwealth Scientific and Industrial Research Organisation's (CSIRO) Centre for Research on Introduced Marine Pests (CRIMP) described by Hewitt and Martin (2001). The sampling methods outlined in the Australian Marine Pest Monitoring Manual are somewhat generic and require modifications to local conditions as implemented for New Zealand Ports (Gust et al., 2001) and tropical applications (Hoedt, 2001).

The sampling techniques chosen for the Norfolk Island survey were based on detecting various life stages and habitat preferences of the 29 IMS based on extensive practical research experience acquired from undertaking over forty IMS surveys around Australia and New Zealand. To best detect the most likely IMS to be present, the following sampling techniques were used:

- Scuba diving and snorkelling
- Benthic sediment cores
- Crab traps
- Intertidal shore searches
- Plankton tows
- eDNA sampling (using metabarcoding, next generation sequencing and qPCR).

A summary of the different sampling methods used and their likelihood of detecting different IMS are provided in **Table 2**. Further descriptions of each sampling technique and the methods used are provided below (Sections 2.3.1-2.3.6).

2.3.1 Scuba Diving and Snorkelling

A combination of Scuba diving and snorkelling was used to undertake visual surveys for potential IMS at each of the four primary sites (namely Cascade Bay, Ball Bay, Emily Bay and Slaughter Bay) and three opportunistic sites (including Anson Bay, Elephant Rock and Duncombe Bay) (**Table 3**). Local knowledge and experience were obtained from Norfolk Island Diving, namely Mitch Graham, to assist with planning and undertaking the surveys at Cascade Bay, Ball Bay, Elephant Rock and at Duncombe Bay. The remaining

locations were accessed and surveyed from land (Emily Bay, Slaughter Bay, and Anson Bay) (see **Appendix 1**).

Table 2. List of different sampling methods used to detect various Invasive Marine Species (IMS), cryptic and/or species displaying invasive characteristics during the survey of Norfolk Island. ✓ = method is capable of detecting certain IMS. Green highlights refer to those IMS which were assessed as having the greatest likelihood of being present and detected during the survey (see Biofouling Solutions, 2021a).

Phylum	Genus/Species	Common Name	Diving/ Snorkelling	Benthic Cores	Crab Traps	Beach Walks	Plankton Tows	eDNA
Algae	Centric diatom	*Chaetoceros concavicornis*					✓	
	Toxic dinoflagellate	*Dinophysis norvegica*					✓	✓
	Japanese wireweed	*Sargassum muticum*	✓			✓		
	Japanese seaweed	*Undaria pinnatifida*	✓			✓		✓
Coelenterata	Comb jelly	*Mnemiopsis leidyi*				✓	✓	✓
Annelida	Red-gilled mudworm	*Marenzelleria neglecta*	✓					✓
	Mediterranean fanworm	*Sabella spallanzanii*	✓					✓
Mollusca	Asian date mussel	*Arcuatula senhousia*	✓	✓		✓		✓
	Pacific oyster	*Magallana gigas*	✓			✓		✓
	New Zealand screwshell	*Maoricolpus roseus*		✓		✓		✓
	Soft shelled clam	*Mya arenaria*	✓			✓		✓
	Black-striped false mussel	*Mytilopsis sallei*	✓			✓		✓
	New Zealand green-lipped mussel	*Perna canaliculus*	✓		✓	✓		✓
	Brown mussel	*Perna perna*	✓			✓		✓
	Asian green mussel	*Perna viridis*	✓			✓		✓
	Asian brackish-water clam	*Potamocorbula amurensis*		✓		✓		✓
	Rapa whelk	*Rapana venosa*	✓			✓		✓
	European clam	*Varicorbula gibba*		✓		✓		
	Atlantic oyster drill	*Urosalpinx cinerea*	✓			✓		✓
Echinodermata	Northern Pacific Seastar	*Asterias amurensis*	✓		✓	✓		✓
Crustacea	Japanese skeleton shrimp	*Caprella mutica*	✓					✓
	European green crab	*Carcius maenas*	✓			✓		✓
	Lady crab / Asian paddle crab	*Charybdis japonica*	✓		✓	✓		✓
	Chinese mitten crab	*Eriocheir sinensis*	✓		✓	✓		✓
	Japanese shore crab	*Hemigrapsus sanguineus*			✓	✓		✓
	Brush-clawed shore crab	*Hemigrapsus takanoi*			✓	✓		✓
	Harris' mud crab	*Rhithropanopeus harrisi*			✓	✓		✓
Chordata	Invasive sea squirt	*Didemnum perlucidum*	✓					✓
	Carpet sea squirt	*Didemnum vexillum*	✓					✓

Of particular interest were the concrete piers at Cascade and Slaughter Bays (Kingston Pier) and the swimming pontoon within Emily Bay. This is because these are the only artificial structures around Norfolk Island and such structures are known to provide unique habitats for marine organisms, including IMS (see Glasby and Connell, 2001; Glasby et al., 2007). Such man-made structures are ideal locations to detect any of the six most likely IMS to be present at Norfolk Island (**Figure 1**). Divers used underwater cameras to photograph the general communities and any suspected IMS. Hand collection/putty-scrapers and specially designed collection bags were used to collect any suspected IMS (**Figure 3**; Figure 4). Any suspected IMS or organisms of interest were photographed, labelled and preserved accordingly (see Section 2.4).

Figure 3. Scuba diving and snorkelling were used to undertake visual surveys for IMS at Cascade Bay, Ball Bay, Emily Bay, Slaughter Bay, Elephant Rock and at Duncombe Bay.

2.3.2 Benthic Cores

Five specially designed tubular hand corers (165 diameter x ~150 mm) constructed from PCV were used while using Scuba to sample benthic sediments to detect potential IMS within this habitat such as the New Zealand screwshell, *Maoricolpus roseus* (**Figure 1**; **Figure 4**). Five replicate cores were collected from each of the four primary locations including Cascade Bay, Ball Bay, Emily Bay and Slaughter Bay (**Table 3**; **Appendix 1**). All sediment samples were then deposited into a sieve with 4 mm[3] holes so that any infauna above this size were retained for closer examination. Any suspected IMS or organisms of interest detected were photographed, labelled and preserved accordingly (see Section 2.4).

Figure 4. Benthic cores were used to sample soft sediments for potential IMS at Cascade Bay, Ball Bay, Emily Bay and Slaughter Bay.

[3] BFS have trialled a variety of methods and found this method to be the most effective, especially when attempting to sample hard encrusting organisms.

Table 3. A summary of the sites, sampling methods and replication used to detect potential IMS, cryptic and/or species displaying invasive characteristics during the survey of Norfolk Island.

Location/ Method	Diving/ Snorkelling	Benthic Cores	Crab Traps	Shore Search	Plankton Tows	eDNA
1. Cascade Bay	2	5	5	1	5	5
2. Ball Bay	1	5	-	1	5	5
3. Emily Bay	3	5	5	1	5	5
4. Slaughter Bay	2	5	5	1	5	5
5. Anson Bay	1	-	-	1	-	-
6. Bumbora Beach	-	-	-	1	-	-
7. Cemetery Bay	-	-	-	1	-	-
8. Elephant Rock	1	-	-	-	-	-
9. Duncombe Bay	1	-	-	-	-	-

2.3.3 Crab Traps

Box traps measuring 850 x 600 x 250 mm with square mesh size of 5 mm were used to target invasive crabs, such as the Asian paddle crab, *Charybdis japonica* (**Figure 1**; **Figure 5**). The intention was to deploy five replicate crab traps at each of the four primary locations namely Cascade Bay, Ball Bay, Emily Bay and Slaughter Bay (**Table 3; Appendix 1**). However, crab traps were unable to be deployed at Ball Bay due to rough seas. Norfolk Island Diving's boat was used to deploy and retrieve the crab traps within Cascade Bay while the traps within Emily and Slaughter Bay were deployed via the shore using Scuba.

A can of fish-flavoured cat food was used as bait to attract crabs by using a pick-hammer to puncture the contents of the can and securing the can to the bottom of the traps using cable-ties inside to the base of each trap. Each trap was weighted with three dive weights (3.0lb) to ensure the traps were negatively buoyant and remained on the seabed. In addition, each crab trap was attached to a 30 m backbone rope (approximately 5 m apart) with three dive weights (3.0lb) and a shot-line attached to each end with a surface buoy used to define the ends of the backbone/traps. All traps were deployed in the late afternoon and recovered as early as possible the following morning to minimise any predation or unwanted mortality of

captured animals. Any suspected IMS or organisms of interest were photographed, labelled and preserved according to Section 2.4, while all other non-target organisms were returned to the sea.

Figure 5. Collapsible box traps were used to target crabs, some of which could be IMS.

2.3.4 Shore Searches

Shore searches along the intertidal zones were undertaken at each of the four primary locations, namely Cascade Bay, Ball Bay, Emily Bay and Slaughter Bay (**Table 3**; **Figure 6**). In addition, shore searches were also undertaken at Anson Bay, Bumbora Beach and Cemetery Bay (**Appendix 1**). Shore searches were timed to coincide with low tide so the intertidal zone could be surveyed for the presence of IMS. Shore searches also included assessment of wrack and washed-up material which is an effective method of detecting any dead IMS which may have washed ashore. The detection of any suspected IMS during searches of beach wrack can help to focus further search efforts if necessary.

Figure 6. Shore searches on low tide were also undertaken at Cascade Bay, Ball Bay, Emily Bay, Slaughter Bay, Anson Bay, Bumbora Beach and Cemetery Bay in search of any IMS.

2.3.5 Plankton Tows

Plankton tows were undertaken to detect the presence of any of the diatom or toxic dinoflagellate IMS. Five replicate plankton tows occurred at the four primary locations namely Cascade Bay, Ball Bay, Emily Bay and Slaughter Bay (**Table 3**; **Appendix 1**). Plankton tows at Cascade Bay and Ball Bay involved using Norfolk Island Diving's boat to deploy a 20-micron plankton net (which was negatively buoyant) towards the seabed, then retrieving it vertically through the water column back to the surface. For plankton tows at Emily and Slaughter Bay, BFS personnel waded into the lagoon up to their waist and trailed the plankton net behind them for approximately 20 m (**Figure 7**). Upon each retrieval, the seawater was allowed to drain slowly then a squirty-bottle filled with seawater was used to gently rinse the inside of the net to wash any remaining plankton into the bottom of the net and into a 70 mL collection jar at the bottom. Each collection jar was then gently unscrewed from the plankton net, labelled and ~15 pipette drops of Lugols solution added,[4] and then placed on ice in an esky. All samples were then returned to Hobart and sent to Professor Gustaaf Hallegraeff at the Institute of Marine and Antarctic Studies, University of Tasmania, Hobart for analysis.

2.3.6 eDNA

For eDNA presence/absence analysis of IMS, five one L seawater samples were collected from each of the four primary locations namely Cascade Bay, Ball Bay, Emily Bay and Slaughter Bay (**Table 3**; **Figure 7**; **Appendix 1**). Such a technique is still evolving, but is capable of detecting the presence of a wide variety of IMS. Where possible, the collection of these samples was spread out over each site to increase coverage. Each seawater sample was stored on ice in an esky while in the field before filtering once back on shore. The samples were filtered on the day of collection using 0.45μm mixed cellulose ester filter with aperistaltic Sentino pump to capture eDNA present in the water. Two rinsate controls were included for quality assurance/quality control purposes. Following filtration, filter paper samples were frozen until departure. For shipment, samples were transferred to a small esky packed with icepacks during transit from the Island to eDNA Frontiers at Curtin University, Perth, Western Australia.

eDNA Frontiers extracted DNA from half of each filter paper and each water sample was assigned an individual combination of index tags and amplified by PCR using three assays: (1) a broad mitochondrial COI assay, (2) a universal 16S assay, and (3) a 16S assay targeting bivalves. Libraries were generated and sequenced using the Illumina MiSeq. Laboratory extraction and PCR controls were included to test for

[4] The amount of acid Lugol's solution which should be added to an algae sample depends on the concentration of algae in the sample, but typically 0.3 ml to 1.0 ml of acid Lugol's solution per 100 ml of sample is sufficient with the larger volume of Lugol's being used for particularly dense algal samples. A good rule of thumb is to add the Lugol's to the sample a few drops at a time until the colour of the water in the sample is that of weak tea allowing a little time between each addition for the Lugol's to be absorbed into the algal cells.

contamination. Bioinformatic tools were used to analyse raw sequence data (Mousavi-Derazmahalleh et al., 2021) generated from the metabarcoding. The sequencing results were demultiplexed and trimmed using Obitools and quality filtered with Usearch v11 for sequencing errors (maxee=1) with an appropriate minimum length used (150 for COI, 100 for the universal 16S, and 150 for the bivalve 16S). Sequences were then dereplicated and unique sequences were transformed into zero radius operational taxonomic units (ZOTUs) to provide sensitive taxonomic resolution (Usearch v11) (Edgar, 2018). ZOTUs, in contrast to OTUs, are a more exact sequence variant, clustering at 99% to improve taxonomic resolution. Generated ZOTUs were queried against the nucleotide database NCBI (GenBank) and assigned to the species-level where possible. Taxonomic assignments were based on an eDNA Frontier Python script which further filters the Blast results (evalue ≤1e-5, %identity ≥95, qCov =100, LULU minMatch =97%), combines them with the ZOTU table results and produces a table containing the taxonomic information available from Blast taxonomy database (accessed May 2022).

Samples of a colonial ascidian suspected to be *Diplosoma virens* (Hartmeyer, 1909) were also collected from Emily Bay and Slaughter Bay due to its smothering characteristics. Samples were placed in 70% ethanol and sent with the water samples to eDNA Frontiers for analysis. eDNA Frontiers amplified the tissue sample by PCR using a COI assay, with the PCR product outsourced for sanger sequencing. Sequencing analysis of the tissue sample was performed in Geneious Prime (version 2021.0.3) where the generated sequence was queried against GenBank, sequences for the closest matches as well as *Diplosoma virens* downloaded, and an alignment produced. Sequences were then trimmed to the same length and pairwise comparisons calculated to determine the percent similarity between them.

The eDNA approach was unable to positively identify three IMS target species, namely *Chaetoceros concavicornis*, *Sargassum muticum* and *Varicorbula gibba* due a lack of COI sequences to match to on GenBank. It is acknowledged that limited sampling and metabarcoding has occurred for the local area, therefore it is notable that IMS may not have been differentiated successfully from unique local species, hence this technique has the potential to mistake a local species for an IMS (i.e. false positives). Unfortunately, there is little that can be done until sufficient ground-truthing of the area can be achieved. Accordingly, a stepwise criterion was used for assigning a sequence to an IMS, as outlined in **Table 4**.

Figure 7. Plankton sampling was achieved using a plankton net deployed from a boat or from shore. eDNA samples were also collected at the same time.

Table 4. Criteria for assigning a sequence to an Invasive Marine Species (IMS).

Criteria				Confidence
1.	Is a sequence ≥ 97%[1] similar to an IMS reference sequence	Yes Go to 2	No	x
2.	Can the assay differentiate the IMS from other species	Yes Go to 3	No	*Inconclusive*
3.	Does the sequence match to an IMS at ≥ 99%[1]	Yes Go to 4	No	*Inconclusive*
4.	Are there available reference sequences for closely related local taxa	Yes Go to 5	No	*Possible*
5.	Has the IMS been reported in the area before	Yes Go to 6	No	*Probable*
6.				*Highly Probable*

2.4 Sample Collection, Import Permit/Declaration and Preservation

Numerous permits were obtained to collect, preserve, and safely transport and import biological samples from Norfolk Island to mainland Australia. These included the various permits and declarations from the DAFF; Parks Australia; Civil Aviation Safety Authority and the Tasmanian Government's Department of Natural Resources and Environment (**Table 5**). Import permits and civil aviation requirements also specify permitted preservation types and concentrations for certain taxa. Hence, these requirements were followed as a priority but ensured that they were also in accordance with the recommended preservation methods for genetic and morphological confirmation (**Table 6**).

Table 5. Various permits, declarations, approvals required to collect, preserve, and safely transport and import biological samples from Norfolk Island to mainland Australia.

Regulator	Requirement	Purpose	Permit No.	Comments
Australian Government – Department of Agriculture, Fisheries and Forestry.	Permit	To access biological resources in Commonwealth Areas	AU-COM2021-540	None
Australian Government - Parks Australia	Permit	To undertake scientific research activities in a Commonwealth Marine Park	PA2021-00139-1	None
Australian Government – Department of Agriculture, Fisheries and Forestry	Declaration, no import permit required	To import biological specimens into Australia	BICON Case: Preserved and fixed animal and human specimens; Phytoplankton samples	Preserved in 70% alcohol, 10% formalin, 4% formaldehyde or 2% glutaraldehyde
Australian Government – Department of Agriculture, Fisheries and Forestry	Declaration, no import permit required	To import algae from Norfolk Island into Australia	BICON Case: Preserved fruit, vegetables or seaweed for human consumption	Preserved in 70% alcohol
Australian Government – Department of Agriculture, Fisheries and Forestry	Declaration, no import permit required	To import oyster shells from Norfolk Island into mainland Australia	Application No. 0005827328	Specific requirement outlined in application.
Australian Government – Department of Agriculture, Fisheries and Forestry	Declaration, no import permit required	To import oyster shells from Norfolk Island into mainland Australia	Application No. 0005827328	Specific requirement outlined in application.
Tasmanian Government – Department of Natural Resources and Environment	Notification	To import biological specimens into Tasmania	N/A	Email correspondence and approval based on above requirements.
Civil Aviation Safety Authority	Declaration	For safe transportation of biological specimens fixed in preservatives	Special Provision A180	Refer to Special Provision A180 of the ICAO Technical Instructions

Table 6. Sample preservation methods used for collected specimens.

Taxon	Narcotisation*	Fixing	Preservation	Comments
Phytoplankton	No	1% Lugol's solution	1% Lugol's solution	Cyst samples to remain cool and dark for transport, no preservatives
Macroalgae		4% formalin	70% ethanol	Preservation may also be achieved by air-drying
Polychaetes		4% formalin	70% ethanol	"default method"
Mollusca	No or F or M*	4% formalin	70% ethanol	-
Crustacea	F*	4% formalin	70% ethanol	-
Ascidians	MC*	4% formalin	70% ethanol	-
All others		4% formalin	70% ethanol	"default method"

* Where M = $MgSO_4$ or $MgCl_2$, MC = menthol crystals, F = freezing and No = narcotisation not needed. Source: Hewitt and Martin (2001).

2.5 Workplace Health and Safety

Workplace Health and Safety is extremely important when conducting field work in remote locations such as Norfolk Island, necessitating careful planning and operation of field equipment to minimise the potential for any incidents. Diving/snorkelling was assessed as posing the greatest potential for serious injury due to the presence of Tiger Sharks or a diving related illness. Consequently, Mitch Graham from Norfolk Island Diving was consulted when planning all diving operations while on the Island. For instance, Norfolk Island does not possess a hyperbaric chamber and the closest facility is likely to be Sydney. Hence, the most conservative dive times and repetitive diving groups within the Scientific Dive Code was adopted. There are also potential hazards associated with operating some of the field equipment. Job Hazard Analyses were completed prior to undertaking each task, each day.

The potential to contract and spread COVID-19 prior to or while on Norfolk Island was assessed as a high risk, therefore, a COVID-19 Risk Management Plan was developed whereby all personnel underwent a COVID-19 PCR test within 48 hours prior to arrival on the Island to ensure they were “negative” to travel to the Island. In addition, all personnel underwent a Rapid Antigen Test for COVID-19 within 24 hours of arrival. All diving activities were prioritised because of the restrictions imposed on diving after contracting COVID-19.

2.6 Lodging of Specimens

Any suspected IMS or specimens of interest were sent to relevant taxonomic authorities as listed in the Australian Marine Pest Monitoring Manual (Version 2.0). Furthermore, all other collected specimens of interest will be lodged with a recognised Australian museum(s) for future taxonomical reference and surveillance of marine pests in activity locations described. These are largely state and territory museum and herbarium collections. However, it will be vital to consult with the relevant taxonomic authority/museum to reach an agreed protocol for specimen preparation and acquiring the necessary permits for any transport of specimens or samples.

2.7 Field Trip Timing and Personnel

Fieldwork was originally planned to occur between 24 January and 4 February, 2022. However, a COVID-19 outbreak on Norfolk Island resulted in the Emergency Management Norfolk Island to close entry to the Island so that the Norfolk Island Health and Residential Aged Care Service could manage the outbreak. Consequently, the survey was rescheduled between 24 April and 5 March, 2022 consisting of a three-person team, Dr Ashley Coutts, Dr Joe Valentine and Antonia Cooper.

3 RESULTS

3.1 Cascade Bay

3.1.1 Diving/Snorkelling

Visual surveys of Cascade Bay occurred on 25 and 27 April, 2022 and consisted of inspecting the Cascade Pier and a rocky reef adjacent to where the crab traps were set (see **Appendix 1**). A thorough inspection of Cascade Pier was achieved due to calm conditions. The structure was dominated by red, brown and green algae with some colonial ascidians (e.g. suspected *Diplosoma virens*), sponges, and stone corals all of which appeared to be suppressed, most likely due to the frequency and strength of the wave action that typifies this location (**Figure 8**). Some mobile taxa were noted within the protected joins between the concrete caissons such as *Drupella* sp. snails; red tipped urchins, *Heliocidaris tuberculata*; red bait crabs, *Plagusia chabrus*; and Green-lined crabs, *Percnon planissimum*. The most significant finding was the detection of a target list species, namely four empty half-shell Pacific oysters, *Magallana gigas* measuring 90-100 mm (shell length). These oyster shells were found on the seabed at the end of the pier (**Figure 8**). The inspection of the rocky reef adjacent to where the crab traps were set were covered in mostly soft and hard corals, although no target IMS were detected (**Figure 9**).

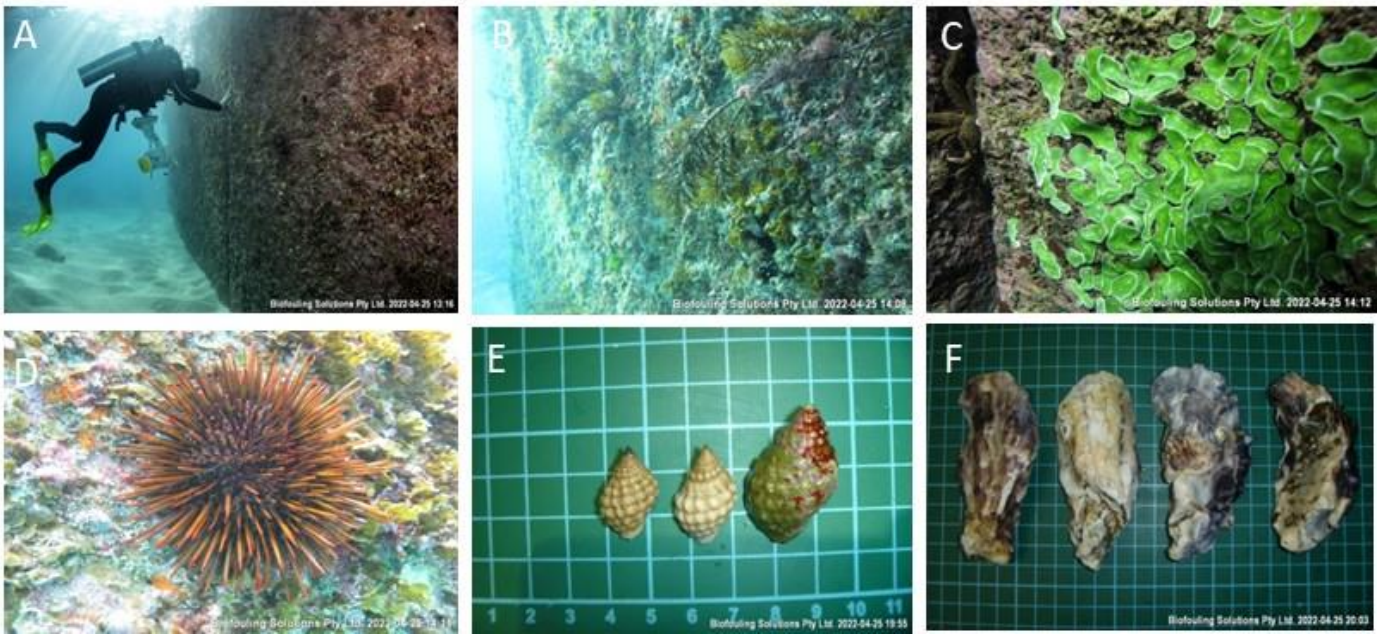

Figure 8. Examples of the marine growth encountered during the inspection of the Cascade Pier. A) Western side of the Pier B) typical growth C) colonial ascidian, suspected *Diplosoma virens* D) red tipped urchins, *Heliocidaris tuberculata* E) gastropods, *Drupella* sp. and F) Pacific oysters, *Magallana gigas*.

Figure 9. Examples of the marine growth encountered while undertaking opportunistic surveys of a neighbouring rocky reef while setting and retrieving the crab traps within Cascade Bay.

3.1.2 Benthic Cores

Five benthic cores were collected from around the end of the Cascade Pier on 25 April, 2022 (**Appendix 1**). Samples consisted of mostly sand with some degraded coral due to the frequent and strong wave action. However, no live organisms above 4 mm were detected amongst the samples collected (**Figure 10**).

Figure 10. Examples of the sediment collected via the benthic corers at Cascade Bay. A) Before washing through the 4 mm sieve B) After washing through the 4 mm sieve.

3.1.3 Crab Traps

Five crab traps were deployed on a large patch of sand on the fringe of a rocky reef within Cascade Bay on the afternoon of 25 April, 2022 and retrieved the following morning (**Appendix 1**). Only one swimming crab, potentially a *Thalamita* sp. was detected within one of the traps. No suspected IMS were detected.

3.1.4 Shore Search

A shore search occurred along Cascade Beach heading both east and west of the Cascade Pier on 25 April, 2022 (**Appendix 1**). The beach consisted of mostly large boulders with some patches of smaller rounded stones/pebbles (**Figure 11**). No suspected IMS were detected.

Figure 11. Examples of the terrain encountered during the shore search at Cascade Bay.

3.1.5 Plankton Tows

Five plankton tows were conducted in a semi-circle between 150-300 m offshore from Cascade Pier (**Appendix 1**). Cascade Bay had the lowest cell concentrations of all locations surveyed, possessing three species of diatoms (*Gyrosigma/Pleurosigma*, *Licmophora,* and *Odontella*) and three species of dinoflagellates (*Gonyaulax, Protoperidinium,* and *Tripos teres*) (**Appendix 2**). None of these are considered IMS.

3.1.6 eDNA

While no IMS were detected amongst the samples collected, the Chaetocerotaceae family was detected which includes one of the target IMS known as *Chaetoceros concavicornis*; a species of diatom. However, the eDNA was unable to positively detect this target IMS, although another non-target species within this family, namely *C. tenuissimus* was detected (see **Appendix 3**).

3.2 Ball Bay

3.2.1 Diving and Snorkelling

Visual surveys of Ball Bay occurred on 26 April, 2022, but were restricted by rough weather and the presence of the *MV Duzgit Venture*, a Chemical/Oil Products Tanker stationed in the middle of the Bay (**Figure 12**; **Appendix 1**). Consequently, the diving surveys were restricted to the northern side of the bay. Despite this, the survey was able to cover a mixture of sand patches with large basalt boulders through to natural coral reef. The sand patches appeared to consist of mostly degraded coral while the coral reef appeared to be consistent with that observed elsewhere around the Island (**Figure 12**). No suspected IMS were detected.

Figure 12. Examples of the visual survey undertaken at Ball Bay.

3.2.2 Benthic Cores

Five benthic cores were collected from a large patch of sand on the edge of the natural rocky reef approximately 200 m from shore on 26 April, 2022 (**Appendix 1**). Samples consisted of mostly sand with

some degraded coral due to the frequent and strong wave action. However, no live organisms above 4 mm were detected amongst the samples collected (**Figure 13**).

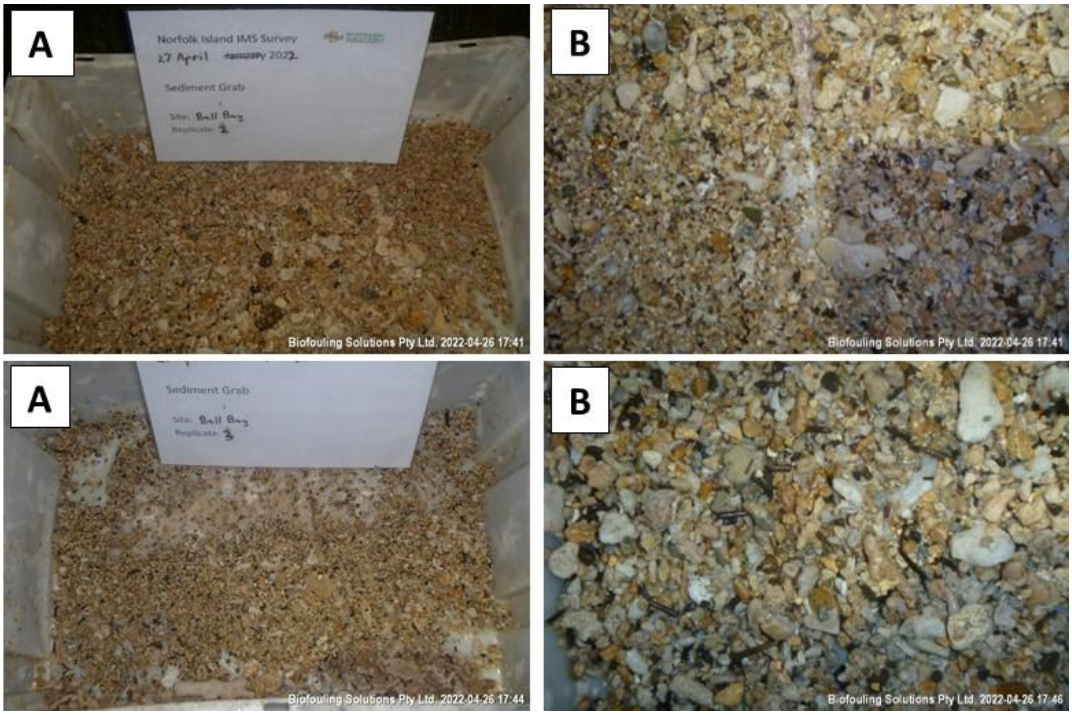

Figure 13. Examples of the sediment collected via the benthic corers. A) Before washing through the 4 mm sieve B) After washing through the 4 mm sieve.

3.2.3 Crab Traps

No crab traps were deployed in Ball Bay due to rough/unsafe sea conditions.

3.2.4 Shore Search

A shore search occurred along the entire length of Ball Bay on 1 May, 2022 (**Appendix 1**). The beach was very exposed and consisted of mostly large boulders with minor patches of smaller rounded stones/pebbles. The intertidal zone consisted of mostly red algae with patches of Eastern Black Crow snails, *Nerita melanotragus* and Yellow clusterwinkles, *Hinea braziliana* (**Figure 14**). No suspected IMS were detected.

3.2.5 Plankton Tows

Five plankton tows were conducted in a semi-circle between ~250-500 m offshore from Ball Bay shoreline (**Appendix 1**). Ball Bay possessed many open ocean phytoplankton species such as the diatoms *Planktoniella sol* and *Rhizosolenia imbricata*, and dinoflagellates *Tripos furca*, *T. fusus*, *T. lineatus*, *T. pentagonus*, and *T. teres* (**Appendix 2**). None of these are considered IMS.

Figure 14. Examples of the terrain encountered during the shore search at Ball Bay. Eastern Black Crow snails, *Nerita melanotragus* and Yellow cluster winkles, *Hinea braziliana* were very frequent and abundant.

3.2.6 eDNA

At Ball Bay no IMS were detected, although the same non-target species, *Chaetoceros tenuissimus* was detected as Cascade Bay. While there is potential for the target IMS *C. concavicornis* to be present, the eDNA was unable to positively detect this IMS (see **Appendix 3**).

3.3 Emily Bay

Visual surveys of Emily Bay occurred on 29, 30 April and 5 May, 2022 (**Appendix 1**). The survey was able to cover the majority of the bay which included a combination of large patches of sand and natural coral reef. Some minor patches of cyanobacteria were noted on the sand patches and the coral reef. Some large patches of *Caulerpa taxifolia* and *C. sertularioides* were noted amongst the sandy areas while patches of *C. lentillifera* and *C. chemnitzia* were observed amongst the coral reef (**Figure 15**). An inspection of the swimming pontoon found the submerged surface to be covered mostly in green, brown and red algae with very few invertebrates present. Of particular interest was the smothering behaviour of a colonial ascidian which resembled the suspected *Diplosoma virens* observed at Cascade Bay. The colonies witnessed along

the southern side of Emily Bay differed from those observed at Cascade Bay and were grey, bulbous and possessed a smothering behaviour which could potentially be confused with the highly invasive *Didemnum perlucidum* (**Figure 15**). No suspected IMS were detected.

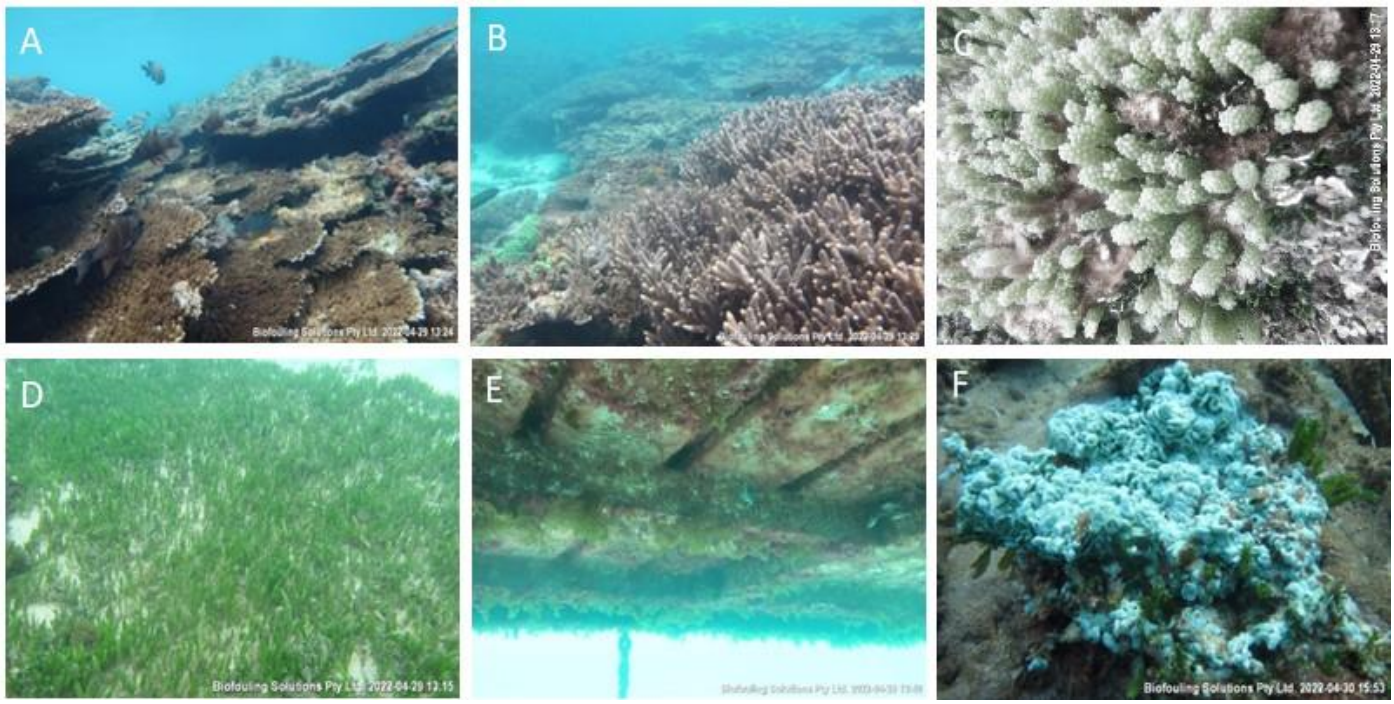

Figure 15. Examples of the marine growth encountered during the inspection of Emily Bay. A) and B) typical natural coral reef C) *Caulerpa lentillifera* and *C. chemnitzia* D) *Caulerpa taxifolia* and *C. sertularioides* E) bottom of the swimming pontoon and F) suspected *Diplosoma virens*.

3.3.1 Benthic Cores

Five benthic cores were collected in 2 m depth of water on the southern side of Emily Bay (i.e. Lone Pine side of the Bay on 29 April, 2022 (**Appendix 1**)). Samples consisted mostly of sand with some degraded coral with occasional sipunculids, tubeworms, *Caulerpa* rhizoids and dead gastropods (**Figure 16**). No suspected IMS were detected amongst the samples collected.

3.3.2 Crab Traps

The five crab traps were deployed on a large patch of sand leading out from the main Slaughter Bay beach on 28 April, 2022 and retrieved the following morning (**Appendix 1**). No crabs or suspected IMS were captured within the traps.

Figure 16. Examples of the contents of the benthic cores collected from Emily Bay.

3.3.3 Shore Search

A shore search occurred along the entire length of Emily Bay on 29 May, 2022 (**Appendix 1**). The beach consisted of a rocky outcrop at the Salthouse Point where numerous crabs (i.e. suspected *Plagsuia squamosa* and *Pachygrapsus* sp.) were noted amongst the rocks followed by a sandy beach where the occasional dead gastropod shell was found (**Figure 17**). An intertidal rocky shore emerges at the southern end of the beach near the Lone Pine and this was also searched. No suspected IMS were detected.

Figure 17. An overview photo of Emily Bay taken near the Lone Pine looking towards Salthouse Point.

3.3.4 Plankton Tows

Five plankton tows were evenly spread out approximately 25 m apart along the beach in approximately 1 meter water depth (**Appendix 1**). Emily Bay possessed mostly benthic diatoms including *Auliscus*, *Diploneis*, *Licmophora*, *Nitzschia, Pleurosigma*/*Gyrosigma*, *Surirella* as well as benthic cyanobacterium *Oscillatoria* (**Appendix 2**). None of these species are IMS.

3.3.5 eDNA

No target IMS were detected within Emily Bay, although the eDNA analysis did detect a toxic dinoflagellate, namely *Gymnodinium catenatum*. This taxon was not on the target list but is a potential IMS of concern. Although, the confidence surrounding this detection was low considering the COI assay detection percentage was only 97.2% and there were equal matches with other species such as *G. impudicum* (97.2%) and even a different genus, *Lepidodinium chlorophorum* (97.2%). Secondly, had *G. catenatum* been present, and in abundance, the phytoplankton sampling should have detected the species presence (see **Appendix 3**). A species of *Sargassum,* namely *S. polycystum* was detected, but not the target IMS *S. muticum.* Analysis of the samples of the suspected *Diplosoma virens* showed that it did not match this species, but rather suggested a ~81% match with *Lissoclinum patella* (Hartmeyer, 1909) (see **Appendix 3**).

3.4 Slaughter Bay

Visual surveys of Slaughter Bay occurred on 28 and 30 April and 3 May, 2022 (**Appendix 1**). The survey was able to cover the majority of the bay, which resembled Emily Bay, consisting of a combination of large patches of sand and natural coral reef. Some larger patches of cyanobacteria were noted on the sand and the coral reef closest to the shore. Large patches of *Caulerpa taxifolia* and *C. sertularioides* were also noted amongst the sandy areas while patches of *C. lentillifera* and *C. chemnitzia* were also noted amongst the coral reef (**Figure 18**). Further patches of a colonial ascidian which resembled *Diplosoma virens* were also noted smothering other benthic organisms in patches on the fringes of the coral and sand margins (**Figure 18**). No suspected IMS were detected.

A further visual survey occurred on the Western side of the Kingston Pier on 2 May, 2022 (**Appendix 1**). The submerged wall was covered in numerous species of green, red and brown algae with some hard corals (**Figure 19**). Some small patches of *Caulerpa* spp. (i.e., C. *taxifolia*, *C. sertularioides, C. lentillifera* and *C. chemnitzia*) and the suspected colonial ascidian, *Diplosoma virens* were also noted on the surrounding reef, although these colonies resembled those witnessed on the Cascade Pier (**Figure 19**). Of particular interest was the detection of two target IMS, namely dozens of empty half-shell Pacific oysters, *Magallana gigas*

measuring 80-110 mm (shell length) and empty New Zealand Greenshell mussel shells, *Perna canaliculus* measuring 90-100 mm (shell length) on the seabed adjacent to the Pier (**Figure 19**).

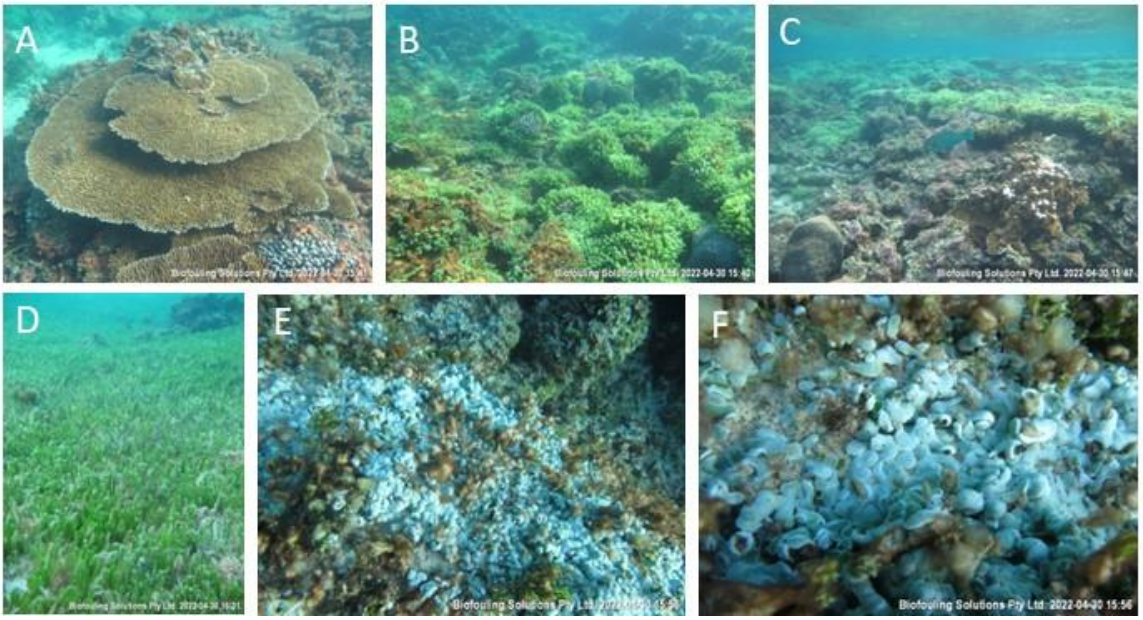

Figure 18. Examples of the marine growth encountered during the inspection of the Slaughter Bay. A) typical natural coral reef B) large patches of *Caulerpa lentillifera* and *C. chemnitzia* present on the coral reef D) *Caulerpa taxifolia* and *C. sertularioides* present on the sand flats E) and F) patches of suspected *Diplosoma virens*.

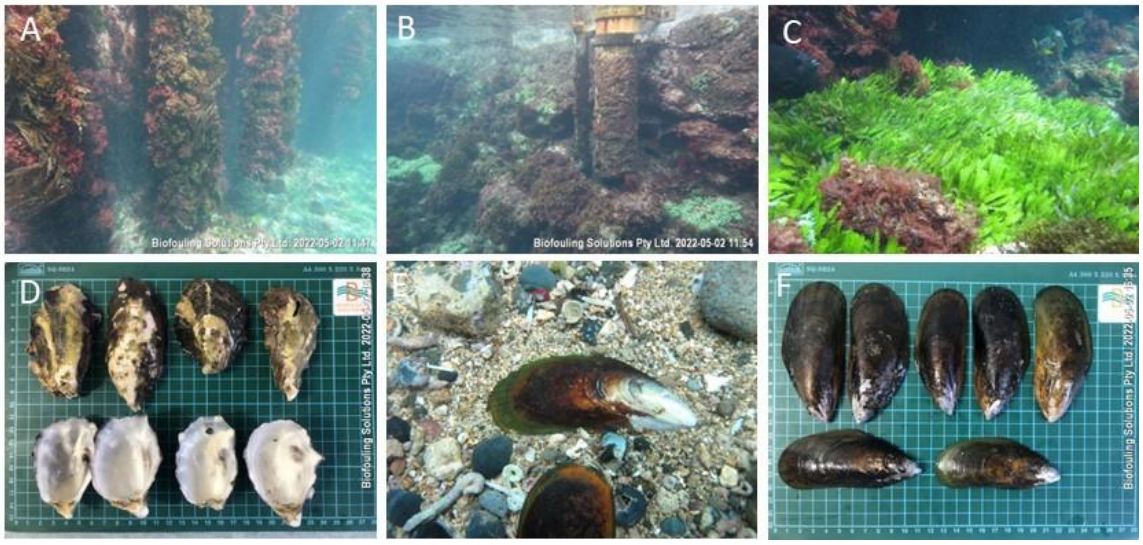

Figure 19. Examples of the marine growth encountered during the inspection of the Kingston Pier. A) and B) typical marine growth on the pier wall C) large patches of *Caulerpa* spp. present on the coral reef D) discarded Pacific oyster shells, *Magellana gigas* found on the seabed E) and F) discarded New Zealand half shell Greenshell mussels, *Perna canaliculus* also found on the seabed.

3.4.1 Benthic Cores

Five benthic cores were collected approximately 40 m from the main Slaughter Bay beach within 2 m depth of water on 28 April, 2022 (**Appendix 1**). Samples consisted mostly of sand with occasional sipunculids, tubeworms, *Caulerpa* rhizoids and dead gastropods (**Figure 20**). No suspected IMS were detected amongst the samples collected.

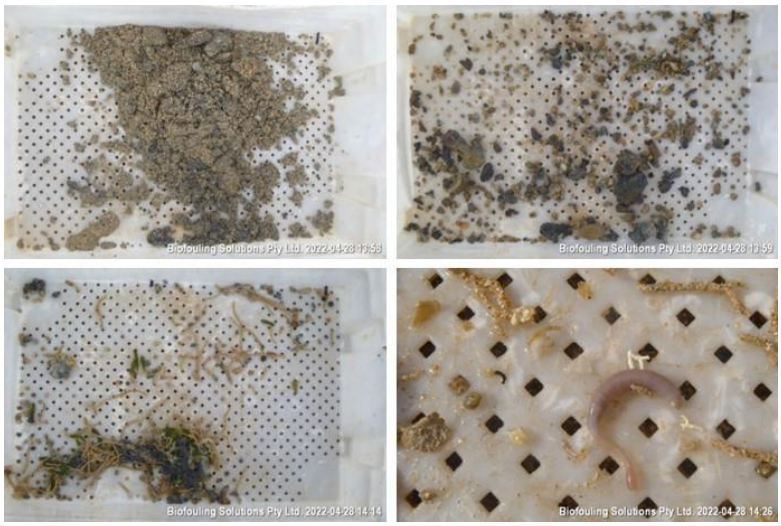

Figure 20. Examples of the contents of the benthic cores collected from Slaughter Bay. Samples consisted of mostly sand with occasional sipunculids, tubeworms, *Caulerpa* rhizoids and dead gastropods.

3.4.2 Crab Traps

Five crab traps were deployed 30-60 m from Slaughter Bay's main beach running perpendicular to the beach on 28 April, 2022 and retrieved the following morning (**Appendix 1**). While no crabs or suspected IMS were captured within the traps, some small gastropods, hermit crabs, holothurians, sea hares, and drift weed were collected (**Figure 21**).

Figure 21. Examples of the contents of the crab traps collected from Slaughter Bay. Samples consisted of some small gastropods, hermit crabs, holothurians, sea hares, and drift weed.

3.4.3 Shore Search

A shore search occurred along the entire length of Slaughter Bay from Kingston Pier to Salthouse Point on 29 May, 2022 (**Appendix 1**). The beach consisted of a rocky shelf with numerous rock pools around the Kingston Pier area through to a sandy beach towards Salthouse Point. The rock pools possessed a wide range of different taxa, many of which were also witnessed while undertaking the dive surveys (**Figure 22**). A single but damaged New Zealand Greenshell mussel, *Perna canaliculus* was detected along the Slaughter Bay side of the Kingston Pier (**Figure 22**). No other suspected IMS were detected.

Figure 22. Examples of the intertidal landscape encountered during the shore search from Kingston Pier to Salthouse Point. A) to D) Kingston Pier E) a damaged New Zealand half shell Greenshell mussels, *Perna canaliculus* found adjacent to the Kingston Pier and F) view looking towards Salthouse Point from Slaughter Bay.

3.4.4 Plankton Tows

Five plankton tows were evenly spread out approximately 20-30 meters apart along the beach within 1 meter of water (**Appendix 1**). Slaughter Bay possessed mostly benthic diatoms including *Auliscus*, *Diploneis*, *Licmophora*, *Nitzschia, Pleurosigma*/*Gyrosigma*, *Surirella* as well as benthic cyanobacterium *Oscillatoria* (**Appendix 2**). None of these are considered IMS.

3.4.5 eDNA

No target IMS were detected within Slaughter Bay, although a species of *Sargassum,* namely *S. polycystum* was detected, but not the target IMS *S. muticum* (**Appendix 3**).

3.5 Other Locations

3.5.1 Diving and Snorkelling

Diving or snorkelling surveys were also undertaken at Anson Bay, Elephant Rock and Duncombe Bay (**Appendix 1**). While no suspected IMS were detected during these dives, patches of the suspected *Diplosoma virens* were noted at Anson Bay, although the colonies were flatter and a vibrant green colour, similar to those witnessed on the Cascade Pier (**Figure 23**).

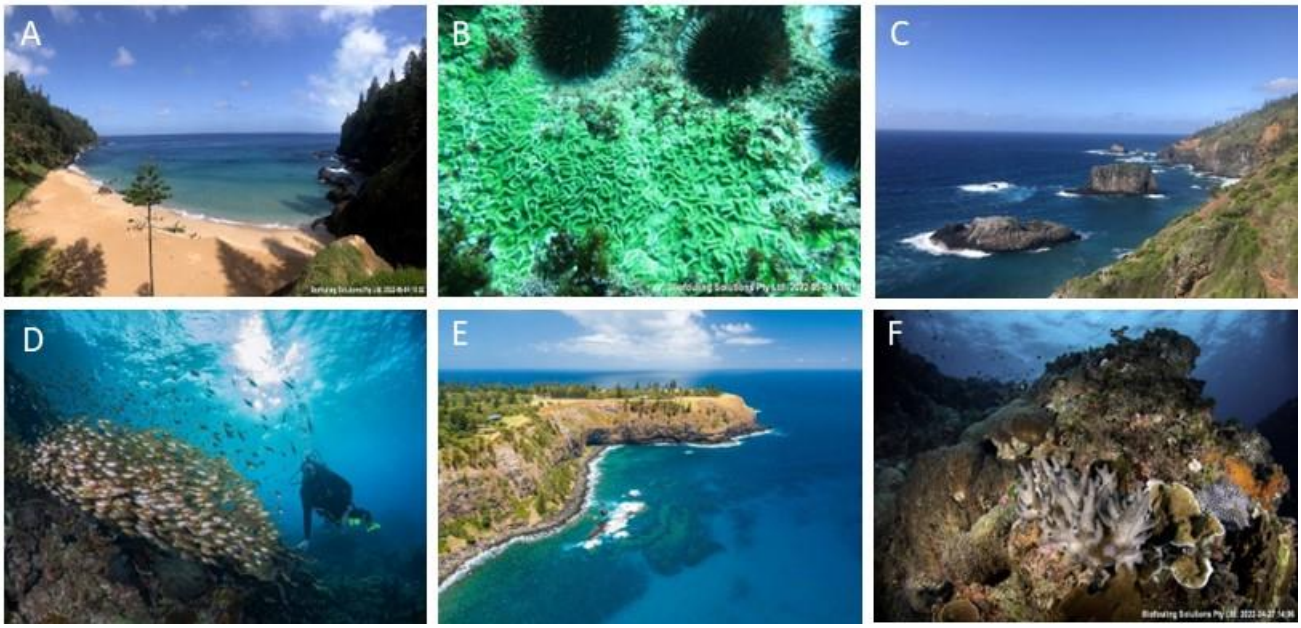

Figure 23. Examples of the scenery encountered during the visual surveys at Anson Bay, Elephant Rock and Duncombe Bay. A) and B) Anson Bay and the colonial ascidian, suspected to be *Diplosoma virens*, C) and D) Elephant Rock, and E) and F) Duncombe Bay.

3.5.2 Shore Search

Shore searches also occurred at Anson Bay, Bumbora Beach, and Cemetery Bay (**Appendix 1**). Anson Bay and Cemetery Bay consisted of golden orange sand while Bumbora Beach consisted of a mixture of large boulders with patches of sand. A variety of different marine organisms such as gooseneck barnacles, *Lepas* sp.; rams-horn squid, *Spirula spirula*; Cowrie shells; and Great violet sea snails, *Janthina janthina* were encountered during the shore searches. No suspected IMS were detected (**Figure 24**).

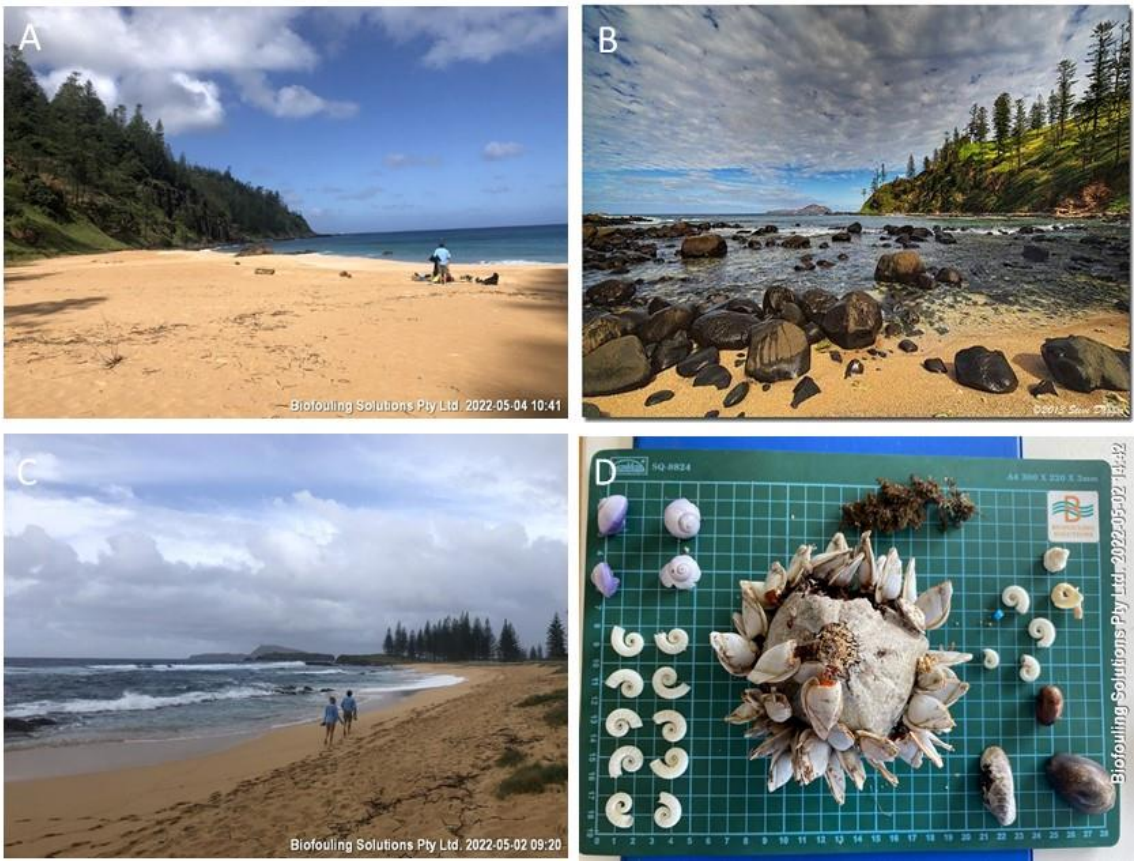

Figure 24. Shore searches also occurred at A) Anson Bay B) Bumbora Beach and C) Cemetery Bay. D) Examples of the various marine organisms (i.e., *Lepas* sp. gooseneck barnacles, rams-horn squid, *Spirula spirula*, Cowrie, Great violet sea snail, *Janthina janthina*) collected during the shore searches.

4 DISCUSSION

4.1 Results

Of the potential six Invasive Marine Species (IMS), namely Japanese seaweed, *Undaria pinnatifida;* New Zealand screwshell, *Maoricolpus roseus;* Japanese skeleton shrimp, *Caprella mutica;* Lady crab/Asian paddle crab, *Charybdis japonica;* and two invasive sea squirts, *Didemnum perlucidum* and *D. vexillum* to be established within the Norfolk Island Marine Park, none of these species were detected during the survey. Two high priority IMS which were on the broader target list, namely Pacific oyster, *Magallana gigas* and New Zealand Greenshell mussels, *Perna canaliculus* were detected during the survey at Cascade and Kingston Pier. However, the specimens detected were all dead half-shells measuring 80-110 mm which matches the commercial food consumption market size for these species. No other live or whole specimens

were detected during the surveys; therefore, it is highly likely that these species were imported frozen for human consumption and discarded at these locations.

While the two invasive sea squirts, *D. perlucidum* and *D. vexillum* were not detected during the surveys, another colonial ascidian, which resembles *Diplosoma virens* (Hartmeyer, 1909) was witnessed on the Cascade Pier, in Emily and Slaughter Bay, on the reef adjacent to the Kingston Pier and at Anson Bay. This particular species is a photosymbiotic sea squirt, hence it has a symbiotic relationship with photosynthetic microalgae (zooxanthellae) similar to corals. While this species is not considered an IMS, the species appeared to be behaving differently within Emily and Slaughter Bay relative to those colonies witnessed outside the lagoon on Cascade Pier, on the natural rocky reef adjacent to the Kingston Pier and at Anson Bay. That is, the size, colour and shape of the colonies witnessed on Cascade Pier, adjacent to the Kingston Pier and at Anson Bay were bright iridescent green colour and relatively flat while those witnessed inside Emily and Slaughter Bay were white/cream colour and more rounded in shape (see **Figure 25**). It remains possible these could be different species. eDNA analysis was conducted on samples collected from Emily and Slaughter Bay which suggested that the samples matched *Lissoclinum patella* (Hartmeyer, 1909). However, this match was only ~81% which is very low.

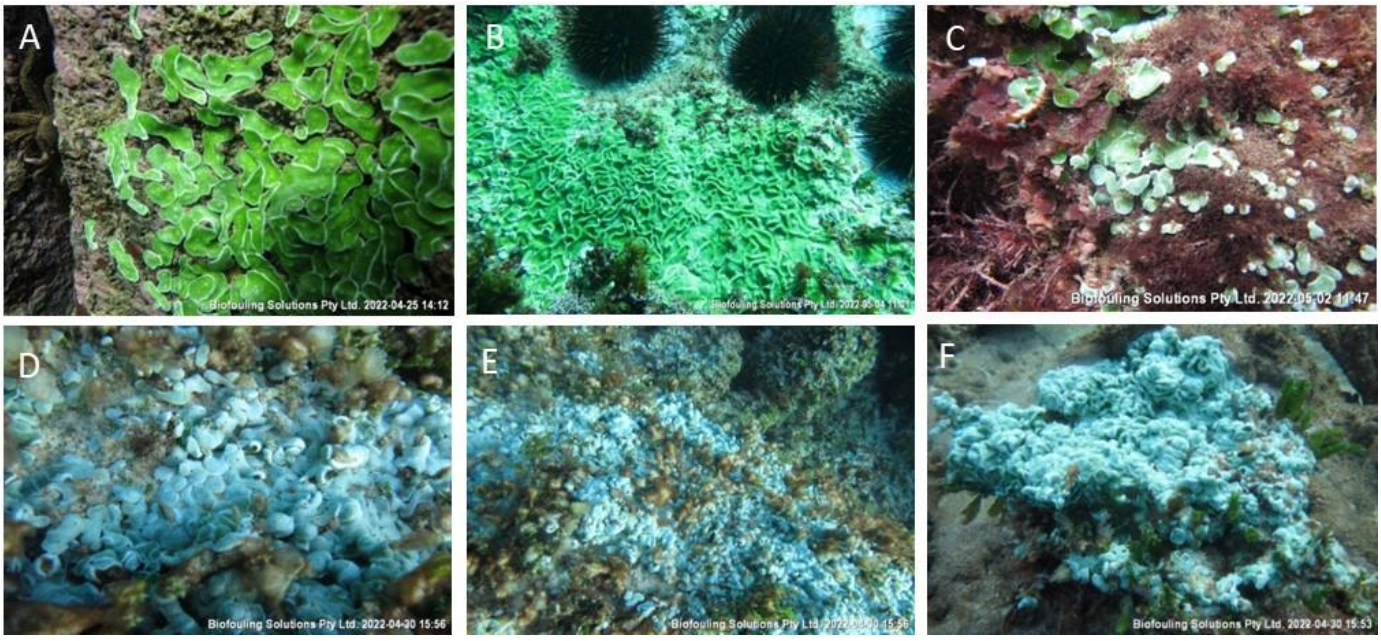

Figure 25. Suspected *Diplosoma virens* colonies at A) Cascade Bay B) Anson Bay C) Kingston Pier D to F) within Emily and Slaughter Bay. Note the difference in colour and shape.

Close inspection of the colonies within Emily and Slaughter Bay showed that some outer margins of the colonies resembled the colour and growth form witnessed on Cascade Pier, adjacent to the Kingston Pier

and at Anson Bay. If these colonies are the same species, namely *D. virens,* it is possible that a combination of differences in water clarity, nutrients and/or hydrodynamic forces could be contributing to these differences in growth forms. That is, colonies at Cascade, Kingston Pier and Anson Bay are likely to be subjected to harsh hydrodynamic forces which may limit their growth forms relative to Emily and Slaughter Bay. Furthermore, there is a possibility that the colonies within Emily and Slaughter Bay are subjected to greater nutrients, hence leading to increased growth with less reliance on the photosynthetic microalgae leading to the change in colour.

The rationale for highlighting the differences in the growth forms is because some colonial ascidians such as *D. perlucidum* and *D. vexillum* have become invasive and caused unwanted impacts. Given that *D. virens* colonies were observed to be growing on and smothering native species in some cases, there is potential for the species to smother native coral and becoming problematic within the lagoon. Interestingly, Susan Prior (of Norfolk Island Reef organisation), has also noticed the species and the size of some of the colonies within the lagoon. Hence, the species should be monitored within the Emily and Slaughter Bay area in case the species responds to a change in environmental conditions which cause it to increase in size and abundance.

Another possible detection included the toxic dinoflagellate, *Gymnodinium catenatum* within Emily Bay via eDNA analysis. While this species is not considered an IMS, it is the only naked dinoflagellate known to be responsible for paralytic shellfish poisoning (PSP), a neurotoxic poisoning syndrome which affects human consumers of contaminated shellfish. Hence, it has a long history of causing catastrophic impacts on shellfish industries around the world affecting thousands of people each year. However, this finding is questionable for a variety of reasons. Firstly, while the COI assay detected a sequence that matched the target species at 97.2%, there were equal matches to other species such as *G. impudicum* (97.2%) and even a different genus, *Lepidodinium chlorophorum* (97.2%). Secondly, had *G. catenatum* been present, and in abundance, the phytoplankton sampling should have detected the species presence.

The likelihood of *G. catenatum* being introduced to Norfolk Island is considered extremely low given that most vessels import commodities, and therefore uptake ballast water rather than discharge it. Although, a vessel ran aground at Ball Bay a number of years ago and was forced to discharge the ballast water to lighten the vessel so it could be retrieved from the rocks (Mitch Graham, Norfolk Island Diving, *pers comm*). While it is acknowledged that the vessel is likely to have complied with Australia's Mandatory Ballast Water Management Requirements, an emergency ballast water discharge has the potential to resuspend and discharge sediments retained within the bottom of tanks which often harbour numerous IMS. Nevertheless,

if *G. catenatum* is present within Emily Bay, it is unlikely to pose a human health risk given there is a lack of shellfish and human consumption of filter-feeding organisms within the lagoon.

The lack of detection of any established IMS at Norfolk Island is not surprising for several reasons. Firstly, the translocation and establishment of IMS around the world is largely associated with vessel biofouling and highly modified and protected port environments. For example, research suggests that up to 69% of IMS introductions are likely to have occurred via organisms attached or associated with vessel hulls (i.e., biofouling) rather than dry/wet ballast, aquaculture, etc (Hewitt et al., 2004; Hewitt and Campbell 2010). In addition, IMS appear to thrive in port environments because they are highly modified environments, possess degraded water quality (Piola and Johnston, 2007; Dafforn et al., 2009a), are often enclosed/protected restricting water exchange (Floerl and Inglis, 2001), with numerous fixed and floating man-made infrastructures (Glasby et al., 2007; Dafforn et al., 2009b).

Given Norfolk Island lacks any substantial port infrastructure, this prohibits vessels from residing close and for prolonged periods, which significantly limits the ability for IMS to transfer and establish. For instance, while many different vessel types have and continue to visit the Island (such as general cargo, oil and gas tankers, cruise ships, tug and barges, yachts, naval vessels, etc; see Biofouling Solutions, 2021a) from mainland Australia, New Zealand and other international locations, all of these vessels either anchor or remain idle off Cascade Bay, Ball Bay or Kingston Pier and rely on smaller vessels for the interactions. Hence, the likelihood of any IMS associated with vessel biofouling departing their hulls and establishing at Norfolk Island is extremely low. It is also unlikely that IMS would be introduced to Norfolk Island via ballast water discharge because: a) the vast majority of vessels which visit the Island import commodities, hence tend take on ballast water rather than discharge, and/or b) all vessels visiting the Island are required to comply with Australia's Mandatory Ballast Water Management Requirements.

4.2 Recommendations

4.2.1 Pre-Border Management Measures – Ballast and Biofouling

Despite the low likelihood of IMS arriving and establishing at Norfolk Island, it is vital that any proposed changes to maritime operations and/or infrastructure around the Norfolk Island carefully consider the potential for IMS introductions. This is because once IMS are introduced and established, there are very few examples of their successful eradication, especially in an open marine environment. Hence, prevention should always be the goal.

Ballast Water Management Considerations

There are proposed plans to export refuse from the Island and such an activity may expose the Island to new IMS risks as such an activity may require vessels to discharge ballast water. Whilst such vessels will be required to abide by Australia's Mandatory Ballast Water Management Requirements prior to entering Norfolk Island waters (i.e. Australian territorial seas), there may be merit in undertaking a specific risk assessment surrounding the proposed vessels, ports of origin and management measures to be adopted and whether these achieve an Appropriate Level of Protection.

Biofouling Management Considerations

The greatest threat of introducing IMS to the Island is likely to be via vessel biofouling. Fortunately, DAFF has introduced Mandatory Biofouling Management Requirements on 15 June, 2022 which requires internationally arriving vessels to demonstrate which of the following three accepted proactive biofouling management options they have adopted to manage their vessel's biofouling:

1) implementation of an effective biofouling management plan; or

2) cleaning of all biofouling within 30 days prior to arriving in Australian territory; or

3) implementation of an alternative biofouling management method pre-approved by DAFF.

DAFF will be focussing on providing education and advice to ship managers with the aim of minimising unintentionally incorrect pre-arrival reporting between 15 June, 2022 and 15 December, 2023. Although such measures may be insufficient to reduce the potential arrival of IMS via vessel biofouling if there are any proposed activities which will require new or novel vessel arrivals at the Island during this period. In addition, it is questionable as to whether these new requirements will apply to domestic vessels arriving from mainland Australia. For instance, a temporary groyne was erected in Ball Bay to facilitate the importation of soil, tarmac, concrete, etc for the Norfolk Island Airport Runway Upgrade during 2020/21. This enabled domestically sourced barges and tugs to have frequent and close interactions within Ball Bay. Without knowing the nature and extent of the operations and whether any biofouling management measures were implemented, such an event had the potential to expose Norfolk Island to IMS established in coastal waters of mainland Australia. If Ball Bay is being considered as a potential location for the building of a temporary or permanent berthing facility, such an activity will increase the Island's potential exposure to IMS in the future.

There may be an opportunity to incorporate specific biofouling management requirements into future tenders for such projects to ensure contracted vessels pose a low likelihood of introducing any biofouling

related IMS risks. For instance, all vessels should possess an effective Biofouling Management Plan and Record Book which demonstrates how their vessel proposes to manage their biofouling during the vessel's in-service period, which could include the following management measures prior to vessels commencing on the project and visiting the Island for the first time:

1) Vessels will be dry-docked, thoroughly cleaned and antifouling coating systems are completely renewed (if less than 12 months old), including within all niche/vulnerable areas.

2) All internal seawater systems will be protected by an effective Marine Growth Prevention System (MGPS) installed preferably within sea chests or at least within internal sea strainer lids.

3) Vessels will undergo an in-water inspection if vessels have remained stationary in coastal waters outside the Norfolk Island Marine Park for more than 21 days to ensure that no IMS or unacceptable risks are present. Such verifications should be conducted by or supervised by a suitably qualified and experienced biofouling inspector.

4) Vessels which remain at Norfolk Island for more than 75 consecutive days should be inspected to ensure that no IMS or unacceptable risks are present. Such verifications should be either be conducted by or supervised by a suitably qualified and experienced biofouling inspector.

5) Any in-water cleaning operations must abide by Australia's Antifouling and In-water Cleaning Guidelines and not occur within the Norfolk Island Marine Park.

4.2.2 Post-Border Measures – On-going Surveillance and Monitoring

An effective biosecurity management system should also include post-border measures such as on-going surveillance for any newly established IMS. While some surveillance programs can be expensive, a cost-effective surveillance program can be implemented at Norfolk Island based on the work BFS has achieved to date. That is, the most likely locations for IMS to arrive and establish include Cascade Bay, Ball Bay, Emily Bay and Slaughter Bay (including Kingston Pier). Fortunately, there is weekly surveillance occurring within Emily and Slaughter Bay by enthusiastic locals from Norfolk Island Reef https://www.norfolkislandreef.com.au/ who regularly snorkel Emily and Slaughter Bay and are likely to notice any changes or newly established species.

The most likely locations for biofouling vectored IMS to establish in the future is at Cascade and Kingston Piers. While it is acknowledged that these two locations are subject to heavy seas and are therefore difficult to access, it may be possible for Norfolk Island Diving to undertake the occasional dive (i.e., every 3-6

months) using a GoPro to record the nature and extent of the biofouling present. This footage could then be sent to an IMS specialist for review. There may be an opportunity to involve a university student who may be interested in using the recordings for monitoring the changes in the community structure over time.

Moreover, there may be potential to include Dr Katherine Dafforn's (Associate Professor and Environmental Scientist at Macquarie University) "Concrete Walls and Living Seawalls" concept. Dr Dafforn and her team have been designing and testing ecologically engineered surfaces for enhancing the establishment of native marine species which in turn increases the immunity to IMS recruitment (see https://www.livingseawalls.com.au/mission). Hence, if some living seawall trials could occur on both the Cascade and Kingston Piers, these would need to be monitored which could also include the surrounding areas. Furthermore, it would be worth incorporating this concept into any proposed extensions or new infrastructure developments around the Island.

Given the present lack of artificial structures at Ball Bay, such a location is less vulnerable to potential IMS recruitment. Furthermore, it is also the most difficult and expensive location to monitor considering it would require a boat, divers or a Remotely Operated Vehicle. Therefore, if there are budgetary constraints, routine monitoring should focus on Kingston Pier, Slaughter Bay, Emily Bay, and Cascade Bay (in order of priority). It is also recommended that a major survey similar to the one, as outlined in this report, occur every 2-3 years which would include Ball Bay and incorporate any other changes or developments.

Should there be any commitment towards the installation of any temporary or permanent infrastructure to facilitate vessel berthing at Ball Bay, then such an activity will not only attract a wide range of vessels and increase disturbance, but it will also potentially increasing the likelihood of IMS exposure and potential establishment. Therefore, it will be vital to design and implement a surveillance program in the Bay to detect IMS. This may involve setting up different settlement arrays to detect any newly released IMS, however the specific surveillance design and methods would need to be tailored to the nature and extent of the proposed project to increase the likelihood of detecting any newly released IMS. In addition, there may be potential to include the design, implementation and on-going surveillance in the tender so that the successful tenderer for building the facility is responsible for ensuring that they do not introduce and IMS into the Bay as a result of their operations.

5 ACKNOWLEDGEMENTS

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- Mitch Graham – Norfolk Island Diving
- Norfolk Island Reef Organisation
- Oxley Travel
- Norfolk Island Holiday House, Ferny Lane House
- NIHH Rental Cars
- Prinke Eco Store

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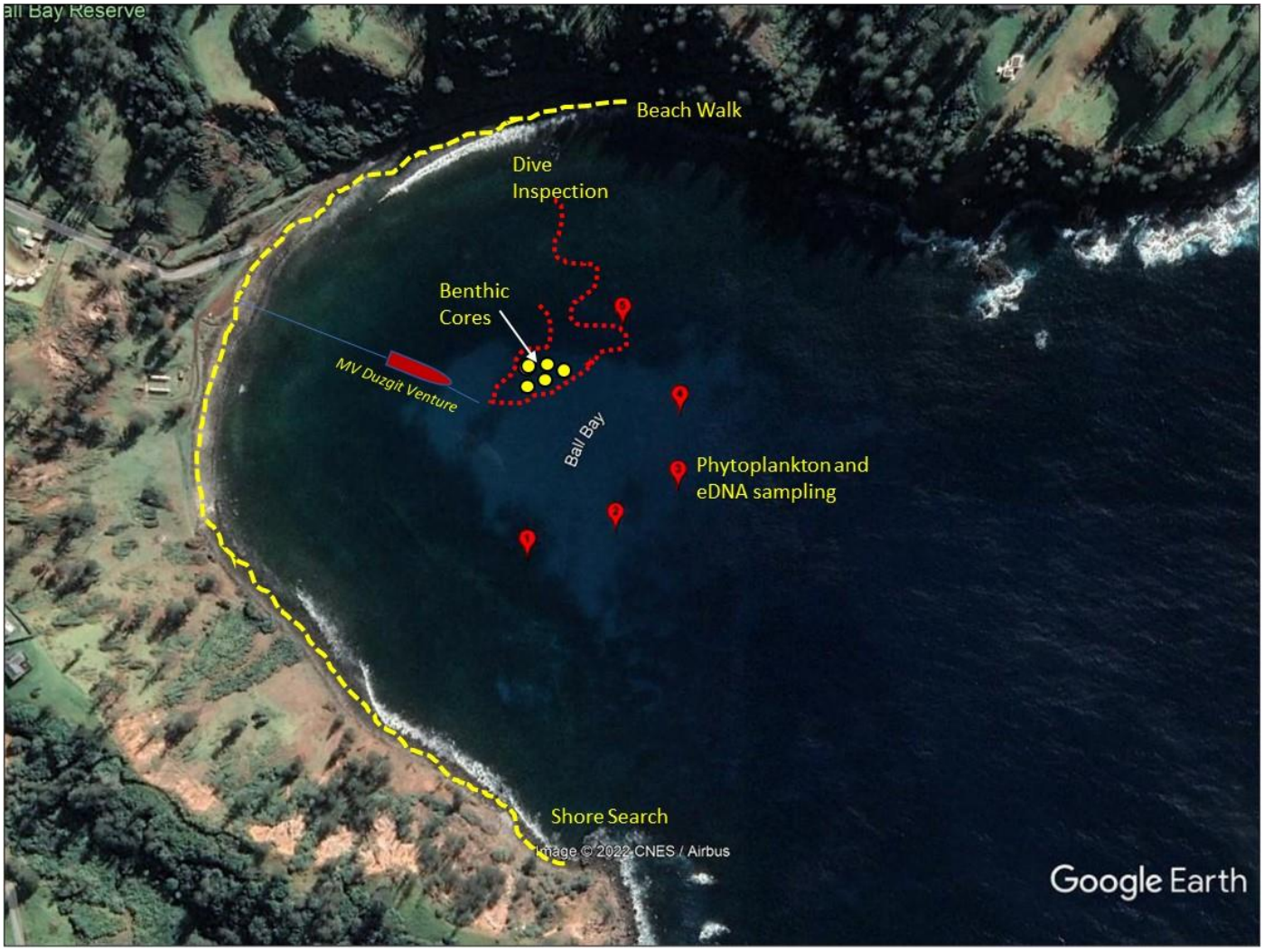
APPENDIX 1 – LOCATION OF DIFFERENT SAMPLING METHODS

Cascade Bay

Method	Details	Latitude and Longitude
Diving/Snorkelling	Cascade Pier - Start	-29.021924° / 167.973332°
	Cascade Pier - Finish	-29.021761° / 167.973451°
	Outer Cascade Bay – Start/Finish	-29.018781° / 167.972799°
Crab Traps	Start of backbone	-29.018781° / 167.972799°
	End of backbone	-29.018768° / 167.972161°
Sediment Core	5 x cores around this location	-29.021501° / 167.973167°
Shore Search	Start	-29.020714° / 167.970080°
	Finish	-29.021785° / 167.976276°
Phytoplankton tows and eDNA	Sample No. 1	-29.020300° / 167.972120°
	Sample No. 2	-29.019767° / 167.972433°
	Sample No. 3	-29.019050° / 167.973100°
	Sample No. 4	-29.019217° / 167.974067°
	Sample No. 5	-29.019583° / 167.974950°

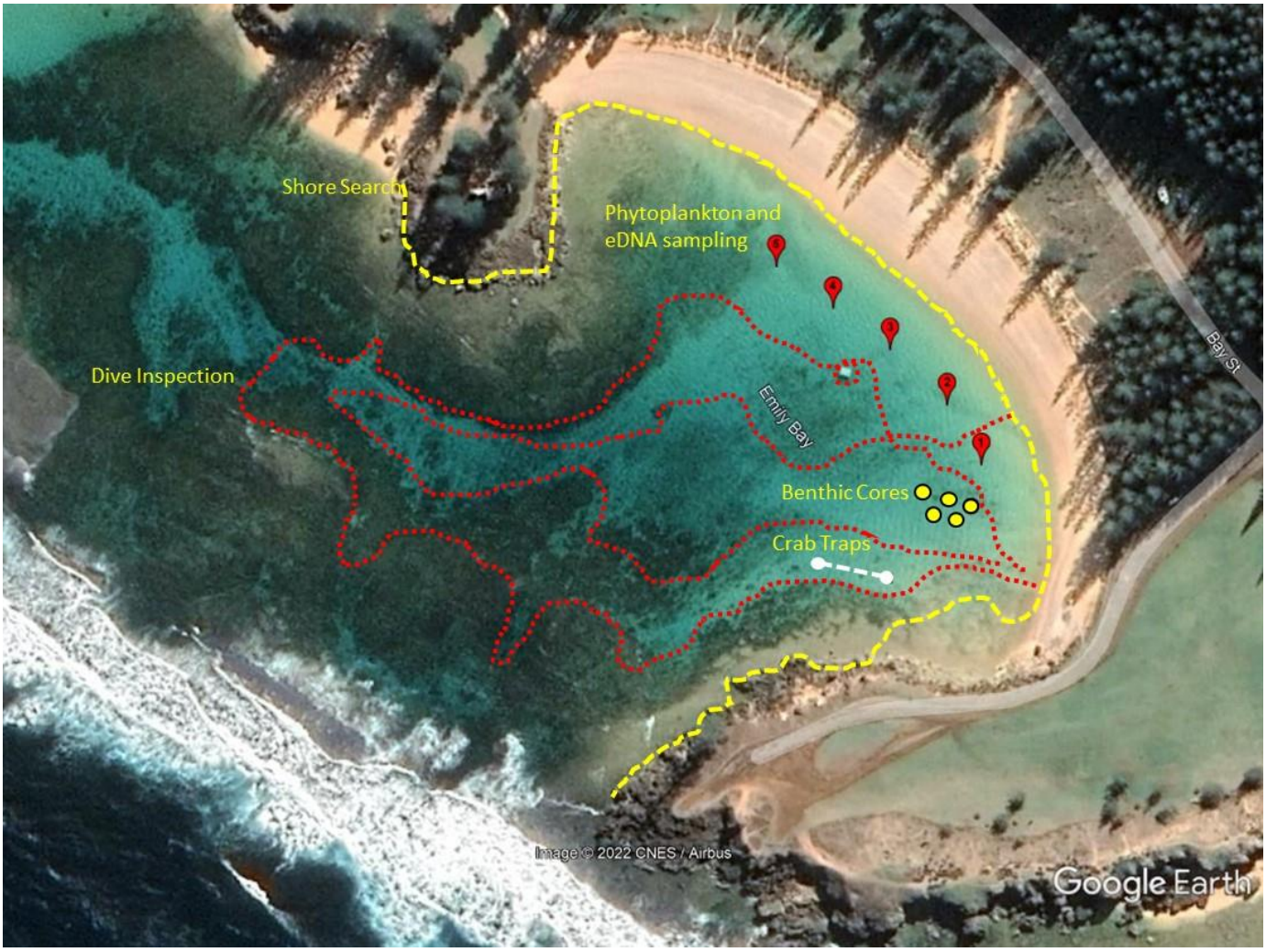
Ball Bay

Method	Details	Latitude and Longitude
Diving/Snorkelling	Start	-29.050540° / 167.985710°
	Finish	-29.047855° / 167.985891°
Crab Traps	N/A	
Sediment Core	5 x cores around this location	-29.050540° / 167.985710°
Shore Search	Start	-29.046773° / 167.986537°
	Finish	-29.053121° / 167.986131°
Phytoplankton tows and eDNA	Sample No. 1	-29.050540° / 167.985710°
	Sample No. 2	-29.050310° / 167.986570°
	Sample No. 3	-29.049950° / 167.987180°
	Sample No. 4	-29.049310° / 167.987200°
	Sample No. 5	-29.048560° / 167.986640°



Emily Bay

Method	Details	Latitude and Longitude
Diving/Snorkelling	Start	-29.061191° / 167.963181°
	Finish	-29.060861° / 167.963076°
Crab Traps	Start of backbone	-29.061390° / 167.962590°
	Finish of backbone	-29.061379°/ 167.962152°
Sediment Core	5 x cores around this location	-29.061140° / 167.962920°
Shore Search	Start	-29.060032° / 167.960682°
	Finish	-29.062156° / 167.961517°
Phytoplankton tows and eDNA	Sample No. 1	-29.061002° / 167.962958°
	Sample No. 2	-29.060792° / 167.962828°
	Sample No. 3	-29.060595°/ 167.962609°
	Sample No. 4	-29.060446° / 167.962388°
	Sample No. 5	-29.060299° / 167.962161°



Slaughter Bay (including Kingston Pier)

Method	Details	Latitude and Longitude
Diving/Snorkelling	Slaughter Bay - Start	-29.058819° / 167.958262°
	Slaughter Bay - Finish	-29.058819° / 167.958262°
	Kingston Pier - Start	-29.057791° / 167.954268°
	Kingston Pier - Finish	-29.058420° / 167.953109°
Crab Traps	Start of backbone	-29.059149° / 167.958104°
	Finish of backbone	-29.059412° / 167.958036°
Sediment Core	5 x cores around this location	-29.061140° / 167.962920°
Shore Search	Start	-29.060032° / 167.960682°
	Finish	-29.060210° / 167.960703°
Phytoplankton tows and eDNA	Sample No. 1	-29.059280° / 167.959500°
	Sample No. 2	-29.059160° / 167.959080°
	Sample No. 3	-29.059020°/ 167.958670°
	Sample No. 4	-29.058950° / 167.958470°
	Sample No. 5	-29.058870° / 167.957970°

Anson Bay

Method	Details	Latitude and Longitude
Diving/Snorkelling	Start	-29.009641° / 167.922469°
	Finish	-29.009641° / 167.922469°
Shore Search	Start	-29.009228° / 167.922381°
	Finish	-29.010399° / 167.922946°

Bumbora Beach

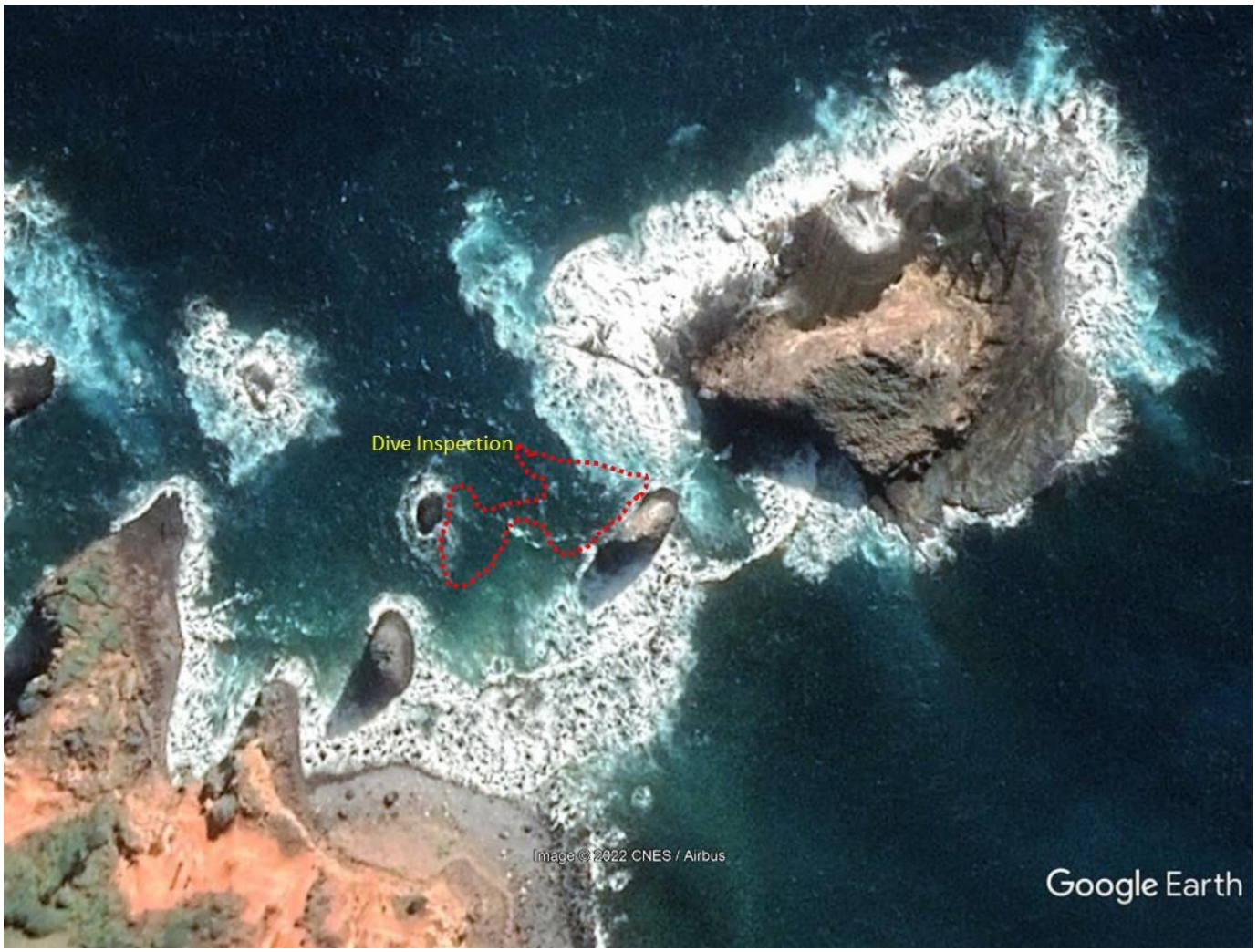
Method	Details	Latitude and Longitude
Shore Search	Start	-29.058024°/ 167.944431°
	Finish	-29.059808° / 167.943147°

Cemetery Bay

Method	Details	Latitude and Longitude
Shore Search	Start	-29.057098° / 167.969596°
	Finish	-29.062593° / 167.963522°

Elephant Rock

Method	Details	Latitude and Longitude
Diving/Snorkelling	Start	-29.005400° / 167.955873°
	Finish	-29.005400° / 167.955873°

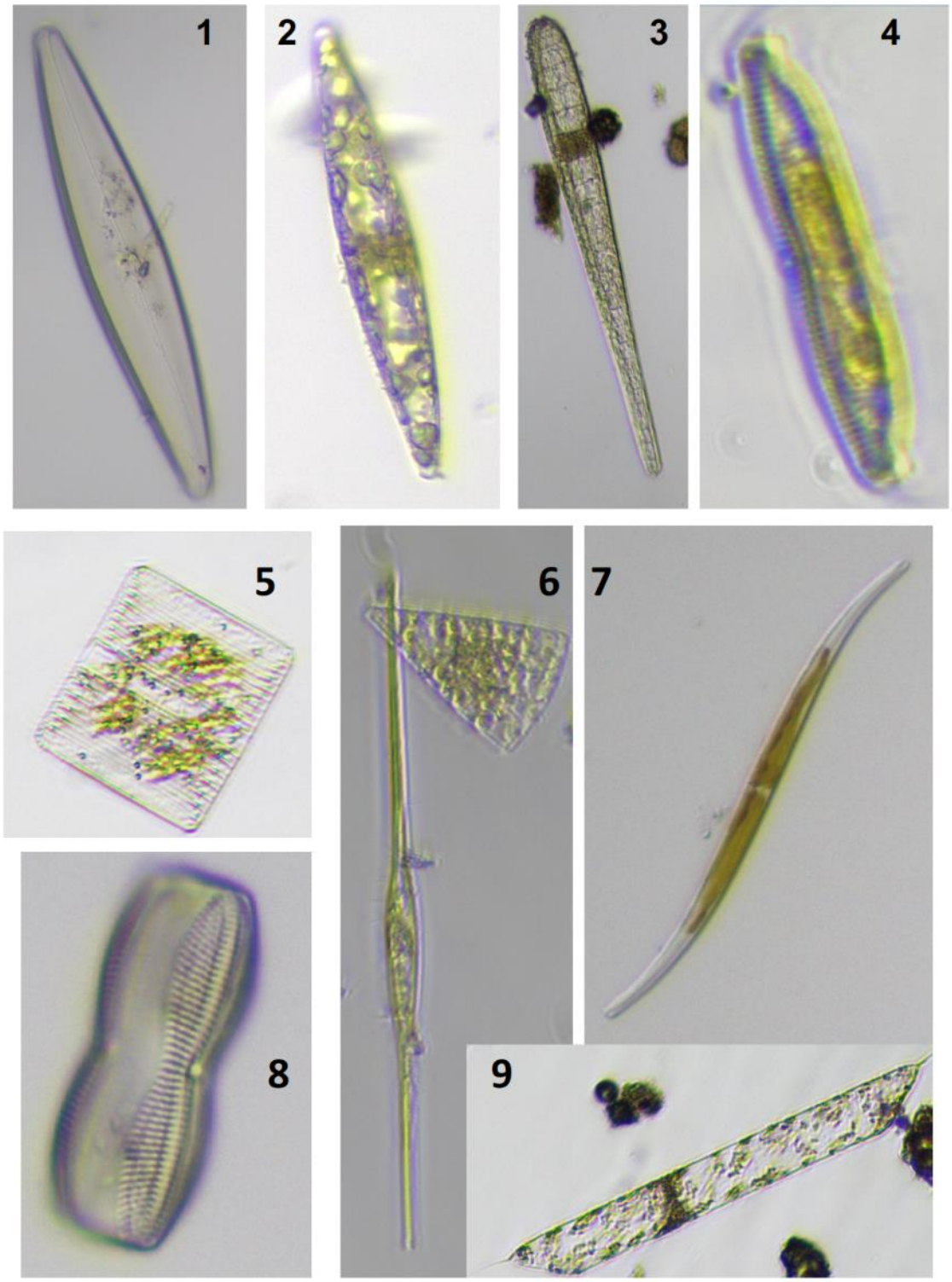


Duncombe Bay

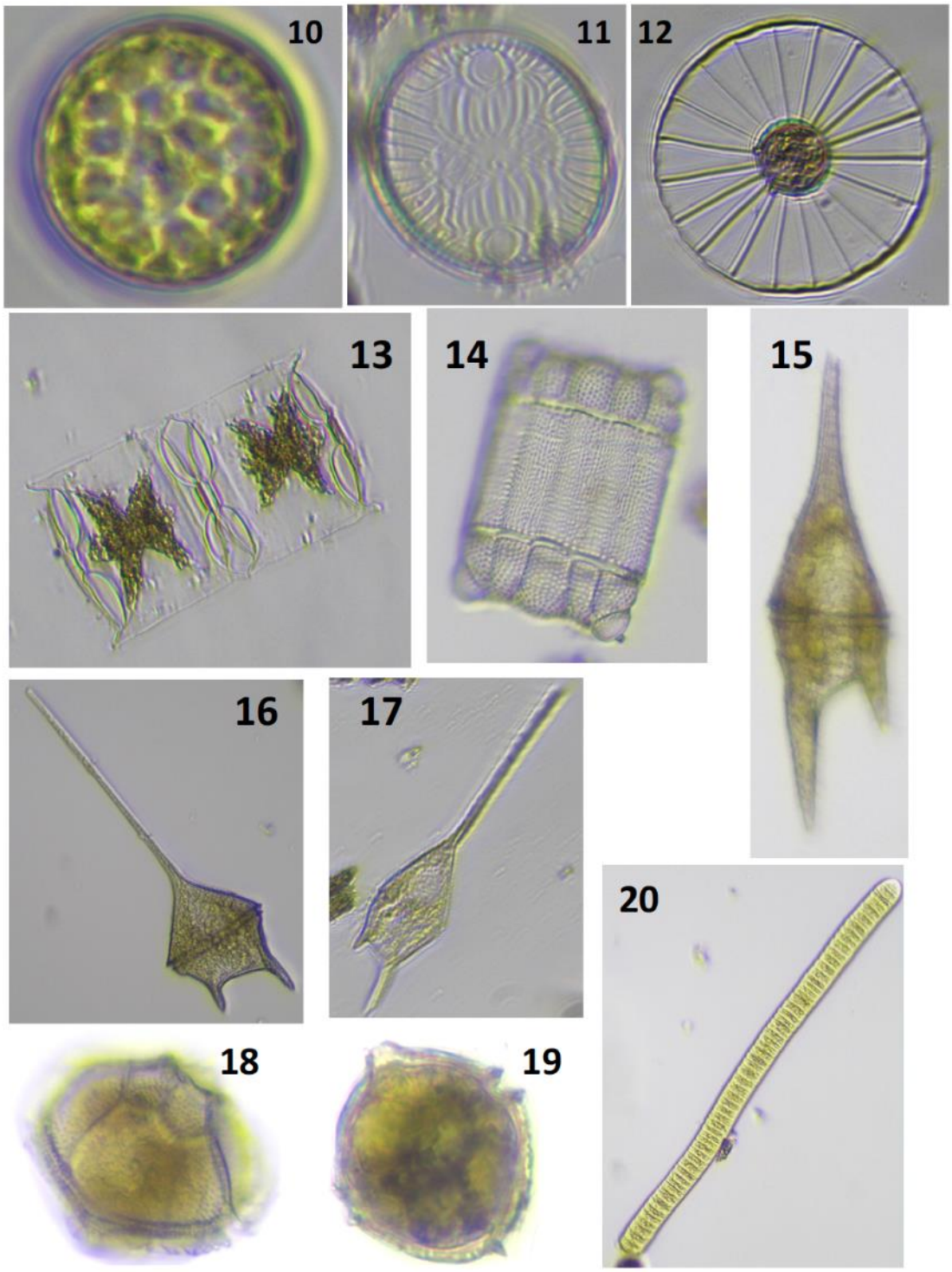
Method	Details	Latitude and Longitude
Diving/Snorkelling	Start	-28.996906° / 167.932326°
	Finish	-28.996906° / 167.932326°

APPENDIX 2 – PHYTOPLANKTON RESULTS

Norfolk Island 25/4/22 to 29/4/22	**Cascade Bay** Composite of CB-1,2,3,4,5	**Ball Bay** Composite of BB-1,2,3,4,5	**Slaughter Bay** Composite of SB-1,2,3,4,5	**Emily Bay** Composite of EB-1,2,3,4,5
Diatoms				
Auliscus				1
Ceratoneis closterium				1
Biddulphia			1	
Diploneis			1	
Gyrosigma/Pleurosigma	2	2	9	2
Licmophora	1	5	15	5
Nitzschia sp.		8		13
Nitzschia sigmoides			1	
Odontella	6	8		
Planktoniella sol		1		
Rhizosolenia imbricata		1		
Surirella		1		
Striatella				1
Thalassiosira				1
Dinoflagellates				
Goniodoma		1		
Gonyaulax	1			
Protoperidinium	1			
Scrippsiella			1	
Tripos furca		1		
Tripos fusus		1		
Tripos lineatus		1		
Tripos pentagonus		1		
Tripos teres	1	1		
Cyanobacteria				
Oscillatoria		7	7	5
Total cells examined 115	12	39	35	29



Diatoms: *1,2. Pleurosigma/Gyrosigma; 3. Licmophora; 4. Nitzschia; 5. Striatella; 6. Ceratoneis +Licmophora; 7. Nitzschia sigmoides; 8. Diploneis; 9. Rhizosolenia imbricata.*



Diatoms: *10. Thalassiosira; 11. Auliscus; 12. Planktoniella sol; 13. Odontella; 14. Biddulphia*;
Dinoflagellates: *15. Tripos furca; 16. Tripos lineatus; 17. Tripos teres; 18. Goniodoma; 19. Gonyaulax;* Cyanobacterium *20. Oscillatoria.*

APPENDIX 3 – eDNA FRONTIERS RESULTS

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REPORT OF eDNA ANALYSIS

Scope of Work:	EF-171		
Project Title:	Marine invasive species baseline audit of the coastal regions of Norfolk Island, South Pacific Ocean using eDNA metabarcoding.		
Client:	Biofouling Solutions (ABN: 62 134 732 193) 244 Summerleas Road, Kingston, Tasmania Australia 7050		
Contact Details:	Dr Joe Valentine Environmental Scientist E: jvalentine@aquenal.com.au | T: +61 (3) 6229 2334		
Test Facility	eDNA frontiers Curtin University (ABN: 99 143 842 569) 303.194 Kent Street Bentley WA 6102 Phone: +61 8 9266 4119 Email: ednafrontiers@curtin.edu.au		
Study Author:	Dr Kat Dawkins eDNA frontiers | Curtin University Email: kat.dawkins@curtin.edu.au		
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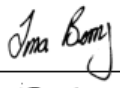
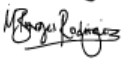
Report Reference:	EF171_Aquenal_RevA		
Laboratory Start Date:	27/05/2022	**Laboratory End Date:**	16/06/2022
Report Issue Date:	30/06/2022		

APPROVALS

	Author Name	Signature	Date (DD/MM/YYYY)
Author	Dr Kathryn Dawkins		30/06/2022
Author	Dr Tina Berry		30/06/2022
Reviewer	Melissa Borges Rodriguez		30/06/2022

DISCLAIMER

Note: If this eDNA report has specific parts reproduced and cited within a wider report on field work, results displayed should be attributed to eDNA frontiers (Curtin University) and the report included in an appendix in its entirety for referencing purposes.

EXECUTIVE SUMMARY

Objective:
This report details the processing and analysis of water samples collected from the coastal regions of Norfolk Island in the South Pacific Ocean. Using environmental DNA (eDNA) metabarcoding, the eDNA frontiers laboratory was tasked with the detection of any DNA signatures attributable to Alien Invasive Species (AIS).

Results:
Across the three metabarcoding assays applied to water samples, a wide range of taxa were detected; however, the primary interest of this study was the detection of AIS species rather than biodiversity.

Because reference DNA databases are not complete (i.e., not all native and invasive species have been sequenced), it is not always possible to confidently assign species identity. Therefore, sequence data was screened for potential AIS detections and flagged for further investigation where appropriate. Potential AIS were highlighted for further investigation from five AIS families, with all detected by the COI assay. However, it should be noted that species across both the Western Australia and Queensland Target AIS Lists (as well as some additional species of interest) were investigated in this study and, as such, not all highlighted detections may be of relevance to the study area.

In addition to the eDNA metabarcoding study, a tissue sample of suspect *Diplosoma virens* was submitted for species identification purposes. This sample was sanger sequenced and identified to be most similar to *Lissoclinum patella*.

1.0 SAMPLE DETAILS

Table 1. Sample receipt details

Date received:	12/05/2022
Transport:	Frozen
Number of samples:	23
Storage:	All samples were stored at -20°C prior to analysis.

Table 2. Supplied sample details.

eDNA frontiers ID	Client Sample ID	Collection Location	Sample Type	Collection Date
E-171-001	CB-1	Cascade Bay, Norfolk Is	Water filter	25/04/2022
E-171-002	CB-2	Cascade Bay, Norfolk Is	Water filter	25/04/2022
E-171-003	CB-3	Cascade Bay, Norfolk Is	Water filter	25/04/2022
E-171-004	CB-4	Cascade Bay, Norfolk Is	Water filter	25/04/2022
E-171-005	CB-5	Cascade Bay, Norfolk Is	Water filter	25/04/2022
E-171-006	BB-1	Ball Bay, Norfolk Is	Water filter	26/04/2022
E-171-007	BB-2	Ball Bay, Norfolk Is	Water filter	26/04/2022
E-171-008	BB-3	Ball Bay, Norfolk Is	Water filter	26/04/2022
E-171-009	BB-4	Ball Bay, Norfolk Is	Water filter	26/04/2022
E-171-010	BB-5	Ball Bay, Norfolk Is	Water filter	26/04/2022
E-171-011	Rinsate #1	-	Water filter	-
E-171-012	SB-1	Slaughter Bay, Norfolk Is	Water filter	28/04/2022
E-171-013	SB-2	Slaughter Bay, Norfolk Is	Water filter	28/04/2022
E-171-014	SB-3	Slaughter Bay, Norfolk Is	Water filter	28/04/2022
E-171-015	SB-4	Slaughter Bay, Norfolk Is	Water filter	28/04/2022
E-171-016	SB-5	Slaughter Bay, Norfolk Is	Water filter	28/04/2022
E-171-017	EB-1	Emily Bay, Norfolk Is	Water filter	29/04/2022
E-171-018	EB-2	Emily Bay, Norfolk Is	Water filter	29/04/2022
E-171-019	EB-3	Emily Bay, Norfolk Is	Water filter	29/04/2022
E-171-020	EB-4	Emily Bay, Norfolk Is	Water filter	29/04/2022
E-171-021	EB-5	Emily Bay, Norfolk Is	Water filter	29/04/2022
E-171-022	Rinsate #2	-	Water filter	-
E-171-023	Ascidian	-	Tissue in ethanol	-

2.0. METHODS

2.1 Sample Collection (Aquenal staff)

Water samples were collected at four locations by Aquenal staff between 25th and 29th April 2022. Five replicates were collected at each sampling point, giving a total of 20 samples. Water samples were collected and filtered using 0.45μm mixed cellulose ester (MCE) with a peristaltic Sentino pump to capture eDNA present in the water. All filtering was carried out by Aquenal staff, with two rinsate controls included to test for contamination due to the use of common filtration equipment. Additionally, a tissue sample of a suspect AIS (*Diplosoma virens*) was also collected. Samples were transported frozen (filter papers) or in ethanol (tissue specimen) to eDNA frontiers laboratories where they were stored at -20°C until scheduled for DNA extraction.

2.2 eDNA Extraction and Analysis

DNA was extracted from half of each filter paper and the tissue sample using a Qiagen DNeasy blood and tissue kit, following the eDNA frontiers lab's SOPs and detailed in Koziol *et al.,* (2018), Stat *et al.,* (2017), and Stat *et al.,* (2018). Each water sample was assigned an individual combination of index tags and amplified by PCR using three assays: (1) a broad mitochondrial COI assay, (2) a universal 16S assay, and (3) a 16S assay targeting bivalves. Libraries were generated and sequenced using the Illumina MiSeq. Laboratory extraction and PCR controls were included to test for contamination. The tissue sample was amplified by PCR using a COI assay, with the PCR product outsourced for sanger sequencing.

2.3 Bioinformatics and Taxonomic Assignments

Bioinformatic tools were used to analyse raw sequence data (Mousavi-Derazmahalleh *et al.,* 2021) generated from the metabarcoding. The sequencing results were demultiplexed and trimmed using Obitools and quality filtered with Usearch v11 for sequencing errors (maxee=1) with an appropriate minimum length used (150 for COI, 100 for the universal 16S, and 150 for the bivalve 16S). Sequences were then dereplicated and unique sequences were transformed into zero radius operational taxonomic units (ZOTUs) to provide sensitive taxonomic resolution (Usearch v11) (Edgar, 2018). ZOTUs, in contrast to OTUs, are a more exact sequence variant, clustering at 99% to improve taxonomic resolution. Generated ZOTUs were queried against the nucleotide database NCBI (GenBank) and assigned to the species-level where possible. Taxonomic assignments were based on an in-house Python script which further filters the Blast results (evalue ≤1e-5, %identity ≥95, qCov =100, LULU minMatch =97%), combines them with the ZOTU table results and produces a table containing the taxonomic information available from Blast taxonomy database (accessed May 2022).

Sequencing analysis of the tissue sample was performed in Geneious Prime (version 2021.0.3) where the generated sequence was queried against GenBank, sequences for the closest matches as well as *Diplosoma virens* downloaded, and an alignment produced. Sequences were then trimmed to the same length and pairwise comparisons calculated to determine the percent similarity between them.

It is important to note that while sequences recovered are converted to the lowest possible taxon based on similarities and differences to a DNA database (NCBI's GenBank), this database, and the taxonomic framework that underpins it, may contain errors. Accordingly, the DNA taxon identifications should be interpreted as the best available assignment based on currently available information and that errors are possible. It is beyond the scope of this present study, but the fidelity of the taxonomic identifications presented here could be further optimised based on specific knowledge and expertise in the taxa of interest.

Following stringent quality control filtering as described by eDNA frontiers' standard operating procedures, the final DNA sequences were screened to determine presence and absence of target alien invasive species (Appendix 1).

Environmental DNA is a powerful and novel method for detection of AIS. However, like morphological analysis, one of the key steps is to differentiate the (genetic) features of an AIS from local taxa that might be 'mistaken' for an AIS (i.e., a false positive). Until suitable reference barcodes are obtained from local closely related taxa, conservative assignments must be made. Accordingly, matches that indicate further investigation may be warranted have been highlighted, with a percent identity match provided.

3.0 RESULTS

3.1 Invasive Species Detections

All sequences were screened for assignment to families that contain potential AIS. Because the assays employed are discriminatory to a species level, only sequences that match the AIS genera have been reported. Detections were highlighted for further investigation where the criteria outlined below were met (Tables 4 and 5).

- There was a direct match to an AIS at ≥97%, or
- There was a match to an AIS genus at ≥95% and the target AIS is either unlikely to be or will not be detected by the assay.

From the water samples, six ZOTUs were highlighted for further investigation by the COI assay. This includes three detections from families that are only listed for Western Australia (not Queensland) and therefore may not be priority detections.

Only one AIS genus was detected using the universal 16S assay, but this detection was not highlighted for further investigation. No AIS genera were detected using the bivalve 16S assay. The remaining sequences matched non-target taxa.

Pairwise comparisons were calculated between the suspect *Diplosoma virens* tissue sample and closest GenBank matches. After sequences were trimmed to the same length, the closest match for the tissue sample was to *Lissoclinum patella* (Table 3); however, this match was still at a very low percent (~81%) indicating that the sample likely matches a species that does not have a reference sequence available.

Table 3. Pairwise similarity between COI sequences generated from the unknown tissue sample, *Diplosoma virens*, and sequences of most similar species retrieved from GenBank.

Most similar/target species	Pairwise similarity to sample
Lissoclinum patella	81.32%
Ciona intestinalis	76.92-77.47%
Ciona robustus	76.37-77.47%
Diplosoma virens	75.27-75.82%

Table 4. Taxa found within AIS families using the COI assay for water samples collected off the coast of Norfolk Island. Genus/species in red text are direct matches to an AIS; matches in highlighted rows may require further investigation; cells are filled with yellow where only a single sequence read was returned; an * next to the family name indicates the family is listed as having invasives for Western Australia but not Queensland. Likelihood of detection is based on whether reference sequence(s) are available in GenBank and whether the assay matches to it *in silico*; Y=yes, P=possible, U=unlikely, N=no.

Target AIS Family	Target AIS	Likelihood of Detection	OTU	Species	% match	CB	BB	SB	EB
Bacillariaceae	*Pseudo-nitzschia seriata*	N	Zotu2901	*Pseudo-nitzschia sabit*	99.7	*	*	*	
			Zotu4832	*Pseudo-nitzschia cuspidata*	99.7	*	*		*
				Pseudo-nitzschia pseudodelicatissima	99.4				
Chaetocerotaceae	*Chaetoceros concavicornis* *Chaetoceros convolutus*	N N	Zotu6829	*Chaetoceros tenuissimus*	95.5	*	*		
Clionaidae*	*Cliona thoosina*	N	Zotu7701	*Cliona delitrix*	95.8			*	
				Cliona sp. 1 PRT-2020	95.5				
				Cliionaopsis platei	96.2				
Gymnodiniaceae*	*Gymnodinium catenatum*	P	Zotu1372	*Gymnodinium impudicum*	97.2				*
				Gymnodinium catenatum	97.2				
				Lepidodinium chlorophorum	97.2				
				Margalefidinium polykrikoides	96.8				
Niphatidae*	*Gelliodes fibrosa*	N	Zotu8948	*Gelliodes cf. gracilis MTR-2018*	98.7			*	
				Haliclona amboinensis	98.7				
Sargassaceae	*Sargassum muticum*	P	Zotu1211	*Sargassum polycystum*	99.4			*	*
			Zotu1980	*Sargassum vachellianum*	100.0				
				Sargassum hemslowianum	100.0				
				Sargassum natans	99.7			*	
				Sargassum fluitans	99.4				
				Sargassum sp.	99.4				

Table 5. Taxa found within AIS families using a universal 16S assay for water samples collected off the coast of Norfolk Island. Genus/species in red text are direct matches to an AIS; matches in highlighted rows may require further investigation; an * next to the family name indicates the family is listed as having invasives for Western Australia but not Queensland. Likelihood of detection is based on whether reference sequence(s) are available in GenBank and whether the assay matches to it *in silico*; Y=yes, P=possible, U=unlikely, N=no.

Target AIS Family	Target AIS	Likelihood of Detection	OTU	Species	% match	CB	BB	SB	EB
Grapsidae*	*Pachygrapsus fakaravensis*	Y	Zotu196	*Pachygrapsus laevimanus*	100.0				*
				Planes minutus	100.0				

4.0 SUMMARY

This report documents the findings of an AIS investigation using eDNA metabarcoding of water samples collected from the coastal regions of Norfolk Island, and sanger sequencing of a suspect invasive species. Detections within five AIS families were highlighted for further investigation using metabarcoding; however, the detection list used includes both AIS for both Western Australia and Queensland and, as such, not all detections may be relevant to the study area. Analysis of the tissue sample showed that it did not match the AIS *Diplosoma virens*, with the closest match to *Lissoclinum patella* at ~81%. Such a low similarity match suggests that the sample matches a species that does not have a reference sequence available on GenBank.

ARCHIVING OF STUDY DATA

The DNA extracts derived from this study will be stored within eDNA frontiers' premises for a period of 12 months. If samples are required to be stored longer a sample archiving service can be provided.

All electronic data relating to the study is stored in an offsite secure server. This includes; all laboratory raw data; personnel records; and the study report. Hard copy documents are archived by study number into a locked area of the test facility located in eDNA frontiers, Curtin University administration area.

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Mousavi-Derazmahalleh M, Stott A, Lines R, Peverley G, Nester G, Simpson T, Zawierta M, De La Pierre M, Bunce M, Christophersen CT (2021). eDNAFlow, an automated, reproducible and scalable workflow for analysis of environmental DNA (eDNA) sequences exploiting Nextflow and Singularity. *Molecular Ecology Resources*.

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Stat M, John J, DiBattista JD, Newman SJ, Bunce M, Harvey ES (2018) Combined use of eDNA metabarcoding and video surveillance for the assessment of fish biodiversity. *Conservation Biology* 0, 1-10.

APPENDIX 1

Families of marine pests that are screened against the metabarcoding assays data output. An * indicates where the AIS is listed on Queensland's and/or Western Australia's target species list. Those in the 'Request' column are of particular interest in this study.

Phylum	Family	Species	Target AIS		
			Qld	WA	Request
Annelida	Sabellidae	*Sabella spallanzanii*	*	*	*
	Serpulidae	*Hydroides dianthus*		*	
	Spionidae	*Marenzelleria spp.*	*	*	
		Marenzelleria neglecta			*
Arthropoda	Acartiidae	*Acartia tonsa*		*	
	Archaeobalanidae	*Hesperibalanus fallax*		*	
	Balanidae	*Amphibalanus improvisus*	*	*	
		Amphibalanus eburneus			
		Balanus glandula		*	
		Megabalanus rosa			
		Megabalanus tintinnabulum			
	Caprellidae	*Caprella mutica*			*
	Carcinidae	*Carcinus maenas*	*	*	*
	Chthamalidae	*Chthamalus proteus*		*	
	Gammaridae	*Dikerogammarus villosus*		*	
	Grapsidae	*Pachygrapsus fakaravensis*		*	
	Limulidae	*Carcinoscorpius rotundicauda*		*	
	Panopeidae	*Rhithropanopeus harrisii*	*	*	*
	Portunidae	*Charybdis japonica*	*	*	*
		Callinectes sapidus		*	
	Pseudodiaptomidae	*Pseudodiaptomus marinus*		*	
	Tortanidae	*Tortanus dextrilobatus*		*	
	Varunidae	*Eriocheir sinensis*	*	*	*
		Hemigrapsus sanguineus			
		Hemigrapsus takanoi			
		Eriocheir spp.	*	*	
		Hemigrapsus penicillatus			
Chlorophyta	Caulerpaceae	*Caulerpa taxifolia*	*	*	
	Codiaceae	*Codium fragile subsp. fragile*	*	*	
Chordata	Cionidae	*Ciona intestinalis*		*	
		Ciona robusta			
		Ciona savignyi			
	Didemnidae	*Didemnum perlucidum*	*	*	*
		Didemnum vexillum			
		Didemnum spp.	*	*	
		Diplosoma virens			*
	Gobiidae	*Neogobius melanostomus*	*	*	
		Acanthogobius flavimanus			
		Tridentiger barbatus		*	
		Tridentiger bifasciatus			
		Tridentiger trigonocephalus			
	Siganidae	*Siganus rivulatus*	*	*	
		Siganus luridus		*	
Cnidaria	Blackfordiidae	*Blackfordia virginica*		*	
Ctenophora	Beroidae	*Beroe ovata*		*	
	Bolinopsidae	*Mnemiopsis leidyi*	*	*	*
Echinodermata	Asteriidae	*Asterias amurensis*	*	*	*
Mollusca	Anomiidae	*Monia nobilis*		*	
	Arcidae	*Anadara transversa*		*	
	Calyptraeidae	*Crepidula fornicata*	*	*	

Phylum	Family	Species	Target AIS		
			Qld	WA	Request
	Corbulidae	*Varicorbula gibba*	•	•	•
		Potamocorbula amurensis			
	Cyrenidae	*Corbicula fluminea*		•	
	Dreissenidae	*Congeria spp.*			
		Dreissena polymorpha		•	
		Dreissena rostriformis bugensis			
		Mytilopsis leucophaeata			
		Mytilopsis sallei	•	•	•
		Mytilopsis spp.	•		
	Muricidae	*Rapana venosa*	•	•	•
		Urosalpinx cinerea			•
	Myidae	*Mya arenaria*	•	•	•
	Mytilidae	*Arcuatula senhousia*	•	•	•
		Mytella charruana	•	•	
		Brachidontes pharaonis			
		Geukensia demissa		•	
		Limnoperna fortunei			
		Mytella strigata			
		Perna perna	•	•	•
		Perna viridis			
		Perna canaliculus		•	•
	Ostreidae	*Crassostrea virginica*		•	
		Magallana ariakensis			
		Magallana bilineata	•	•	
		Magallana gigas	•	•	•
	Pharidae	*Ensis leei*	•	•	
	Turritellidae	*Maoricolpus roseus*	•	•	•
Myzozoa	Dinophysaceae	*Dinophysis norvegica*	•	•	•
	Gymnodiniaceae	*Gymnodinium catenatum*		•	
	Ostreopsidaceae	*Alexandrium monilatum*	•	•	
		Alexandrium catenella			
		Alexandrium minutum		•	
		Alexandrium tamarense			
	Pfiesteriaceae	*Pfiesteria piscicida*	•	•	
Ochrophyta	Alariaceae	*Undaria pinnatifida*	•	•	•
	Bacillariaceae	*Pseudo-nitzschia seriata*	•	•	
	Chaetocerotaceae	*Chaetoceros concavicornis*	•	•	•
		Chaetoceros convolutus	•	•	
	Fucaceae	*Fucus evanescens*		•	
	Sargassaceae	*Sargassum muticum*	•	•	•
Porifera	Clionaidae	*Cliona thoosina*		•	
	Niphatidae	*Gelliodes fibrosa*		•	
Rhodophyta	Bonnemaisoniaceae	*Bonnemaisonia hamifera*		•	
	Dasyaceae	*Grateloupia turuturu*	•	•	
		Grateloupia imbricata		•	
	Gracilariaceae	*Gracilaria vermiculophylla*		•	
	Rhodomelaceae	*Womersleyella setacea*		•	