Evaluating eDNA and Visual Surveillance as Complementary Methods for Detecting Benthic Marine Species

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Statement of responsibility

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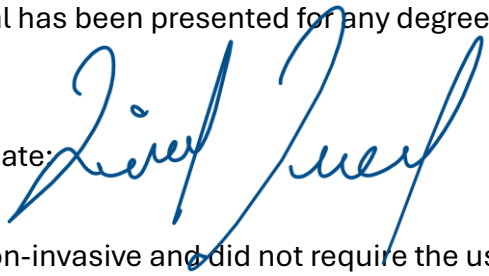
Signature of candidate:  Date: 06/11/2025

As this project is non-invasive and did not require the use of or handling of live animals, it did not require approval from an Institutional Ethics Committee or a permit from an external organisation. The potential movement of genetic material of noxious species is covered under the following permit:

Name of Ethics Committee or External: Victorian Fisheries Authority
Organisation Project Title: Victorian Fisheries Authority
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ABSTRACT

As of 2000, there was over 165 million individual *Asterias amurensis* (Northern Pacific Sea Star) in Port Phillip Bay. *A. amurensis* is a top priority marine pest requiring cost-effective monitoring approaches. No population assessment has been conducted since 2011, highlighting the need for cost-effective monitoring approaches. This study evaluated environmental DNA (eDNA) analysis and drop camera surveys as complementary methods for detecting *A. amurensis*, to inform future monitoring strategies for this established invasive species.

Both methods were deployed concurrently at 150 sites across three locations in Port Phillip Bay during November 2024 (post-spawning period). Water samples were filtered and analysed using quantitative PCR with species-specific primers (Muha et al., 2019), Drop-camera surveys recorded benthic habitat and visible sea stars surrounding a $1m^2$ quadrat. Environmental variables (depth, temperature, substrate characteristics) were recorded at each site.

Visual surveys detected *A. amurensis* at 8 of 150 sites, while eDNA detected them at 23 sites, yet methods agreed at only one site. McNemar's test confirmed systematically different detection patterns with eDNA detecting sea stars at more than 3 times more sites than cameras when methods disagreed. Combined approaches detected *A. amurensis* at 30 sites, representing a substantial increase over either method alone, demonstrating complementary rather than redundant detection.

Exploratory analysis suggested possible associations between detection probability and environmental factors, though findings require cautious interpretation given limited statistical power. eDNA likely integrates signals from multiple life stages and broader spatial areas via hydrodynamic transport, while cameras provide precise localisation of visible adults. Results demonstrate that eDNA and visual surveys provide complementary rather than interchangeable information. Effective monitoring programs should integrate both approaches strategically based on management objectives: eDNA for broad-scale regional screening, visual surveys for precise population characterisation and targeted management interventions.

Keywords: Asterias amurensis, eDNA, QPCR, Drop Camera, Port Phillip Bay

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This thesis follows APA7th edition referencing and formatting guidelines

List of Abbreviations

DNA	Deoxyribonucleic Acid	qPCR	Quantitative Polymerase Chain Reaction
eDNA	Environmental DNA	NTC	Non-Template Control
Asterias amurensis	Asterias amurensis	LOD	Limit of Detection
CT	Cycle Threshold	CI	Confidence Interval
PCR	Polymerase chain reaction	COI	Cytochrome c oxidase subunit 1
DF	Degrees of Freedom	OR	Odds Ratio
FoR	Field of Research	GPS	Global positioning system
p	Probability	PES	Polyether sulfone

INTRODUCTION

Marine invasive species

Invasive species represent one of the greatest challenges to global biodiversity, increasingly recognized as a primary driver of recent extinctions (Bellard et al., 2016; IPBES, 2019; Otani et al., 2017). This threat is particularly acute in marine environments, where changing climate conditions may create new opportunities for non-native species establishment and range expansion (Bellard et al., 2016; Dommisse & Hough, 2004). The interface between climate change and biological invasions presents a complex challenge for conservation, as habitat suitability modelling indicates that many problematic species will find increasingly favourable conditions in both northward and southward range expansions (Bradshaw et al., 2021; Byrne, 2016; Byrne et al., 1997).

As global trade continues to increase so too does the threat of alien species incursions. Australia's total goods and services imports increased by 425% from $110b in 2000 to $614b in 2024 (ABS, 2006, 2007, 2024), with substantial volumes transported through international shipping. Australia receives more than 29,000 vessels (Richey, 2021) to its ports and harbours each year, with each vessel posing a potential biosecurity risk. Early detection of invasive species is critical, as once established; they become extremely difficult or impossible to eradicate, result in costly management interventions, and can have significant ecological and economic impacts. Furthermore, there is a lack of effective tools to help manage species once they have become established in novel environments. It is vital that there are robust strategies put in place to monitor for the introduction and spread of exotic pests (Townhill et al., 2017)to enable effective management and minimise impacts on native ecosystems and the blue economy, valued at over $100b in Australia alone (Voyer et al., 2017).

The impacts of *Asterias amurensis*

Asterias amurensis (Northern Pacific Sea Star*)* is a top 3 established pest on the Australian Priority Marine Pest List (ABARES, 2019) and is a noted dangerous pest on the UICN Global Invasive Species Database for its potential for ecological and

economic harm (ABARES, 2018; Luque G et al., 2025). *A. amurensis* are native to the northern region of the Pacific Ocean including Japan, Korea, China, and Russia (Bruce et al., 1995; Du et al., 2021). First detected in the Derwent River estuary near Hobart, Tasmania in 1986 and believed to be introduced via ballast water, *A. amurensis* larvae are thought to have been translocated to Naarm (Port Phillip Bay), around 1995 (Aquenal, 2008; Parry, 2001) when a few hundred individuals were detected. By 2000, the population had exploded to over 165 million individuals (Parry, 2004), with biomass equalling 56% of the total demersal fish biomass in the central region of the bay (Parry, 2004). *A. amurensis* have established large populations in Tasmania's Derwent River and Port Phillip Bay (Deagle et al., 2003), but incursions have also been reported along the Victorian coast including San Remo, Wilsons Prom and Lakes Entrance (Australian & Government, 2022; Hayward, 2018). These populations have been largely controlled before *A. amurensis* could establish through hand removal of individuals.

Why is *A. amurensis* so successful?

A. amurensis has several key traits that enhance adaptability to their surroundings. First, they are highly fecund; females are able to become reproductive within a year and produce 5-20 million eggs in their first reproductive season (Byrne et al., 1997; Du et al., 2021). Second, *A. amurensis* larvae remain viable in the water column for up to 120 days (Australia, 2025; Bruce et al., 1995; Richardson et al., 2016) until conditions become favourable to continue development. This extended larval phase significantly increases dispersal potential and range expansions to new areas (Ellis et al., 2022). Third, *A. amurensis* can be found across a broad range of environments, inhabiting a depth range of less than 1m to more than 200m (Byrne, 2016). They can cope with a broad range of salinities, temperatures, and pH, suggesting they are capable of adapting to changing oceanic conditions (Barrett et al., 2020). Fourth, *A. amurensis* can outcompete many native species aggregating in huge numbers, reducing available food stocks, with a preference for bivalves, but also consuming gastropods, sea squirts, sponges, ascidians and even conspecifics, exploiting currents to spread to new locations (Australia, 2025; Bellard et al., 2016; Charko & Blake, 2022; Hutson et al., 2005; Ross et al., 2002). The fact that *A. amurensis* has

limited effective natural predators in its introduced range (Barrett et al., 2020), combined with high fecundity, has contributed to their rapid establishment and population expansion. These traits align with r-selected species characteristics – organisms that prioritise rapid reproduction and population growth over competitive ability (Caswell, 1982; L. Hünicken, S et al., 2019; L. Hünicken et al., 2019; Quadros & Araujo, 2008; Winemiller, 2005). This reproductive strategy makes *A. amurensis* particularly successful as an invasive species, able to quickly exploit available resources and establish populations in novel environments.

Port Phillip Bay represents a compelling case study of how *A. amurensis* can exploit both their inherent adaptability and favourable environmental conditions to achieve rapid establishment (Ford & Hamer, 2016; Winstanley, 1995). Invasive species often establish more successfully in disturbed or degraded environments where native communities have been disrupted. While *A. amurensis* hold the life history traits necessary to invade diverse marine environments, Port Phillip Bay presented a particularly conducive scenario – a 'perfect storm' of conditions that accelerated their success. The bay has experienced profound ecological degradation since European settlement, with the loss of over 95% of its shellfish reefs fundamentally altering competitive dynamics (Ford & Hamer, 2016). These reefs, composed primarily of native flat oyster (*Ostrea* angasi) and blue mussel (*Mytilus planulatus*), would have supported complex communities. Their loss fundamentally altered the bay's competitive and trophic dynamics (Ford & Hamer, 2016; Ross et al., 2002). The loss of native shellfish reefs reduced habitat complexity and native competition, creating conditions that facilitated rapid *A. amurensis* establishment. The simplified benthic environment following reef decline, combined with reduced native competition and *A. amurensis'* inherent adaptability, creates conditions that facilitated the explosive population growth observed. The invasion also coincided with the millennium drought (Parry, 2016) providing additional environmental disruption.

The current state of Port Phillip Bay, supporting over 180 introduced species (Hewitt et al., 2004), demonstrates how effective invaders like *A. amurensis* can capitalise on anthropogenic ecosystem modifications to establish dominant populations.

Monitoring the presence and abundance of established populations

A. amurensis monitoring in Port Phillip Bay was initially conducted via bottom-set trawl surveys between 2000-2011 (Parry, 2001, 2004, 2016). While this method was not specifically designed for sea star population estimates, the surveys were largely opportunistic through fish trawl data and provided valuable historical data on the population dynamics of benthic species in Port Phillip Bay. This approach allowed for some estimate and quantification of the abundance and distribution in the bay and enabled the detection of the significant increase and spread of this species over a short time frame. However, the annual trawl data collection was stopped in 2011, and there have been no formal assessments of the distribution and abundance of this species since.

Survey methods are highly variable and need to be developed with an understanding of their bias and limitations – no method is perfect. Trawl surveys, while providing important baseline information (Jarvis & Brennan, 2024; Kumar, 2022; Schönke et al., 2022), are not only costly and labour-intensive but also damaging to benthic communities. The destructive nature of trawl sampling highlights the urgent need for developing alternate methods that can effectively assess abundance and distribution while minimising ecosystem impact. Fortunately, in recent years developments in technologies have enabled new methods such as in-situ visual surveillance (including drop camera, camera tows, and time lapse cameras) as well as new molecular tools that allow for biosecurity surveillance that is less cost and labour intensive, more sensitive and less ecologically damaging.

eDNA as a potential solution for *A. amurensis* monitoring

Environmental DNA (eDNA) is the DNA fragments shed into the environment by an organism (e.g., Though excretion, and decomposition). This DNA can then be sampled and analysed using species-specific genetic markers to identify target organisms (Muha et al., 2019; Rees et al., 2014). Simple water samples can be taken quickly and more cost effectively than putting divers, cameras or remotely operated vehicles into the water, allowing one to sample more rapidly over larger areas (Von Ammon et al., 2023). eDNA is a promising alternative to visual or trawl surveys as it can pick up species not detected visually due to its high sensitivity (Gold et al.,

2021). eDNA methods are gaining popularity for detecting elusive or exotic species with applications sometimes offering additional information about organism abundance and spatial patterns through non-destructive sampling (Gold et al., 2021; Monuki et al., 2021; Rees et al., 2014). In the case of species which are more difficult to visually observe from the surface, such as benthic marine invertebrates, DNA fragments can be extracted from water samples collected in the water column (Antich et al., 2021), providing a means of detection that does not rely on direct visual observation or physical capture of specimens. This molecular approach provides a sensitive method for detecting *A. amurensis* presence, potentially including life stages not visible in camera surveys, such as microscopic larvae or recently settled juveniles.

eDNA detection systems are highly sensitive and therefore great for early detection of invasive species (Jerde et al., 2013; Stat et al., 2017), potentially identifying new introductions before populations reach levels detectable by conventional survey methods or by detecting presence of free larvae in the water column. Species-specific assays have been developed and validated for *A. amurensis* (Bruce et al., 1995). Primers targeting the mitochondrial cytochrome c oxidase subunit I (COI) gene—specifically the primers Ast-F and Ast-R developed for *A. amurensis* — enable precise molecular detection from environmental samples (Byrne, 2016; Kashenko, 2005; Villier & Loïc, 2014), with a limit of detection/quantification established at 1.44 copies p/µL (Ellis et al., 2022). However, eDNA is not good at estimating numbers or biomass (although some studies are looking at how to do this) and can be transported from an area some distance away from where it was sampled. It is thus increasingly recognised as a complementary survey approach and doesn't replace more traditional survey approaches (Tulloch et al., 2025).

In contrast to molecular detection methods, advances in underwater imaging technology have made image-based benthic surveys increasingly practical and cost-effective. Population assessments are commonly done via visual or acoustic methodologies, so a visual drop camera component was included as fair comparison against eDNA data (Suarez-Bregua et al., 2022). Drop camera systems, which have been successfully employed for monitoring Atlantic Sea scallop populations

(Bethoney & Stokesbury, 2018), can potentially be adapted for surveying other benthic invertebrates, including *A. amurensis*. These systems typically consist of a waterproof camera mounted on a frame that is lowered to the seafloor, capturing images or video of benthic organisms and habitat features (Clayton & Dennison, 2017). The decreasing cost of equipment and improved data storage capabilities have removed many historical barriers to widespread implementation of visual monitoring programs (Clayton & Dennison, 2017; Von Ammon et al., 2023). However, cameras take time to deploy and can only visualise a relatively small area. They are also limited by visibility such as depth (light attenuation) and suspended sediments that increase turbidity. Visual surveys provide direct confirmation of species presence and life stage (Bethoney & Stokesbury, 2018), contextual information about habitat associations (De Mendonça & Metaxas, 2021), and quantitative data on population density and size structure (De Mendonça & Metaxas, 2021). Unlike trawl surveys, drop camera methods minimize physical disturbance to benthic communities, allowing for repeated sampling of the same locations without negatively impacting the benthos.

The integration of multiple sampling approaches is particularly valuable given the dynamic nature of marine environments and the mobility of *A. amurensis* populations across life stages (Nester et al., 2024). Traditional monitoring techniques have often relied on single-method approaches that may fail to capture the full spectrum of species distribution and life-stage presence (Parry, 2004, 2016). By combining eDNA sampling with visual documentation through drop camera surveys, researchers can potentially establish a more comprehensive understanding of *A. amurensis* populations. However, whether these methods detect the same biological signals or capture different aspects of sea star presence remains an open question.

The relationship between molecular and visual detection is not straightforward. eDNA concentrations can be influenced by numerous factors beyond simple presence/absence, including organism density, proximity, physiological state (e.g., spawning), life stage, water movement patterns, and DNA degradation rates (Miller et al., 2013; O'Donnell et al., 2017). Similarly, visual detection depends on population density, spatial distribution patterns, camera field of view, and visibility conditions.

These methods may operate at fundamentally different temporal and spatial scales: eDNA potentially integrates signals across hours to days and tens to hundreds of meters via water movement, while cameras capture instantaneous snapshots of a direct area. Whether this scale mismatch represents a critical limitation or an opportunity to capture complementary information depends on the specific management questions being addressed.

Study Aims and Hypothesis

This study evaluates eDNA analysis and drop camera surveys as complementary approaches for detecting *A. amurensis* in Port Phillip Bay, to inform future monitoring strategies for this established invasive species. We address two primary questions:

1. Do these methods provide comparable detection of *A. amurensis* at the site level? And
2. If detection patterns differ, what factors might explain these differences and what are the implications for monitoring program design?

We hypothesise that eDNA and visual surveys may capture different aspects of sea star presence due to: (a) differences in spatial integration (eDNA transported by currents vs. cameras surveying 1 m^2 quadrats), (b) differences in temporal integration (eDNA persisting after spawning events vs. cameras capturing current adult presence), and (c) differences in life-stage detection (eDNA capturing microscopic larvae vs. cameras detecting visible adults only).

By collecting water samples for eDNA analysis concurrently with drop camera surveys at 150 sites, we directly compare detection rates and evaluate whether eDNA concentrations correspond with visually observed populations. We expect that detection patterns will reveal whether these methods provide redundant information (indicating one could replace the other) or complementary information (indicating both are needed for comprehensive assessment). Methods are complementary if they detect different aspects of species presence, providing additive information value; they are redundant if detecting the same signals with different sensitivities. We also examine whether environmental variables (depth, temperature, substrate

type, prey availability) influence detection probability differently for each method, which could indicate scale-dependent of mechanism-dependent detection patterns.

This methodological comparison is critical for developing effective, non-destructive monitoring protocols of established *A. amurensis* populations and preventing further range expansion into new areas of Port Phillip Bay and beyond (Richardson, 2015; Sorte et al., 2010). Understanding the relative strengths and limitations of each approach will enable evidence-based decisions about monitoring program design, optimising the balance between spatial coverage, detection sensitivity, cost-effectiveness, and ecological impact.

METHODS AND MATERIALS

To compare the efficacy of eDNA analysis and drop-camera for detecting *A. amurensis,* water samples and drop camera surveys were undertaken from 150 sites across three locations in Port Phillip Bay from November 25-26 (late spring), 2024. The sample collection period followed the broadcast spawning season and free larvae may have remained in the water column and contribute to the eDNA detections.

Study site and data collection

Both water samples and drop camera imagery were collected simultaneously from each of the 150 sites. Three study locations within Port Phillip Bay were selected based on historical distribution data (Hewitt et al., 2004; Parry, 2001, 2004, 2016) indicating previous *A. amurensis* aggregations, local ecological knowledge, and preliminary observations (Figure 1). Each location covered approximately 1 square kilometre of benthic habitat. Within each location, five transects parallel to the shoreline were established at 250-meter intervals apart (± 30m), with the first transect positioned parallel to, and closer to the shoreline (Figure 2). Along each transect, 10 sampling points were designated at 100-meter intervals (± 30m), resulting in 50 sampling points per location and a total of 150 sampling sites. This sample design provided approximately one sampling site per 20,000 m^2 within each 1km^2 location (50 sites per location), balancing broad spatial coverage for detecting patchy distribution with logistical constraints of concurrent eDNA and visual sampling. All sampling was

conducted over two consecutive days with similar weather conditions, ensuring the results between locations were comparable. At each sampling site, GPS coordinates were recorded using differential GPS to allow for potential future resampling. eDNA water sampling times were recorded relative to tidal state. Complex hydrodynamic patterns within Port Phillip Bay remain an unmeasured variable that could affect results.

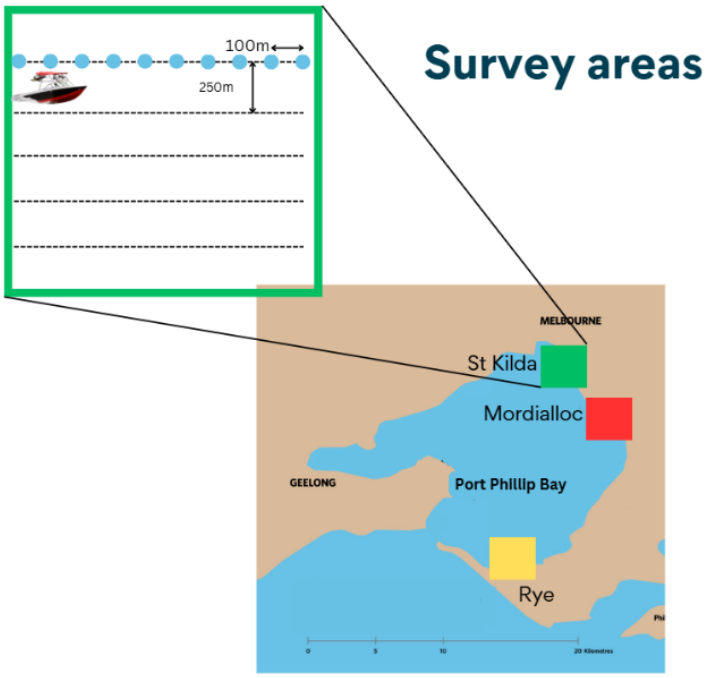

Figure 1: Map of Survey Locations in Port Phillip Bay, Victoria, Australia. Three survey locations were selected based on historical A. amurensis distribution data and local ecological knowledge: St Kilda = green, Mordialloc = red, Rye = yellow. Each location covered approximately 1km^2 of benthic habitat. Inset box shows the grid design with five transects (250m spacing) running parallel to the shoreline, each containing 10 sampling sites (100m spacing), for a total of 50 sites per location (150 sites total).

Figure 2: Example survey design at Mordialloc location. Five transects run parallel to shoreline at 250m intervals (±30m), with 10 sample sites along each transect at 100m intervals (±30m). Transects extend progressively offshore into deeper water. This standardised grid design was replicated at all three survey locations to ensure consistent spatial sampling.

Drop Camera Sampling

Drop camera footage of the benthos was captured using a pyramidal steel quadrat with a 1m × 1m square quadrat (Figure 3). A GoPro camera with wide-angle lens was securely mounted at the apex of the frame, oriented downward to record the sea floor within and beyond the frame's footprint. The frame served as both a protective housing for the camera and a standardized reference for size estimation during subsequent analysis.

The camera frame was lowered to the sea floor and allowed to rest on the benthos for approximately 15 seconds before retrieval, allowing sufficient time to capture a clear image of the 1m x 1m quadrat on the sea floor.

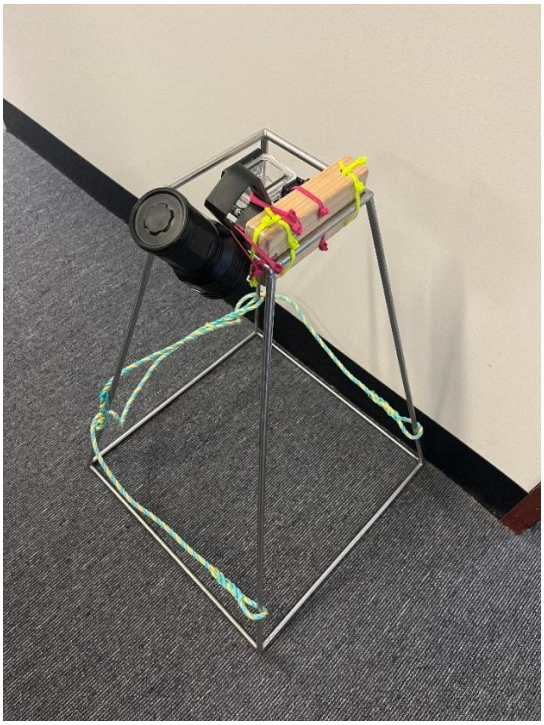

Figure 3: Drop camera deployment system: Pyramidal steel frame with a 1m x 1m quadrat base, equipped with a wide-angle GoPro camera mounted at the apex and oriented downward. The frame serves as both a protective housing and standardised size reference for subsequent video analysis. The frame was lowered to the seafloor and allowed to rest for approximately 15 seconds to capture clear imagery of benthic organisms and substrate type.

Drop camera footage was subsequently analysed in Windows media player to identify and count *A. amurensis* individuals. The analysis included areas both within and outside the steel frame's footprint, with the frame serving primarily as a size reference. For each sampling site, data were recorded on *A. amurensis* abundance (number of individuals per square meter), water depth, substrate type (categorized as sand, shell/rubble, or seagrass), and the presence of other identifiable species.

Environmental DNA Collection

Water samples for eDNA analysis were collected concurrently with drop camera footage at each sampling site. A 1-liter plastic bottle attached to an extendable pole was submerged to approximately 0.5 meters below the water surface (Figure 4). A new clean bottle was used for all sample collections. Drop camera samples covered a range of depths from as shallow as 4.4m up to 14.7m at the deepest. For this study it was deemed impractical to retrieve water samples closer to the benthos given logistical constraints of concurrent camera deployment and vessel operations, so 0.5m below the surface was considered a consistent sampling depth which could be maintained across all points to ensure standardization. Each bottle was filled to 1L, capped

securely, and immediately placed in a cooler with ice. Using new, or sterilised bottles for each sample and wearing gloves reduced the potential for cross-contamination between water samples. Samples were kept on ice for up to 5 hours, until they could be transferred to a -20°C freezer for storage until laboratory processing which occurred between 2 and 3 months later.

Figure 4: Water sampling apparatus: Extendable pole attachment for 1L plastic bottles, marked at 0.5m to ensure consistent sampling depth across all sites. A new or sterilized bottle was used for each sample to prevent cross-contamination. Water samples were collected concurrently with drop camera footage at each survey site.

Prior to extraction, eDNA water samples were thawed in a darkened cool room over 48 hours. Samples were then filtered through 1.2 µm polyether sulfone filters using a MasterFlex L/S peristaltic pump in batches of seven based on the capacity of the peristaltic pump system. Prior to each batch processing, the peristaltic pump system was thoroughly decontaminated with an 8% bleach solution, followed by extensive rinsing with fresh water and a final flush with ultrapure Milli-Q water to remove any residual bleach that could degrade DNA.

Water samples were filtered through 1.2µm, 47mm polyethersulfone (PES) membrane filters using X19 filter holders. This filter pore size was selected to capture the majority of environmental DNA while maintaining practical filtration rates (Brandt et al., 2021; Dass et al., 2024; Pastor-Rollin et al., 2024). Following filtration, membrane filters were carefully transferred into 2.0mL free-standing tubes (SSIbio 2340-00), labelled, placed

into zip-lock bags, double-bagged for added protection, and stored in a -80°C freezer until eDNA extraction.

DNA extraction was performed using a modified QIAGEN DNeasy Blood & Tissue Kit protocol specifically optimized for marine environmental samples (Chevrinais et al., 2023; Nester et al., 2024; Qiagen, 2023). Premixed lysis buffer (540 µL ATL Buffer and 20 µL proteinase K per reaction) was added to each filter membrane. This buffer combination facilitates the breakdown of cellular material and release of DNA. Samples were vigorously shaken in a vortex mixer for 3 seconds to ensure complete coverage of the filter with lysis buffer, then incubated at 55°C for 2-3 hours with intermittent mixing to maintain enzyme distribution while preventing DNA shearing (Dass et al., 2022; Ellis et al., 2022). Following incubation, 500 µL of lysate was combined with 500 µL Buffer AL and 500 µL of 100% ethanol, which precipitates the DNA. The mixture was then transferred in 600 µL aliquots to a DNeasy Mini spin column containing a silica membrane that selectively binds DNA while allowing contaminants to pass through during centrifugation at 8,000 rpm (6,000 g) for 1 minute (Sanches & Schreier, 2020). The bound DNA was subsequently washed with two specialized buffers (AW1 and AW2) to remove PCR inhibitors and other potential contaminants while retaining the target DNA on the membrane.

The purified DNA was eluted from the membrane by adding 100 µL Buffer AE directly onto the membrane, allowing it to incubate for 2 minutes at room temperature, followed by centrifugation. This elution step was repeated once to maximize DNA recovery, resulting in approximately 200 µL of purified DNA extract per sample. This extraction protocol effectively isolates DNA from marine water samples while minimizing the co-extraction of PCR inhibitors that could interfere with subsequent analysis (Sanches & Schreier, 2020).

Quantitative Polymerase Chain Reaction (PCR)

Quantitative PCR (qPCR) analysis was performed using an Aria MX real-time PCR system (Agilent Technologies) with 96-well plates. For each individual sample, three technical replicates were run to reduce the likelihood of false positives or false negatives. Species-specific primers [Ast-F TGGAACTGGCTGAACGATTTCT Ast -R

AATAGAAGAAGCCCCTGCCGAA](Yang et al., 2024) targeting the mitochondrial cytochrome c oxidase subunit I (COI) gene of *A. amurensis* were used to amplify and quantify DNA fragments from the environmental samples. Each qPCR reaction contained 0.5 µL forward primer (20 µM), 0.5 µL reverse primer (20 µM), 0.5 µL probe (10 µM), 5 µL TaqMan master mix, 1.5 µL Milli-Q water, and 2 µL of DNA sample, for a total reaction volume of 10 µL.

To establish a standard curve for quantification, synthetic DNA standards (G-block) (Huang et al., 2024) were prepared at known concentrations ranging from 10^0 (1 copy) to 10^8 (100,000,000 copies) of target DNA p/µL. With standard concentrations established, they were subsequently included in each qPCR run to allow for the quantification of unknown samples based on their cycle threshold (CT) values. Non-template controls (NTC) using water instead of DNA sample were also included in each run to verify the absence of contamination in the reagents.

Quantification was based on the cycle threshold, which represents the number of amplification cycles required for the fluorescent signal to exceed background levels. A standard curve was generated from the known standards producing a linear function, with cycle threshold values plotted against known quantities to determine the concentration of target DNA in unknown samples. Standard curves on each plate demonstrated acceptable performance with $R^2 > 0.99$ and amplification efficiencies ranging from 82.2% to 94.1% (slopes: -3.463 to -3.838), confirming the reliability of DNA quantification across all runs (Table 1). By comparing the cycle threshold values of environmental samples with those of the known standards, the concentration of *A. amurensis* DNA in each water sample can be determined.

Early cycle threshold values (below 29) indicate high abundance of target DNA, moderate cycle threshold values (30-35) suggest moderate abundance, and late cycle threshold values (above 35) indicate low abundance. The relationship between cycle threshold values and initial DNA quantity follows an exponential function ($Q \sim e^{-CT}$) (Pfeifer, 2022), where Q represents the initial target gene quantity and e represents the amplification efficiency, ideally approaching 2 (indicating that the target sequence doubles with each cycle).

By comparing the cycle threshold values of environmental samples with those of the known standards, the concentration of *A. amurensis* DNA in each water sample can be determined.

Table 1: Summary of Standards: Standards curves for all six qPCR plates showing slope, R^2, and amplification efficiency. Mean efficiency of 87.3% ± 4,2% confirms reliable DNA quantification across all runs.

Summary table of standards				
Plate	**Standards**	**Slope**	**R^2**	**Efficiency %**
1	STD 4 & 9	-3.556	1	90.8
2	STD 2 & 8	-3.463	1	94.1
3	STD 1 & 6	-3.838	1	82.2
4	STD 3 & 7	-3.645	1	87.3
5	STD 1 & 5	-3.733	1	84.9
6	STD 4 & 9	-3.744	1	84.6

Methodological Constraints and Study Scope

Several methodological constraints inherent to this honours-level project should be acknowledged. Water samples were collected at 0.5m depth rather than near the benthos due to logistical and cost constraints of deploying subsurface sampling equipment across 150 sites. This surface sampling approach tests whether benthic organism signals can be detected in surface waters – a practical consideration for cost-effective large-scale monitoring programs (Foote et al., 2012; Nester et al., 2024), though it may reduce detection sensitivity through vertical dilution. Samples were collected over two consecutive days under similar whether conditions to maintain comparability between locations, though temporal variation in eDNA persistence and hydrodynamic transport between days remains a potential source of variability. Each site was sampled once for each method. (n=1 water sample, n=1 camera deployment per site) to maximise spatial coverage across Port Phillip Bay within project constraints. This spatial extent was prioritised over intensive replication at fewer sites, as broad-scale distribution patterns were of primary interest for informing monitoring strategy. These design decisions reflect pragmatic trade-offs between spatial coverage, sampling intensity, and project feasibility, and are typical of initial comparative assessments that inform more targeted future research.

STATISTICAL ANALYSIS

All statistical analysis were performed in R version 4.2.0 (R et al., 2023), with significance assessed at $\alpha = 0.05$ for all tests. The following R packages were used: ggplot2 for data visualisation (Wickham, 2016a), stats for basic statistical tests, exact 2x2 for exact McNemar's test (Fay, 2010), psych for Cohen's Kappa (Cohen, 1960; Kvalseth, 1989), DescTools for pseudo R^2 and binomial confidence intervals, and readxl for data import (Wickham, 2016b; Wickham & Bryan, 2023)

Detection Threshold Determination

For eDNA analysis, the limit of detection (LOD) was defined following Ellis et al. (2022), who established 1.44 copies/µL as the minimum reliably detectable concentration for *A. amurensis* in Port Phillip Bay water samples using these primers. Our standard dilution series showed cycle threshold (CT) values <37 corresponded to approximately 1-10 target DNA copies, representing extremely low but reliably detectable quantities. Sites were classified as eDNA-positive when at least 2 out of 3 technical replicates showed amplification with CT <37. Replicates with CT≥ 37 or no amplification were considered negative. This conservative threshold (requiring 2/3 positive replicates rather than 1/3) reduces false positive risk from stochastic amplification while maintaining detection sensitivity.

Comparative Analysis of Detection Methods

Binary presence-absence data (1=detected, 0=not detected) were compiled for both methods across all 150 sites. Detection rates were calculated as the proportion of positive sites, with 95% confidence intervals estimated using exact binomial methods (Clopper-Pearson).

To assess whether methods agreed beyond chance expectation, we calculated Cohen's Kappa coefficient (κ) using the psych package (Cohen, 1960). Kappa ranges from -1 to +1, where values near 0 indicate agreement no better than chance, positive values indicate agreement beyond chance, and negative values indicate systematic disagreement. We constructed a 2x2 contingency table cross-classifying sites by

detection status for each method, allowing calculation of overall agreement and conditional detection probabilities.

The key statistical question was whether detection patterns differed systematically between methods. Standard chi-squared tests of independence are inappropriate for paired data (both methods applied to the same sites), as they do not account for within site correlation. Instead, we applied McNemar's test for paired binary data, which specifically tests whether methods disagree in a systematic direction by focusing on discordant pairs (sites where only one method detected sea stars). Both asymptotic and exact McNemar's tests were conducted using the stats and exact 2x2 packages (Fagerland et al., 2013). The odds ratio of discordant pairs quantifies the tendency for one method to detect more sites that the other when they disagree.

Environmental Associations with Detection

To explore whether environmental factors influenced detection probability, we fitted separate binary logistic regression models for each method. Five environmental predictors were considered: temperature (°C), depth (m), and binary indicators for seagrass presence, seaweed presence and observed prey availability from camera footage. This exploratory analysis aimed to generate hypotheses about ecological or methodological factors affecting detection rather than definitively test causal relationships, given limited statistical power from sparse positive detections (8 camera, 23 eDNA positives across 150 sites).

Preliminary correlation analysis revealed several multicollinearities between temperature and depth (Pearson's r = -0.93, $p<0.001$), reflecting the physical oceanography of Port Phillip Bay where shallow waters are consistently warmer. Including both variables simultaneously in regression models creates numeric instability, inflates standard errors, and produces unreliable coefficient estimates. To address this, we fitted separate model sets for each detection method:

- **Temperature models:** Including temperature, seagrass, seaweed, and prey (excluding depth)
- **Depth models:** Including depth, seagrass, seaweed, and prey (excluding Temperature)

Because temperature and depth are so tightly coupled in this system (r = -0.93), they effectively represent alternate measures of the same shallow-warm versus deep-cool environmental gradient. Comparing models with each variable separately allows us to assess whether this gradient associates with detection, while acknowledging we cannot statistically separate temperature from depth effects.

Models were fitted using binomial generalised linear models with logit link functions. For significant predictors (p<0.05), odds ratio and 95% confidence intervals were calculated to quantify effect sizes. Model fit was assessed using Nagelkerk's pseudo-R^2, which approximates the proportion of variation explained and ranges from 0 (no explanatory power) to 1 (perfect prediction). We report pseudo-R^2, values to provide context for model performance, recognising that values of 10-20% are typical for ecological models with binary outcomes and substantial unmeasured variation.

Combined Detection Analysis

To assess whether using both methods together improved overall detection compared to either alone, we classified sites as positive if either method (or both) detected *A. amurensis*. This combines detection approach was analysed using the same logistic regression framework to explore environmental associations with sea star presence independent of detection method, potentially increasing statistical power by pooling detections (30 positive sites vs. 8 or 23 separately).

RESULTS

Detection Rates and Basic Comparison

Visual surveys of the benthos positively confirmed the presence of *A. amurensis* at 8 sites out of 150 (5.3%, 95% CI:2.3-10.2%) across the three locations (Figure 5). No *A. amurensis* were detected at Rye. At St Kilda, single individuals were detected at five sites (STK018, STK021, STK034, STK40, STK042, and STK046). At Mordialloc, two sites showed notable aggregations: MOR040 (15-18 individuals) and MOR044 (approximately 60 individuals).

eDNA analysis showed a larger number of detections, with *A. amurensis* DNA detected at 23 sites (15.3%, 95% CI: 10.0-21.9%) (Figure 5). eDNA detections occurred at all

three locations, with Mordialloc showing the greatest number: 18 detections out of 50, St Kilda had 3 detections out of 50 sites, and Rye had 2 detections out of 50 sites. eDNA concentrations at positive sites ranged from 1.69 to 38.74 copies/µL (median CT = 36.15), near the median for all positive sites. Mordialloc, which contained the highest visual densities (up to 60 individuals at MOR040), showed higher median eDNA concentrations (2.49 copies/µL, n=18 positive sites) compared to St Kilda (1.78 copies/µL, n=3) and Rye (1.87 copies/µL, n=2), though differences were not statistically significant (Kruskal-Wallis test: H=0.5, p=0.79). The generally low eDNA concentrations detected (median 2.38 copies/µL) are consistent with the post-spawning sampling period and surface water collection, where dilution effects reduce detectable signals.

The non-overlapping confidence intervals between detection methods indicate significantly different detection frequencies, with eDNA detecting sea stars at more than 3 times the rate of drop camera surveys.

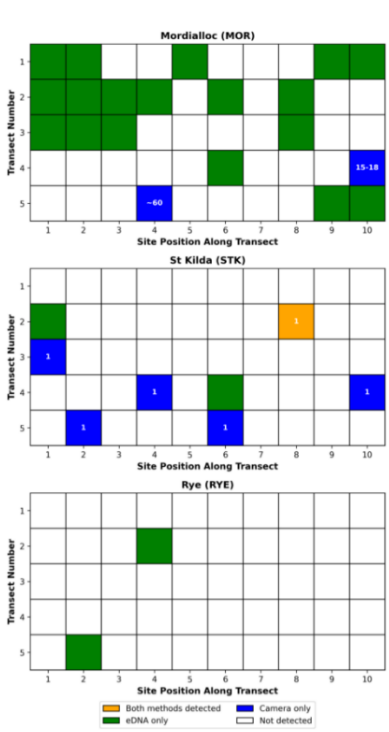

Figure 5: Spatial distribution of Asterias amurensis detections across three survey locations in Port Phillip Bay. Each panel represents one survey location with 50 sites arranged in a 5x10 grid, where rows represent transects parallel to the shoreline and columns represent sites along each transect (100m spacing). Colours indicate detection method: Green = eDNA only, blue = drop camera only, orange = both methods, white = no detection. Numbers in blue and orange squares indicate the count of individual asteria observed by drop camera at that site. With greater aggregations of asteria at the Mordialloc site, greater eDNA detections are observed demonstrating a spatial "halo" or molecular detection around high-density populations.

Comparative Analysis of Methodology for Detecting Invasive Sea Stars

Although both methods successfully detected the presence of sea stars, there was not a strong relationship between detection methods at the site level. Only 1 site (STK018) was positive for both eDNA and drop camera detections. Neither method detected sea stars at 120 sites (80.0% of sites), only drop camera detected sea stars at 7 sites (4.7%), and only eDNA detected sea stars at 22 sites (14.7%). Overall agreement between methods was 80.7%, primarily driven by concordant negative detections where both methods failed to detect sea stars (Table 2).

Table 2: Contingency table showing agreement between methods of detection. Of 150 sites surveyed, methods agreed at 121 sites (80.7%): 1 site where both detected A. amurensis and 120 sites where neither method detected the species. Methods disagreed at 29 sites (19.3%), with eDNA detecting 22 sites that cameras missed and cameras detecting 7 sites that eDNA missed.

	Camera Positive	Camera Negative	Total
eDNA Positive	1	22	23
eDNA Negative	7	120	127
Total	8	142	150

Notably, of the eight sites where camera detected sea stars, only one corresponded with eDNA detection at that same site (STK018). The single site with dual detection showed one visible adult and positive eDNA signal (CT = 36.15), providing the only instance where both methods concurred. This minimal overlap suggests the methods may be capturing different spatial or temporal signals of *A. amurensis* presence rather than simply differing in sensitivity for the same target.

Cohens Kappa coefficient was calculated to assess agreement beyond that expected by chance alone. Cohens Kappa ranges from -1 to 1 where -1 indicates no agreement, 0 indicates agreement due to chance and 1 indicates perfect agreement (Kvalseth, 1989). The kappa value was 0.03 (95% CI:- 0.14-0.09), indicating agreement no better than would be expected by chance. This very low kappa value highlights that despite high overall agreement percentage, the methods rarely concurred on positive detections.

When examining conditional probabilities, drop camera detection was followed by eDNA detection at the same site in only 12.5% of cases (1 of 8 camera-positive sites). Conversely, eDNA detection was followed by drop camera detection at the same site in

only 4.3% of cases (1 of 23 eDNA-positive sites). These low conditional probabilities demonstrate minimal overlap in positive detections between methods.

McNemar's Test for Paired Method Comparison

McNemar's test was applied to determine if the methods disagreed in a systematic way, specifically testing whether one method consistently detected more positives than the other when they disagreed. The test focuses on discordant pairs where only one method detected sea stars (eDNA alone: 22 sites vs drop camera alone: 7 sites).

McNemar's test demonstrated a significant difference in detection patterns between methods (x^2 = 10.125, *df* = 1, *p* = 0.002), with the exact test yielding similar results (*p* = 0.001). This confirms that eDNA systematically detects sea stars at different sites than drop camera, rather than the disagreement being random. The odds ratio of discordant pairs was 3.14, indicating that eDNA-only detections were approximately three times more common than drop camera-only detections.

Environmental Factors and Detection Probability

Logistic regression models were fitted separately for each detection method to examine how environmental variables influenced detection probability. Due to severe multicollinearity between temperature and depth (r = -0.93), separate models were fitted using either temperature or depth (but not both) as predictors, along with seagrass presence, seaweed presence, and available prey (Figure 6).

For eDNA detection, seaweed presence was the only significant predictor in both the temperature model (*Odds Ratio* = 0.32, 95% *CI*: 0.11-0.92, *p* = 0.035) and the depth model (*Odds Ratio* = 0.29, 95% *CI:* 0.10-0.78, *p* = 0.014). Sites with seaweed present had approximately 70% lower odds of eDNA detection compared to sites without a seaweed. Neither temperature nor depth showed significant associations with eDNA detection (*p* > 0.05). Environmental factors explained approximately 9.5-9.7% of the variation in eDNA detection probability (Nagelkerke's R^2).

For drop camera detection, the environmental gradient represented by temperature and depth showed significant associations, but only when modelled separately due to multicollinearity. In the temperature model, warmer temperatures were associated with

increased detection probability (*Odds Ratio* = 6.77, 95% *CI*: 1.60-39.82, *p* = 0.014). In the depth model, greater depth was associated with decreased detection probability (*Odds Ratio* = 0.62, 95% *CI*: 0.37-0.91, *p* = 0.026) indicating higher detection in shallow waters. Because temperature and depth are so highly correlated in this system (r = 0.93), these results represent the same underlying environmental gradient: shallower, warmer waters had higher camera

detection rates. Environmental factors explained approximately 15.0-16.7% of the variation in camera detection probability (Nagelkerke's R^2). These low R^2 values are typical for ecological field studies where unmeasured factors (e.g., fine-scale habitat heterogeneity, organism behaviour, hydrodynamic variability) contribute substantial unexplained variation. The limited number of camera detections (n=8) constrains statistical power, and results should be considered hypothesis-generating rather than definitive.

When environmental variables were categorised into quartiles, eDNA detection rates showed minimal variation across temperature quartiles. Drop camera detection rates were 3.1% compared to 5.1% where prey (anything *A. amurensis* are known to eat) was absent. Conversely, eDNA detection rates were higher where prey was present (21.9%) compared to sites where prey was absent (17.1%). However, prey availability was not a significant predictor in either model ($p > 0.05$).

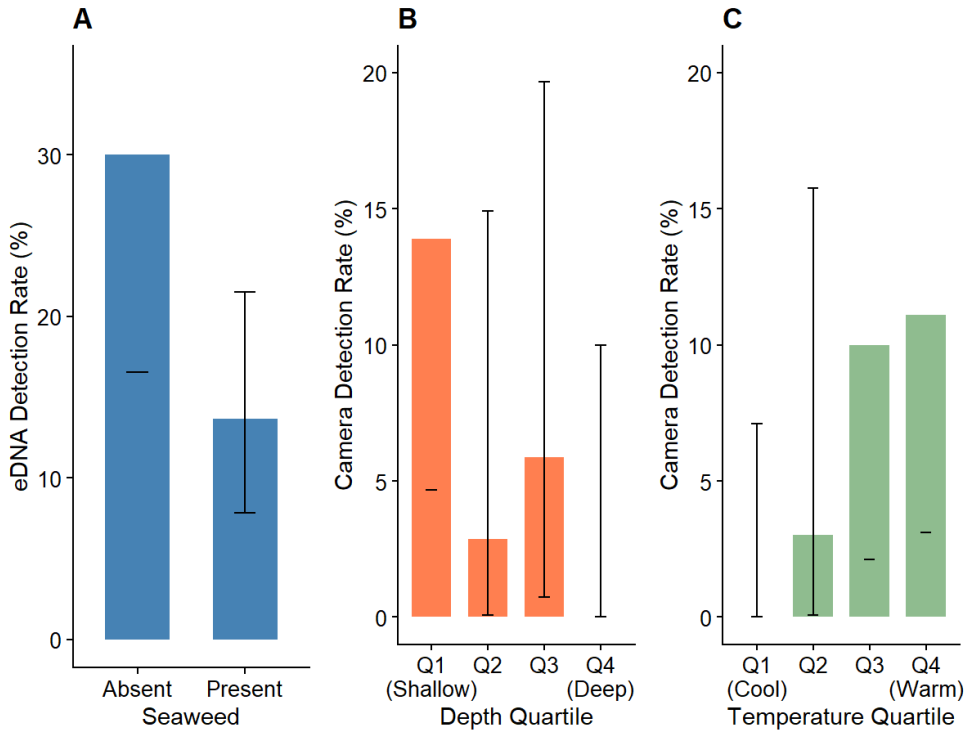

Figure 6: Exploratory analysis of environmental variables and detection probability. a) eDNA detection rates by seaweed presence/absence (any visible microalgae vs none). Seaweed presence showed statistical association with lower eDNA detection, though this coarse binary measurement may not reflect genuine ecological pattern. B-c) camera detection across depth and temperature quartiles (highly correlated, r=0.93). Error bars show 95% binomial confidence intervals. Environmental associations should be interpreted cautiously given limited statistical power (n=8 camera, n=23 eDNA detections) and coarse categorical measurements.

Combined Detection Analysis

To examine environmental associations with *A. amurensis* presence independent of detection method, an additional analysis pooled detections from both methods. Sites were classified as positive if either eDNA, drop camera (or both) detected sea stars. Among the 140 sites with complete environmental data, 30 sites (21.4%) showed positive detection by at least one method. This combined detection rate was higher than either method alone (eDNA: 15.3%, camera: 5.3%), demonstrating the complementary nature of the two approaches (Hinz et al., 2022).

Of the 30 sites with positive detections, only 1 site (3.3%) was detected by both methods concurrently, 22 sites (73.3%) were detected by eDNA only, and 7 sites (23.3%) were detected by camera only. This pattern confirms that using both methods together captures substantially more occupied sites than either method alone with the dual-method approach detecting 30.4% more sites than eDNA alone and 275% more sites than drop camera alone would have identified.

Due to the severe multicollinearity between temperature and depth (r = -0.93), separate models were fitted using either temperature or depth as predictors, along with seagrass, seaweed and prey availability.

In the combines analysis, seaweed presence was the only significant predictor in both models (temperature model: Odds Ratio: = 0.29, 95% *CI*: 0.10-0.79, p = 0.020; depth model Odds Ratio: = 0.32, 95% *CI*: 0.12-0.82, p = 0.017). Sites with seaweed presented approximately 70% lower odds of *A, amurensis* detection by either method compared to sites without seaweed. Neither temperature or depth showed significant associations with combined detection probability ($p > 0.05$). Environmental factors explained approximately 9.6-10.1% of variation in overall sea star presence. (Nagelkerke's R^2).

The robust negative association between seaweed presence and detection across eDNA-only, drop camera-only, and combined analysis suggests this relationship reflects a genuine ecological pattern rather than a method-specific artefact. The combined analysis, utilising increased statistical power from 30 detections rather than 8 (drop camera) or 23 (eDNA) separately, reinforces that integrated monitoring using both methods is essential for comprehensive assessment of *A, amurensis* distribution, as single-method approaches would miss a substantial proportion of occupied sites.

DISCUSSION

Our concurrent deployment of eDNA analysis and drop camera surveys across 150 sites in Port Phillip Bay revealed a striking pattern: both methods successfully detected *Asterias amurensis yet* agrees on specific site-level presence no better than expected by chance (κ =0.03, 95% CI: -0.14 to 0.09). This minimal overlap, rather than indicating methodological failure, suggests these approaches capture fundamentally different aspects of sea star ecology. eDNA detected *A. amurensis* at 18% of sites compared to 5.3% for cameras, but only one site showed concordant detection by both methods. This pattern has significant implications for monitoring program design (Von Ammon et al., 2023): the choice between methods depends critically on the specific management question being addressed and the scale at which information is needed.

What Each Method Actually Detects: Temporal, Spatial, and Biological Scale Differences

The divergent detection patterns likely reflect fundamental differences in what each method measures across three key dimensions: temporal integration, spatial integration, and target life stages.

Temporal Scale and Spawning Legacy

The most parsimonious explanation for widespread eDNA detection with minimal visual confirmation involves the timing of our sampling immediately following the broadcast spawning season. Females may become reproductive within a year and produce up to 20 million eggs in their first year (Byrne et al., 1997; Du et al., 2021), and larvae can remain viable in the water column for up to 120 days (Bruce et al., 1995; Richardson et al., 2016). This broadcast spawning releases enormous quantities of gametes, fertilisation products, larval exuviae, and associated cellular material into the water column – all containing target DNA. Importantly, this genetic material persists after the spawning event, creating a temporal "echo" of reproductive activity the eDNA captures but visual surveys miss entirely.

This temporal integration may represent a valuable signal rather than a limitation. Detecting spawning activity provides information about reproductive potential and larval recruitment that adult counts alone cannot capture. However, it also means eDNA-positive sites may not indicate current adult presence at that specific location. The single site where both methods detected sea stars (STK018, one visible adult, CT = 35.2) demonstrates that some eDNA signals do correspond to adult presence, but this was the exception rather than the rule in our post-spawning survey. Future studies conducted outside the larval window, when spawning-derived eDNA has degraded and adults are more sedentary, would help disentangle these temporal signals and clarify whether eDNA more reliably indicates adult populations during non-reproductive periods.

Spatial Scale and Hydrodynamic Transport

The methods also operate at fundamentally different spatial scales, Drop-cameras survey only 1 m^2 per deployment, creating substantial potential for false negatives when

A. amurensis occur in patchy distributions. This limited spatial coverage provides precise confirmation of presence at specific coordinates but may miss nearby individuals outside the narrow field of view.

Conversely, eDNA integrates signals over larger spatial scales through hydrodynamic processes. Port Phillip Bay experiences semi-diurnal tidal cycles reaching 0.5m/s, creating complex three-dimensional flow patterns that transport particulate matter considerable distances from source locations (Jensen et al., 2022; O'Donnell et al., 2017). Surface currents often differ substantially from benthic flows due to density stratification and wind forcing (Llebot et al., 2014; Richardson et al., 2016), further coupling the relationship between benthic populations and surface eDNA concentrations.

The spatial pattern at Mordialloc provides compelling evidence for this transport effect. Drop cameras detected substantial *A. amurensis* aggregations at MOR040 (15-18 individuals) and MORE044 (~60 individuals), representing the highest densities observed across all locations. Yet these high-density populations did not correspond with eDNA detections at those exact coordinates. Instead, Mordialloc showed significantly elevates eDNA detection rates across the broader location (32% of sites vs. 12% at St Kilda and 10% at Rye), creating a spatial "halo" of molecular detection around population centres. This pattern strongly suggests hydrodynamic transport of genetic material from areas of high biomass to surrounding waters, with eDNA providing a regional signature of sea star presence rather than pinpoint localisation.

Importantly, not every site within Mordialloc showed positive eDNA detection despite elevated regional rates, suggesting the eDNA signal reflects localised population sources rather than uniform bay-wide dispersion. This heterogeneity indicates eDNA methods can provide meaningful information about regional population patterns, though with compromised spatial precision compared to visual surveys.

Vertical Sampling Mismatch

The substantial vertical separation between sampling approaches also likely contributed to detection differences. While drop cameras surveyed benthic habitats where *A. amurensis* reside, water samples were collected 0.5m below the surface – up

to 14.2m above the target organisms at deeper sites (Antich et al., 2021). This vertical displacement represents a pragmatic compromise: surface sampling is logistically simpler and more cost-effective for large-scale programs but may dramatically reduce detection sensitivity for strictly benthic species unless active mixing transports genetic material upward.

Marine eDNA exhibits strong vertical gradients, with highest concentrations near source organisms (Jeunen et al., 2020; Monuki et al., 2021). The low conditional probability of eDNA detection at sites where cameras confirmed adult sea star presence (12.5%) likely reflects this vertical dilution effect. Near-benthic water sampling would presumably improve correspondence between methods and provide more reliable spatial information about adult populations, though at increased cost and logistical complexity. The surface sampling approach employed here should be viewed as testing whether benthic organism signals can be detected in surface waters – a question relevant to practical monitoring program design (Foote et al., 2012)– rather than as an optimised detection protocol.

Biological Scale: Life Stage Detection

The methods target different life stages: camera detect only visible adults (typically >7cm diameter in our footage), while eDNA potentially captures genetic material from microscopic larvae, recently settled juveniles, gametes, and adults. Post-spawning sampling maximises the likelihood that eDNA detections include signals from multiple life stages simultaneously, particularly given the extended larval duration (up to 120 days) characteristic of *A. amurensis* (Bruce et al., 1995). This life-stage integration represents both an advantage (comprehensive detection of reproductive activity and recruitment potential) and a complication (difficulty attributing eDNA signals to specific demographic components).

Synthesis: Complementary Information Across Scales

Rather than viewing these factors as simply "limitations," they highlight that eDNA and visual surveys provide fundamentally complementary information (Hinz et al., 2022; Tulloch et al., 2025). eDNA offers broad-scale screening capable of detecting multiple life stages and integrating signals across larger spatial and temporal windows, making it

valuable for identifying regions with *A. amurensis* activity – whether from adults, recent spawning, or larval presence. However, this integrated signal compromises spatial precision for locating specific populations and temporal precision for determining current adult distributions.

Visual surveys provide precise confirmation of adult presence at exact coordinates and offer contextual habitat information (substrate type, prey availability, population density) that cannot be inferred from molecular data alone. However, limited spatial coverage (1m^2 per deployment) may miss patchy populations, and the method is blind to microscopic life stages and temporal signals of recent spawning.

The 30.4% increase in total detected sites when methods were pooled (30 combined positives vs. 23 eDNA-only or 8 camera-only) demonstrates clear value in integrated monitoring. Each method captured sites the other missed: sites detected by eDNA alone likely represent transported signals, spawning legacy, or larval presence, while sites detected by camera alone may reflect localised adult aggregations in areas where surface eDNA signals were diluted or degraded (Foote et al., 2012). The single site with dual detection demonstrates that correspondence can occur but was exceptional in this study's sampling design.

For management applications requiring precise locations of high-density aggregations for targeted removal efforts, visual surveys remain essential. For broad-scale surveillance to detect new incursions, track regional patterns, or assess reproductive activity. eDNA provides efficient screening. An effective monitoring program would integrate both approaches strategically based on management objectives (Von Ammon et al., 2023), with eDNA identifying priority regions for focussed visual surveys.

Environmental Associations with Detection: Exploratory Insights

Our exploratory analysis of environmental predictors identified significant associations with detection probability for each method, though interpretation requires caution given limited statistical power from sparse detections and the inherent constraints of observational field data where unmeasured confounding factors inevitably exist.

Seaweed and eDNA Detection

eDNA detection showed a negative statistical association with seaweed presence, though this finding warrants substantial scepticism. Seaweed presence was scored as a simple binary variable (visible/not visible in camera frame) without quantifying density, percent coverage, or distinguishing sparse fragments or dense beds. In practice, "seaweed presence" indicated any visible microalgae in frame – often just small fragments or pieces of drift algae rather than substantial seaweed beds or structured habitat. This extremely coarse measurement provides minimal information about actual habitat conditions.

Given this measurement limitation, combined with limited statistical power, this assessment may well represent a statistical artefact rather than a genuine ecological pattern. While it is theoretically plausible that dense seaweed beds could affect eDNA detection through altered flow dynamics reducing transport (Jeunen et al., 2020) or through biochemical degradation processes, our binary scoring system was insufficiently precise to test such hypotheses. The association should not be interpreted as evidence that *A. amurensis* avoid seaweed habitat or that seaweed meaningfully influences eDNA persistence in Port Phillip Bay.

Future studies seeking to understand habitat associations would require quantitative macroalgae measurements (e.g., percent cover, biomass, canopy height) rather than simple presence/absence scoring. Given the substantial measurement limitations, we do not consider this finding reliable for ecological inference and include it only to fully report our analytical results.

Camera Detection and the Shallow-Warm Gradient

For drop camera detection, the depth-temperature gradient showed significant associations (warmer/shallower sites had higher detection), though these two variables could not be modelled simultaneously due to severe multicollinearity. In Port Phillip bay's shallow, well-mixed waters, depth and temperature are tightly coupled: shallow waters are consistently warmer, creating an essentially one-dimensional environmental gradient rather than two independent factors.

The pattern observed – higher camera detection in shallow, warm waters versus deeper, cooler waters – could reflect several processes that our data cannot distinguish. *A. amurensis* may genuinely prefer shallower habitats in Port Phillip Bay, consistent with their known depth range preference in invaded regions (Byrne et al., 1997). Alternatively, the pattern may reflect post-spawning behaviour, as this species undergoes seasonal migrations to deeper waters following reproduction (Barrett et al., 2020), potentially explaining low detection rates if adults had retreated from shallow sites when surveys were conducted.

The minimal temperature variation across sites falls well within the known thermal tolerance of this species (Kashenko, 2005) suggesting any observed relationship more likely reflects seasonal behaviour or methodological factors rather than fundamental physiological constraints.

Importantly, the limited environmental variation across study sites – all within relatively homogenous shallow marine environment renders these statistical relationships of uncertain ecological significance. The R^2 values of 15-17% indicate environmental variables explained a modest proportion of detection variation, with most variation attributable to unmeasured factors (e.g., fine-scale habitat heterogeneity, stochastic encounter probability, organism behaviour, hydrodynamic variability).

The environmental association analysis, while yielding some statistically significant relationships, should be viewed as hypothesis-generating rather than hypothesis-testing given substantial constraints: sparse positive detections limiting statistical power, coarse predictors failing to capture quantitative environmental variation, limited environmental gradients across study sites, and inability to control for confounding factors in observational field studies. These exploratory findings may inform future targeted research with larger sample sizes, quantitative environmental measurements, and sampling across broader gradients, but do not provide reliable evidence for causal relationships between environmental factors and detection in this study.

Broader Implications for Marine eDNA Applications

The findings highlight fundamental challenges for eDNA applications in dynamic marine environments that extend beyond *A. amurensis* monitoring. The spatial displacement of

molecular signals through hydrodynamic transport, the confounding effects of complex life cycles and seasonal reproduction, and the difficulties of interpreting surface-water samples for benthic targets represent systematic issues for marine eDNA studies in general (Jeunen et al., 2020; O'Donnell et al., 2017).

These challenges are particularly acute in semi-enclosed bays and estuaries where complex circulation patterns, vertical stratification and variable mixing create heterogenous conditions that influence eDNA persistence and transport. Future marine eDNA studies must incorporate hydrodynamic considerations, depth-stratified sampling, and temporal replication outside reproductive periods to provide meaningful ecological inference. The assumption often implicit in eDNA literature – requires critical evaluation in each study system, particularly for broadcast-spawning marine invertebrates in advective environments.

Conversely, the broad spatial integration and life-stage sensitivity that complicate interpretation for localisation purposes may represent advantages for different management questions. Early detection of new incursions via planktonic larvae, assessment of reproductive activity and recruitment potential and regional-scale screening for presence/absence are applications where eDNA's integrative properties align well with management needs. The key is matching method properties to management objectives rather than expecting any single method to serve all purposes optimally.

Study Limitations and Future Research Priorities

Several constraints inherent to this honours-level project influence interpretation and suggest priorities for future work. The decision to sample surface waters rather than near-benthos represented a trade-off between spatial coverage and vertical sampling precision. This approach successfully demonstrated that benthic organism signals can be detected in surface waters – relevant for practical monitoring logistics – but likely reduced detection sensitivity and spatial precision through vertical dilution. Future studies should compare near-benthic versus surface eDNA sampling at sites with known adult densities to quantify correspondence with visual surveys to justify increased cost and complexity.

The post-spawning timing maximised eDNA detection but confounded interpretation by mixing signals from multiple life stages and temporal windows. Field blanks were not collected during sampling, though laboratory controls (non-template controls in qPCR) confirmed absence of reagent contamination, and the use of new sterile bottles for each sample minimised cross-contamination risk between sites. Multi-seasonal sampling is essential to disentangle spawning-derived eDNA from signals associated with current adult presence. Baseline studies establishing seasonal variations in both *A. amurensis* abundance and eDNA persistence under varying environmental conditions would clarify when eDNA most reliably indicates adult populations and improve integration of molecular and visual monitoring approaches,

The environmental association analysis, while yielding intriguing patterns, were exploratory rather than definitive given the limited statistical power from sparse detections. Future studies should incorporate: (1) stratified sampling designs targeting known high-density areas to increase positive detections and statistical power; (2) quantitative environmental measurements (e.g., macroalgal biomass, flow velocity precise temperature profiles) rather than coarse gradients to break multicollinearity between depth and temperature and enable evaluation of independent effects.

Finally, controlled validation experiments under known *A. amurensis* densities are critically needed to establish quantitative relationships between organism abundance and eDNA signal strength. Such studies should manipulate density, environmental conditions (temperature flow), and sampling protocol (depth, volume, replication) to develop predictive models linking eDNA concentrations to population metrics relevant for management.

Management Recommendations for *A. amurensis* Monitoring Program

Based on these findings, we recommend a strategic, tiered monitoring framework that leverages the complementary strengths of each method (Hinz et al., 2022). For regional screening and early detection, annual eDNA surveys during non-spawning periods should be conducted across broad spatial scales to identify regions with established populations and detect new incursions before visible populations establish. Surface water sampling while reducing detection sensitivity through vertical dilution, remains

practical for large-scale surveillance programs where cost-effectiveness and spatial coverage are prioritised over precise localisation.

For population assessment and management intervention, targeted visual surveys (drop camera transects or dive surveys) should be deployed at eDNA-positive locations to confirm adult presence, quantify population densities, characterise size structure, and precisely locate high-density aggregations requiring active removal efforts.

For early detection surveillance in high-risk introduction zones (commercial ports, marina, boat ramps), eDNA sampling immediately post-spawning capitalises on the method's sensitivity to larval signals, potentially directing new incursions via planktonic larvae before benthic recruitment occurs. However, managers must recognise that eDNA detections during reproductive periods may reflect transient larval presence of hydrodynamically-transported signals rather than established local populations, necessitating follow-up visual confirmation before committing resources to control efforts.

This integrated approach optimises detection sensitivity while managing costs, providing actionable spatial information for targeted interventions, and balancing the inherent trade-offs between broad surveillance and precise population characterisation. The substantial increase in detection achieved through combined methods (30.4% more sites than eDNA alone, 275% more than cameras alone) demonstrates that neither approach alone provides sufficient information for comprehensive management, and resource allocation should reflect this complementarity.

Conclusions

This study demonstrates that eDNA analysis and drop camera surveys provide complementary rather than interchangeable information for monitoring established *A. amurensis* populations in Port Phillip Bay. The minimal site-level agreement between methods reflects fundamental differences in temporal, spatial, and biological scales of detection rather than simple differences in sensitivity. eDNA excels at broad-scale screening and potentially detecting multiple life stages and reproductive activity, while

visual surveys provide essential ground-truth validation and precise population characterisation needed for targeted management interventions.

The combines approach detected substantially more occupied sites than either method individually, demonstrating clear value in integrated monitoring. However, the disconnect between surface water sampling and benthic target organisms, combined with complex hydrodynamic transport and post-spawning temporal signals, means eDNA spatial precision remains insufficient for applications requiring exact population locations. Future monitoring programs should integrate both approaches strategically: eDNA for targeted visual surveys to locate high-density aggregations and characterise population structure,

The development of effective monitoring protocols for *A. amurensis* and similar marine invasive species requires continued refinement of both methodological approaches and their integration, with particular attention to temporal sampling design, vertical sampling considerations, and hydrodynamic influences. This study provides a foundation for such development by rigorously comparing methods under field conditions and identifying key factors influencing detection patterns. The insights gained here have implications extending beyond *A. amurensis* to broader applications of eDNA for monitoring marine benthic species in dynamic coastal environments.

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