CENTRE FOR
INVASIVE SPECIES SOLUTIONS

REAL TIME eDNA TOOLS TO IMPROVE EARLY DETECTION AND RESPONSE APPROACHES FOR HIGH-RISK PEST ANIMALS

FINAL REPORT FOR PROJECT P01-I-004

AUTHORS

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We are a not-for-profit, member-based organisation formed to address the impact of invasive plants and animals across Australia.

We acknowledge the Traditional Custodians of the lands on which we meet and work and pay our respects to Elders — past, present and emerging.

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Cover Image: Alejandro Trujillo-González tests eDNA in a laboratory.

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EXECUTIVE SUMMARY

This report provides an overall summary of the work completed by the Centre for Invasive Species Solutions. Rapid detection and identification of high-risk invasive animals either at the point of entry or in the field are essential to prevent new incursions and enable a rapid response for successful eradication. Detecting and monitoring species using environmental DNA (eDNA) is recognised as a powerful tool, and has been shown to have greater sensitivity, with less effort and negative effects, compared to traditional survey methods.

The key outcomes of this project have been the following:

- protocols and tools that can be used at point-of-need locations have been developed and evaluated
- real-time assays have tested and validated for two high-risk invasive animals (red-eared slider turtle and Asian black-spined toad) and field validations for each have been carried out
- a multi-species detection framework has been developed with national guidelines and testing protocols published
- a National eDNA Reference Centre has been established at the University of Canberra along with a Collaboration Network of participating laboratories through a partnership with the Department of Agriculture, Fisheries and Forestry (DAFF)
- ongoing projects continuing with DAFF under the Biosecurity Innovation Program (BIP) expanding assays for targeted high-risk species and developing further novel applications.

INTRODUCTION

This project has built on the previous Invasive Animals Cooperative Research Centre (CRC) research (Project 1. W. 2) that demonstrated the effectiveness of environmental DNA (eDNA) as a tool for detecting invasive aquatic species. A quantitative framework for targeted species detection was developed in Australia. With eDNA firmly established as a highly effective method for species detection, it can now be further refined for routine use in biosecurity applications.

The objectives of this project were to (1) provide rapid detection tools using real-time technology that can be used *in situ*, either in the field or at the border; (2) develop new tests for targeted high-risk invasive animals; (3) validate a multi-species detection framework for species of unknown risk; and (4) operationalise eDNA through building capability for routine application by end users and partner agencies.

OBJECTIVE 1: REAL-TIME RAPID EDNA DETECTION TOOLS

The suitability of real-time detection tools for Australian applications, focusing on field tests for high-risk incursions and for border-based applications, were assessed. At the conception of this project there were real-time field tests available for six species, including: New Zealand mudsnail, hellbender salamander, bighead carp and silver carp, grass garp, and sea lamprey. Through the Invasive Animal CRC project (1.W.2) we had validated, peer-reviewed assays available for three invasive species: European carp, redfin perch, and oriental weatherloach. Our approach was to work with the company Biomeme to trial field portable quantitative PCR analysis (qPCR) devices using our custom assay for at least one species.

For multi-species detection, we investigated the suitability of the most recent DNA sequencing technology to emerge that is based on nanopore sequencing. The Oxford Nanopore MinION is a thumb-drive sized device that measures deviations in electrical current as a single DNA strand is passed through a protein nanopore. The size, robustness, and affordability of the MinION is unique, and more akin to a mobile sensor than a traditional sequencer. Initial trials have shown promise for this technology for microbial community assessment but is yet to be expanded for other species.

OBJECTIVE 2: DEVELOP NEW EDNA TESTS FOR AT LEAST TWO HIGH-RISK INVASIVE SPECIES AND APPLY THESE TO FIELD OPERATIONS

The Asian black-spined toad (ABST) (*Duttaphrynus melanostictus),* is considered to be a high-risk invasive species with the most likely pathway of introduction being as a stowaway from South-East Asia. There have been a growing number of ABST detections pre- and post-border across Australia. The risks posed by this species are well documented. The growing numbers of detections of ABST means agencies increasingly need to be operationally prepared to respond to incursions. eDNA is the most promising technology for a cost-effective active and passive surveillance tool and to increase the efficiency of delimitation and post-control.

Red-eared slider turtles (REST) (*Trachemys scripta elegans)* are a high-risk invasive species that have been detected across a number of jurisdictions. Since 2000, 70 REST have been detected in Victoria, with 20 of these found in or adjacent to Victorian rivers and lakes. The source of each animal is exclusively illegal trade, suspected to be either directly from overseas or from those domestically bred and distributed. Without ongoing intervention this species will increasingly be detected in the wild in Victoria and could establish a breeding population like those in New South Wales (NSW) and Queensland. Given this species is illegally brought into Australia from South-East Asia and poses associated exotic animal disease risk, a tool that increases the efficiency and effectiveness of surveillance is required.

Our aim was to develop validated eDNA assays following protocols in Furlan and Gleeson (2016a, 2016b) for both species and include these as trial species for ongoing passive surveillance.

OBJECTIVE 3: VALIDATE MULTI-SPECIES EDNA DETECTION FRAMEWORK TO ENABLE ALL SPECIES TO BE DETECTED FROM A SAMPLE OR LOCATION

Alternative to the single-species-specific approach, analysing eDNA with high-throughput sequencing allows for multiple taxa identification simultaneously from a single sample using metabarcoding. Metabarcoding is especially useful for discerning non-target or unanticipated taxa. Although this approach is promising, and we have demonstrated the ability of this method to detect fish species from eDNA samples in a river system, a quantifiable framework has not been established and validated as is the case with single-species detection. For this method to be applied for surveillance and border-detection, challenges such as identifying discriminatory DNA markers, understanding likely bias from PCR and sequencing needs to be overcome. These then require protocols and guidelines for direct application purposes.

OBJECTIVE 4: DEVELOP EDNA CAPABILITY WITHIN END-USER ORGANISATIONS THROUGH TARGETED TRAINING AND PROVIDE READILY AVAILABLE EDNA SERVICES FOR ONGOING SURVEILLANCE

Currently one of the key limitations in the uptake and application of eDNA is the capability within agencies to be able to access these tools as part of their mainstream surveillance operations. Our aim was to work closely with partner agencies and end users to provide specific training and make available the necessary resources for the collection of eDNA samples. This would involve developing a central portal for specific services as they become available; and the maintenance of well-curated databases and tissue collections, bioinformatics, and modelling capacity for the interpretation of results and timely reporting.

Critically, we aimed to undertake the necessary steps to becoming the first facility to provide eDNA tests that are accredited under ISO 17025, an internationally recognised accreditation for testing laboratories. The 17025 standard evaluates technical expertise and systems so that clients, regulatory authorities, and the courts have confidence in the test results. As eDNA becomes more widely used, the credibility of the results will become more essential. This will be crucial for enforcement and prosecution of Australia’s biosecurity policies. Therefore, at least one facility in Australia will require this level of competence.

OBJECTIVE 1: RAPID EDNA DETECTION TOOLS USING REAL-TIME TECHNOLOGY FOR *IN SITU* APPLICATION

CASE STUDY A: PORTABLE TECHNOLOGIES FOR TARGETED SPECIES DETECTION

This contains information from the publication:

Gonzalez AT, Thuo D, Divi UK, Sparks K, Gleeson D and Wallenius T (2022) 'Detection of Khapra Beetle Environmental DNA Using Portable Technologies in Australian Biosecurity', *Frontiers in Insect Science,* 2:1–10

SUMMARY

- This study tested laboratory and portable molecular technologies to detect khapra beetle environmental DNA extracted from dust samples collected during two biosecurity responses.
- Airborne and floor dust samples were collected opportunistically using handheld vacuum cleaners and eDNA was extracted using either field (Biomeme M1 sample prep kit) or laboratory-based (Qiagen DNeasy Blood and Tissue kit) extraction methods.
- eDNA extracts were tested using two real-time PCR TaqMan assays in benchtop (Applied Biosystems ViiA 7 Real Time PCR system) and portable machines (Biomeme Franklin Real-Time PCR thermocycler) and one LAMP-based assay in a portable machine.
- We successfully collected, extracted, and amplified khapra beetle eDNA from dust samples by qPCR, but failed to amplify khapra beetle (*T. granarium)* eDNA using loop-mediated isothermal amplification (LAMP) .
- There were no significant differences in DNA yield between collection methods or differences in amplification associated to extraction or collection methods in either platform tested in this study.
- Portable technologies tested in this study (Franklin™ Real Time Thermocycler and Genie III) accurately amplified all tissue-derived positive control DNA used during assay optimisation and field testing, highlighting the capacity of these technologies to complement biosecurity requirements in confirming specimen ID.
- Environmental DNA testing could effectively complement current inspection methods in biosecurity responses by providing a tool to triage and prioritise efforts. However, there are still multiple obstacles that must be critically assessed before biosecurity officers could use portable molecular technologies as part of their biosecurity toolbox.
- Legislative, policy and science-based guidelines that would determine how eDNA-based testing is to be undertaken in Australia are yet to be defined. This study provides an early view of how eDNA-based testing could greatly complement Australian biosecurity measures.

BACKGROUND

eDNA-based methods have been reliably shown to be cost-effective methods to inform users of the presence of target species in surveillance applications (Morisette et al. 2021), with studies showing how eDNA-based detection using soil and airborne dust samples can provide valuable data on species presence and diversity (Lennartz et al. 2021; Yasashimoto et al. 2021). When used with portable, point-of-care technologies, eDNA-based detection can offer sensitive detection tools to support management of biosecurity risks (Hole et al. 2019).

Multiple platforms have recently become available for rapid field-based DNA testing to identify species. In this objective, we investigated the suitability of two platforms for Australian applications. One platform was for species-specific assays using Biomeme kits (Biomeme, Inc.) and portable PCR instruments, while the other platform was for multi-species detection using Oxford Nanopore MinION. Both platforms were then compared with standard laboratory-based methods for performance in detection ability.

For the species-specific *in situ* comparison, the research team were fortunate to have access to a biosecurity response in August and November 2020, following the detection of khapra beetle in two separate locations in the Australian Capital Territory (ACT). Khapra beetle is currently considered the most important national priority plant invertebrate pest for the grain industry in Australia (Australian Government 2019). Although this species is not established within Australia, it is a highly invasive quarantine pest, and a widespread incursion could cost the country $15.5 billion over 20 years.

METHODS

ENVIRONMENTAL DNA SAMPLE COLLECTION

Environmental DNA sampling at confirmed *T. granarium* detection sites was subject to government approval and access. Sampling occurred where biosecurity response measures had already been initiated by Department of Agriculture, Fisheries and Forestry (DAFF) under the Emergency Plant Pest Response Deed. As such, testing of eDNA collection methods and portable technologies was opportunistic, and subject to available time frames within which approval was given to sample each location. Samples were collected from two separate ACT retail stores that had received and stored goods infested with khapra beetle in August (Tuggeranong, Canberra) and November (Fyshwick, Canberra) of 2020. Specimens were morphologically identified by DAFF entomologists and by molecular identification. Environmental DNA sampling was undertaken during biosecurity delimitation responses at each site following detection of *T. granarium*.

TUGGERANONG DETECTION EVENT – AUGUST 2020

Environmental DNA samples were collected simultaneously using two methods. In the first method, officers vacuumed each grid using four vacuum cleaners; three handheld (Black+Decker Pet Dustbuster) and one commercial vacuum cleaner provided by the retail store (brand unknown). Vacuum cleaners were used randomly to vacuum each grid by biosecurity officers for approximately four minutes. The content of each vacuum cleaner was then emptied into a plastic bag and labelled for further visual inspection by entomologists of DAFF. Vacuum cleaners were not sterilised between grids and cross-contamination could not be prevented. Three samples were placed directly inside 5 mL tubes with 2.5 mm ball bearings and 3 mL of Biomeme Lysis Buffer (Biomeme, Inc.). The remaining three samples were placed inside 5 mL tubes with 4 mL of 80% ethanol.

The second method involved the use of a separate handheld vacuum cleaner (Dyson V7 cord-free vacuum) attached to a plastic filter casing with a 42 mm, 1.2 µm pore size cellulose nitrate filter paper (Sterlitech, Inc.) to sample airborne dust. This method was based on a previously tested protocol used to sample for dust samples in field conditions (Campbell et al. 2022). This vacuum cleaner had not been previously used by biosecurity officers and was sterilised with 2.5% bleach before the sampling event. Airborne dust within each grid was vacuumed for approximately 15 seconds, approximately 30 cm above the area of each quadrant. Filters were then carefully removed from the casing using sterile single-use forceps and placed inside 5 mL tubes containing 2.5 mm ball bearings and either 3 mL of Biomeme Lysis Buffer (Biomeme, Inc.) or 4 mL of 80% ethanol. The filter casing and vacuum cleaners were then wiped clean using paper towels and one per cent bleach. A total of six airborne samples were planned to be collected from each quadrant; however, time limitations allowed for only two filters to be collected from three out of six quadrants. All samples were immediately taken to the University of Canberra for eDNA extraction.

A total of eight field negative controls were collected. Three were 5 mL tubes 3 mL of Biomeme Lysis Buffer (Biomeme, Inc.), three 5 mL tubes with 4 mL of 80% ethanol; and two consisted of filter papers placed inside the plastic filter casing attached to the handheld vacuum cleaner, placed inside either a 5 mL tube with 3 mL of Biomeme Lysis Buffer or 4 mL of 80% ethanol. Field controls were collected

by opening each tube and walking across all quadrants while officers were vacuuming. Following that, filter paper controls were collected by vacuuming the air at eye-level (approximately 185 cm) while walking across all quadrants while officers were vacuuming.

FYSHWICK DETECTION EVENT – NOVEMBER 2020

In this occasion, officers vacuumed the whole 5-metre area using a large commercial dry vacuum cleaner with internal single-use paper filter bags (VAX®, Australia) for approximately 15 minutes. This vacuum cleaner had been used during a separate *T. granarium* detection event three days prior, and it was unclear if officers used single-use filters during that event. For this reason, two dust samples were taken from the inside filter of the vacuum cleaner using sterile, single-use forceps to determine potential cross-contamination and placed directly inside a 5 mL tube with 2.5 mm ball bearings and 3 mL of Biomeme Lysis Buffer (Biomeme, Inc.). After vacuuming, eight dust samples (approximately 10 g of sample) were collected from the paper filter of the vacuum cleaner using sterile single-use forceps and placed directly inside 5 mL tubes with 2.5 mm ball bearings and 4 mL of Biomeme Lysis Buffer (Biomeme, Inc.).

Environmental DNA samples were processed and analysed at the site to test operational use requirements during this biosecurity response. All samples were processed using the M1 Bulk Sample Prep Kit for DNA-HI (Biomeme Inc.) onsite. Given indications that there was potential for khapra beetle eDNA contamination from a prior biosecurity response, the two samples collected from the vacuum cleaner were tested before any others using two Franklin™ Real Time Thermocyclers. Both samples were run in triplicate in each thermocycler with a separate strip containing two non-template controls and one genomic positive control. Each thermocycler ran one of the two *T. granarium* TaqMan assays tested in this study.

CONFIRMED POSITIVE KHAPRA BEETLE EDNA SAMPLE AND POSITIVE CONTROLS

A separate vacuumed sample was provided by DAFF, collected from the car boot of an individual who purchased a refrigerator infested with *T. granarium* during a third detection event in the suburb of Kambah, Canberra. This sample was collected by biosecurity officers by sweeping and vacuuming using a handheld vacuum cleaner, and was confirmed to contain a live *T. granarium* larva. The larva was sent for molecular identification, while the dust sample was kept at room temperature inside a plastic zip-lock bag at the Commonwealth Scientific and Industrial Research Organisation (CSIRO) Black Mountain site in Canberra for approximately five days prior to being delivered to the University of Canberra for DNA extraction.

Biosecurity officers also provided 10 separate vials with *T. granarium* larvae and adult specimens collected during the initial detection at the Tuggeranong site as positive controls for testing during qPCR amplification. Genomic DNA from each vial was extracted using a DNeasy Blood and Tissue Kit (Qiagen) following the manufacturer's instructions. All samples were stored at –20 °C.

ENVIRONMENTAL DNA EXTRACTION PROCESS

All samples kept in Biomeme lysis buffer collected during the Tuggeranong and Fyshwick detection events were extracted using M1 Bulk Sample Prep Kits for DNA-HI (Biomeme, Inc.). Two extraction negative controls (5 mL tubes with 3 mL of Biomeme lysis buffer) were prepared and processed together with samples to assess extraction-level cross-contamination. Each tube was shaken vigorously for two minutes and 1 mL of lysis buffer from each tube was collected sequentially using a Biomeme syringe filter attached to a sterile 1 mL syringe and processed following the manufacturer's protocol. Each sample was eluted in 500 µL of Biomeme Elution buffer and stored at –20 °C.

All samples kept in 80% ethanol and the confirmed positive sample from Kambah were processed using a DNeasy Blood and Tissue Kit (Qiagen). Two extraction negative controls (1.7 mL Eppendorf tubes with 180 µL of lysis buffer and 20 µL of Proteinase K) were prepared and processed together with samples to assess extraction level cross-contamination. Dust samples from each tube were placed on a sterile glass surface and a small fraction (approximately 5 g) was placed inside a 1.7 mL Eppendorf tube with 180 µL of lysis buffer and 20 µL of Proteinase K. Tubes were then placed on a rocker and incubated at 56 °C inside a hybridising oven for 1 h. Samples were then processed

following the manufacturer's protocol and eDNA eluted in 50 µL of MilliQ water. A total of 15 replicate samples were extracted from the Kambah dust sample, given its importance for assay testing and optimisation. DNA yield of each extract was measured using a Thermo Scientific™ NanoDrop™ One Microvolume UV-Vis Spectrophotometer and then stored at –80 °C.

TROGODERMA GRANARIUM TAQMAN ASSAY OPTIMISATION

Samples collected in this study were tested for the presence of *T. granarium* eDNA using two published probe-based assays (Furui et al. 2019; Olson et al. 2014) (Table 1). The Olson assay targeted a 248bp fragment of the 16s gene region (Olson et al. 2014) while the Furui assay targeted an 83bp fragment of the ND6 (NADH dehydrogenase VI region) (Furui et al. 2019). Both assays were optimised for the purpose of testing environmental samples for the presence of *T. granarium* DNA following minimum quality standards for qPCR testing (Bustin et al. 2009). Both assays were *in silico* tested for specificity using the BLAST search function on the National Centre for Biotechnology Information (NCBI) website. The Olson assay has been validated as a diagnostic assay to confirm the identity of *T. granarium* specimens targeting tissue-derived DNA, having undergone specificity testing against co-occurring and phylogenetically related dermestid species in Australia (*National Diagnostic Protocol, under review*). The Furui assay has not been formally validated as a diagnostic assay but was tested for sensitivity and specificity by the authors (Furui et al. 2019). This study complements prior specificity testing of each assay by testing assays against two separate specimens of *Trogoderma variabile* Ballion, 1878 and native *Trogoderma*, *Anthrenus*, *Anthrenocerus*, *Attagenus* and *Orphinus* (Coleoptera: Dermestidae) tissue samples provided by the Science and Surveillance Group from DAFF.

The analytical sensitivity of each TaqMan assay was assessed by obtaining the limit of detection (LoD) using eDNA and synthetic standards designed for each assay (Supplementary 1). Standard curves were established using dilution series of known concentrations ranging from 10^7 copies/µL and decreasing tenfold down to one copy/µL. The same was done with eDNA extracted from the confirmed positive dust sample collected in Kambah, from starting concentration of 10 ng/µL down to 10^{-6} ng/µL. Six PCR replicates were used in each dilution step to assess LoD. The LoD was assessed as the last dilution of the standard curve wherein the targeted DNA amplified in all qPCR technical replicates. Given that, at the time of testing, there was one confirmed positive eDNA sample collected in Kambah, this study provides a preliminary evaluation of accuracy by examining positive amplification of qPCR technical replicates.

Each TaqMan assay was optimised for use in a ViiA™ 7 Real-Time PCR System (Applied Biosystems, Australia) and in a Franklin™ Real Time Thermocycler (Biomeme Inc., USA) to test eDNA. Each reaction in the ViiA™ 7 Real-Time PCR System consisted of 10 µL TaqMan Environmental Master Mix 2.0 (ThermoFisher), 1 µL of each of primer and probe (10 µM) for the Olson assay or 0.8 µL (10 µM) for the Furui assay, 5 µL of template, and PCR water for a total volume of 20 µL. Reactions in the Franklin™ Real Time thermocycler consisted of 10 µL of 2x LyoDNA 2.0 + IPC Master mix (Biomeme, Inc.), 1 µL of each of primer and probe (10 µM) for the Olson assay or 0.8 µL (10 µM) for the Furui assay, 5 µL of template and PCR water for a total volume of 20 µL.

Cycling conditions differed between platforms. In the ViiA™ 7 Real-Time PCR System, conditions for the Olson assay were: 95 °C (10 minutes), followed by 50 cycles of 95 °C (20 seconds), 50 °C (1 minute) and 72°C (30 s) ramping at 2.42 °C/second, followed by a final holding stage at 4 °C. Conditions for the Furui assay were: 95 °C (10 minutes), followed by 45 cycles of 95 °C (20 seconds) and 60 °C (20 seconds) ramping at 2.42 °C/second, followed by a final holding stage at 4 °C. In the Franklin™ Real Time Thermocycler, conditions for both assays were optimised for field-based operational use. Cycling conditions for the Olson assay were optimised as follows: 95 °C (2 minutes), followed by 50 cycles of 95 °C (10 seconds) and 50 °C (30 seconds). Conditions for the Furui assay were 95 °C (2 minutes), followed by 45 cycles of 95 °C (20 seconds) and 60 °C (10 seconds). Samples in both platforms were run in triplicate with positive and non-template controls. All positive controls in this study were genomic DNA extracted from confirmed *T. granarium* larvae or adults collected by biosecurity officers during each event.

TROGODERMA GRANARIUM LAMP ASSAY OPTIMISATION

An LAMP assay designed to detect a 234 bp fragment in the 16S gene region of *T. granarium* was tested against eDNA (Rako et al. 2021) (Table 1). This assay was designed as a rapid molecular method to identify *T. granarium* specimens. The assay has been tested for specificity against 23 non-target *Dermestidae* species, showing no non-target amplification, and was validated to detect as little as 1.0^{-6} ng/µL of *T. granarium* DNA extracted from larvae and adult specimens (Rako et al. 2021) This assay is currently accepted by DAFF to confirm the identity of *T. granarium* specimens collected during biosecurity responses in laboratory conditions (Trujillo-González et al. 2022). This assay was tested in laboratory conditions against confirmed positive eDNA extracts from samples collected in Kambah. Reactions were undertaken in a handheld Genie III machine (OptiGene, UK) at the University of Canberra. Each reaction consisted of 14 µL Isothermal Master Mix Iso-001 (Geneworks, Australia), 10 µL of primer mix and 1 µL of eDNA or DNA template for a total volume of 25 µL. Each run in the Genie III consisted of six technical replicates of the eDNA positive sample, one positive DNA control and one non-template control. Isothermal amplifications conditions were 65 °C for 25 minutes followed by a ramping step from 98 °C to 73 °C at 0.05 °C/second.

Table 1. TaqMan and Loop-mediated Isothermal Amplification assays used to amplify Trogoderma granarium eDNA

Assay	Primer	5'-Sequence-3'	Size (bp)	Reference
Modified Olson assay	der16SF4	CTAAAATTGAAAATTTCTATACT	248	Olson et al. 2014
	der16SR1	CTAGCCTGCTCCCTGATTGA		
	P1	FAM- TGACTGTGCGAAGGTAGCAT-QSY		
Furui assay	Furui F	CAGCCTTATATGACTTCTCATACC	83	Furui et al. 2019
	Furui R	GATTTCATGTTGGGAATGATG		
	Furui P	FAM- GCAAATGGTGGCGAGTGTTGTC-QSY		
LAMP assay	Khapra_F3	GGTAATTTAATCTTATAATCACAAGATGG	234	Rako et al. 2021
	Khapra_B3	AACTGGAATGAATGGTTGGACGAA		
	Khapra_FIP	TTGTTAGTATAGAAATTTTCAATTTTAGGATC ATCTAATCATAAATCAATGTTTCA TTTAACAATTAAAGAAATAATAAAACTCTTGAT		
	Khapra_BIP	TACTGTCTCTTTTTTATTTTG		
	Khapra_Floop	TTAATTTGGTTGGGGTGACTA		
	Khapra_Bloop	CGTCTTTTAAAAAAATTTGAGCC		

REAL TIME PCR POSITIVE/NEGATIVE DETERMINATION AND STATISTICAL ANALYSES

PCR replicates for the eDNA samples were putatively positive if amplification curves crossed a common fluorescence threshold. The threshold was determined by the inclusion of positive controls within each qPCR run. Replicates where no amplification was observed above a common threshold were deemed negative. Putative positive amplicons and replicates with amplification beyond each assay's limit of detection were purified using a PCR purification Kit (Qiagen) following the manufacturer's instructions and sent for Sanger sequencing to the Biomolecular Resource Facility (BRF) at the John Curtin School of Medical Research at the Australian National University (JCSMR, ANU) for species amplification confirmation. Mean DNA yields were compared between sites and methods using non-parametric Mann-Whitney U tests as data was not normally distributed. Mean cycle thresholds (Ct) of confirmed positive detections were also compared between sites, assays and collection methods using two-way ANOVAs followed by Tukey's HSD tests (SPSS Statistics 23.0.0).

RESULTS

ENVIRONMENTAL DNA EXTRACTION YIELD AND AMPLIFICATION

There was a significant difference in mean DNA yield achieved between the methods used to extract DNA from vacuum and filter samples collected in Tuggeranong. Yields were significantly higher using the Qiagen DNeasy Blood and Tissue Kits with means of 5.52 ± 4.45 ng/µL in vacuum samples and 4.77 ± 1.68 ng/µL in airborne samples, compared to the Biomeme protocol of 1.75 ± 1.17 ng/µL in vacuum samples and 1.36 ± 1.29 ng/µL in airborne samples (Mann-Whitney U Test, $p < 0.001$) (Figure 1A). There were no significant differences in DNA yield between collection methods within each extraction method (Figure 1A). In comparison to the dust eDNA samples collected in Tuggeranong, mean DNA yield from the Kambah sample was 17.27 ± 11.61 ng/µL ($n = 15$ technical replicates). There was no amplification of extraction negative controls.

The ViiA™ 7 Real-Time PCR System showed significantly higher mean Ct values (two-way ANOVA, $F_{1,371} = 197.557$, $p < 0.001$) and significantly higher positive detections for both assays (two-way ANOVA, $F_{1,371} = 9.172$, $p < 0.001$) compared to the Franklin™ Real Time thermocycler. There were no significant differences associated to extraction or collection methods in either platform tested in this study (Figure 1).

Amplification of *T. granarium* eDNA in the ViiA™ 7 Real-Time PCR System showed significant differences between sampling locations (two-way ANOVA, $F_{3,179} = 45.910$, $p < 0.001$) and assays (two-way ANOVA, $F_{1,179} = 12.051$, $p < 0.001$). Samples from Tuggeranong displayed significantly higher Ct values compared to all other sites, with mean Ct ± SD of 47.62 ± 4.01 and 50.05 ± 2.80 with the Furui and Olson assay, respectively, (Figure 1B). Replicates from the Kambah sample showed mean Ct ± SD of 40.03 ± 9.5 and 46.01 ± 6.59 with the Furui and Olson assay, respectively (Figure 1B), while samples collected in Fyshwick showed 36.674 ± 5.85 Ct with the Furui assay and 42.75 ± 5.95 Ct with the Olson assay (Figure 1B). There were no significant differences in mean Ct of samples collected in Kambah and Fyshwick. However, it is important to note that samples from Fyshwick were collected with a contaminated vacuum cleaner used during a separate biosecurity response of khapra beetle.

Similarly, amplification of *T. granarium* eDNA in the Franklin™ Real Time thermocycler showed significant differences between locations (two-way ANOVA, $F_{1,100} = 12.090$, $p < 0.001$), where samples from Tuggeranong displayed significantly higher Ct values than all other sites, with mean Ct ± SD of 34.87 ± 2.88 and 33.69 ± 2.40 with the Furui and Olson assay, respectively (Figure 1C). Samples collected in Fyshwick showed 31.71 ± 4.64 Ct with the Furui assay and 25.76 ± 1.97 Ct with the Olson assay (Figure 1C). Lastly, technical replicates from the Kambah sample showed mean Ct ± SD of 25.60 ± 5.94 and 23.74 ± 5.26 with the Furui and Olson assay, respectively (Figure 1C). There were no significant differences between mean Ct values between assays used in the Franklin™ Real Time thermocycler.

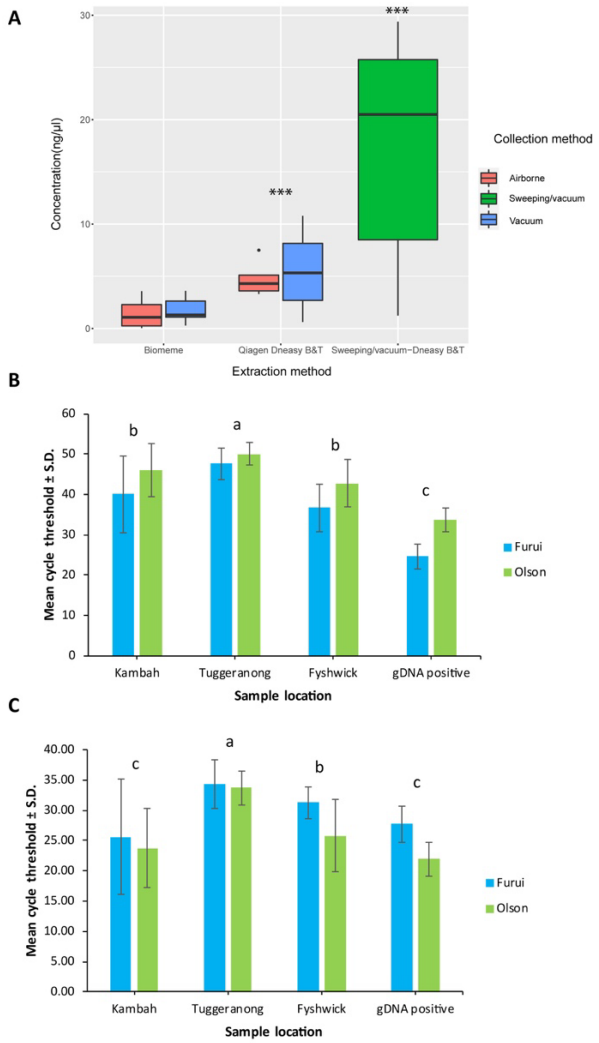


*Figure 1. Mean environmental DNA yield achieved by either Biomeme or Qiagen Dneasy Blood and Tissue kit extraction protocols from dust samples collected during biosecurity responses in Tuggeranong (vacuum) and Kambah (sweeping/vacuum) (A) and mean cycle threshold values of environmental DNA and genomic DNA samples collected during biosecurity responses in Kambah, Tuggeranong and Fyshwick achieved with the Olson and Furui assays in the ViiA™ 7 Real-Time PCR System (B) and in the Franklin™ Real Time thermocycler (C). *** = Statistical differences using non-parametric Mann-Whitney U tests. 'a' and 'b' and 'c' indicate differences between pairs of means determined using Tukey's HSD tests of statistically significant two-way ANOVAs.*

Field negative controls collected in both biosecurity responses showed positive amplification of T. granarium eDNA using both assays in both the ViiA™ 7 Real-Time PCR System and the Franklin™ Real Time thermocycler. Specifically, 12.3% and 50% of qPCR technical replicates from field negative controls collected from Tuggeranong and Fyshwick respectively, were confirmed positive for T. granarium eDNA. The total number of technical qPCR replicates/total number of positive technical qPCR replicates were 8/65 and 6/12 respectively, for the Tuggeranong and Fyshwick sites, as demonstrated in Table 2.

Table 2. Number of confirmed positive technical qPCR replicates for Trogoderma granarium environmental DNA from samples and field negative controls collected during biosecurity responses in Fyshwick and Tuggeranong

Sample type	Location	Assay	Total technical qPCR replicates	Positive qPCR replicates
Dust sample	Fyshwick	Furui	26	24
		Olson	24	14
	Tuggeranong	Furui	221	105
		Olson	226	31
Field control	Fyshwick	Furui	6	5
		Olson	6	1
	Tuggeranong	Furui	29	7
		Olson	36	1

TAQMAN ASSAY OPTIMISATION AND REPRODUCIBILITY

Both TaqMan assays successfully amplified *T. granarium* eDNA extracted from dust samples in laboratory and field conditions. The Olson assay had an LoD of 100 copies/µL (mean CT ± SD = 48.17 ± 1.44) with an R2 = 0.99 and efficiency = 93%. Similarly, the LoD for the Furui assay was estimated to be 10 copies/µL (mean CT ± SD = 40.78± 0.44) an R2 = 0.96 and efficiency = 91%. Positive controls amplified in all plates, while no amplification occurred in the negative template controls. All positive detections of eDNA and genomic DNA samples were confirmed by Sanger sequencing to display 98.7–100% similarity with *T. granarium* sequences in NCBI (NCBI no. MT113335) and a selected number of sequences were accessioned for future studies (NCBI no. MW911673-MW911691). Positive amplicons obtained using the Furui et al. (2019) assay that amplified within assay cut-off values consistently showed poor sequencing quality due to the small size of the fragments, while all positive amplicons outside cut-off values were confirmed to be amplification errors. There was no amplification of any of the provided tissue samples from non-target Australian native specimens with either of the tested assays.

TaqMan assays successfully amplified *T. granarium* eDNA in the confirmed positive eDNA sample provided for this study from the private residence in Kambah. Assay reproducibility for the Olson assay was 66% (positive technical replicates/total technical replicates = 18/27) and 100% (positive technical replicates/total technical replicates = 9/9) in ViiA™ 7 Realtime PCR system and Franklin™ Realtime Thermocycler, respectively. In the same way, the Furui assay had a detection success rate of 90% (positive technical replicates/total technical replicates = 29/32) and 100% (positive technical replicates/total technical replicates = 6/6) in the ViiA™ 7 Realtime PCR system and Franklin™ Realtime Thermocycler, respectively. The LAMP assay failed to detect *T. granarium* eDNA in the confirmed eDNA sample provided (positive technical replicates/total technical replicates = 0/24) and was not selected for further testing. Environmental DNA extracts from this confirmed positive sample were heavily used during assay optimisation, resulting in differences in available technical replicates to assess assay reproducibility in the portable Franklin™ Thermocycler.

The Franklin™ Real Time Thermocycler accurately amplified all tissue-derived positive control DNA used during assay optimisation and field testing of the technology using the Olson assay. Similarly, the LAMP assay tested in this study using a Genie III accurately amplified all DNA positive controls much faster than the qPCR-based assays tested in this study (detection < 25 minutes), highlighting the capacity of these technologies to complement biosecurity requirements in confirming specimen ID.

DISCUSSION

The use of eDNA-based molecular techniques for invasive species identification are increasingly promising tools to inform biosecurity (Zaiko et al. 2018). For these methods to be adopted, sampling, processing and analysing samples must be technically feasible, precise and repeatable (Zaiko et al. 2018). This study presents results from Australian border biosecurity responses to the detection of *T. granarium* specimens contaminating non-agricultural commodities imported from overseas. We demonstrated that *T. granarium* eDNA can be extracted and amplified from dust samples and tested using qPCR TaqMan assays. We highlighted issues regarding false-positive results associated with cross-contamination during sample collection that must be addressed for eDNA-based methods to be implemented as part of operational biosecurity responses and applications.

Sample collection methods used in this study were prone to cross-contamination during sample collection within and between sampled sites at Tuggeranong and Fyshwick. False-positive results were attributed to the opportunistic nature of sample collection, as biosecurity officers were not informed beforehand of the requirements needed to minimise cross-contamination. Officers carried out each biosecurity response as per guidelines and legislation approved by DAFF, which do not yet outline requirements for eDNA testing at these early stages of implementation. As such, the priority at each site was to detect and remove *T. granarium,* followed by cleaning of all contaminated surfaces and fumigation. Within this context, the effect of false-positive detections due to the use of contaminated vacuum cleaners had no impact on the outcome of each response as officers had confirmed the presence of *T. granarium*. Nonetheless, such an obstacle highlighted the need to minimise eDNA cross-contamination in biosecurity.

Further testing is required to better gauge assay reproducibility with both TaqMan assays tested in this study and better assess the capacity of LAMP to amplify insect eDNA in dust samples. There was only one sample from which detection success could be assessed appropriately for the purpose of eDNA testing in this biosecurity setting, which was also used for assay optimisation and assessing analytical sensitivity. As such, both TaqMan and LAMP assays were tested against a single environmental matrix for the purpose of assessing assay reproducibility.

Real-time PCR has been routinely used to detect eDNA from a broad range of surveillance applications (Morisette et al. 2021) and was shown by this study to successfully amplify *T. granarium* eDNA in a biosecurity context. Similarly, LAMP assays have also been developed to amplify DNA from microscopic pathogens and parasites in water samples and other fluid matrices (Ebbinghaus et al. 2012; Wong et al. 2018; Leonardo et al. 2021). Between both molecular methods, qPCR has so far been used to successfully amplify insect eDNA from soil (Yasashimoto et al. 2021) and faecal (Sigsgaard, 2021) environmental matrices. LAMP-based assays had, until this study, not been tested to detect insect DNA in any environmental matrix. In this study, LAMP failed to detect *T. granarium* eDNA in dust samples. Non-detection may be associated with the mechanism used in LAMP to amplify DNA, in which the six primers bind laterally to distinct sites using strand-displacement *Bst* DNA polymerase to amplify a single fragment of DNA (Khan et al. 2018).

When used to target high-quality DNA, LAMP has been shown to offer more timely detection of targeted species with higher sensitivity and accuracy than qPCR (Khan et al. 2018). However, the degraded and inhibited matrix in which *T. granarium* eDNA samples were collected may not offer suitable templates for all primers in the LAMP assay to amplify the target gene region. It is important to highlight that the assay was not developed for the purpose of eDNA-based testing, but it is routinely used in Australia to confirm the identify of *T. granarium* specimens by targeting tissue-derived DNA (Rako et al. 2021). Future testing of TaqMan assays tested in this study for the purpose of eDNA-based detection will require more confirmed positive eDNA sample. The conditions in which this single sample was collected – i.e. a sample from a private residence in Kambah containing alive larva confirmed to be *T. granarium* – may be rare unless officers are actively looking to collect such samples.

Testing of both TaqMan assays using the Biomeme Franklin™ Thermocycler highlighted three important considerations that must be addressed for future field-based testing. Firstly, the technology can test only a small number of samples per day which creates a critical bottleneck during a

biosecurity response. There are other portable technologies that could process a much greater number of samples per run in the field (e.g. Biomolecular systems Mic qPCR Cycler = 48 separate qPCR reactions), and larger numbers of samples could also be sent for laboratory-based testing if needed. In this study, the Franklin™ Thermocycler and the Genie III were suitable to determine the identity of collected specimens by qPCR and LAMP respectively, while qPCR-based assays were suitable to determine the presence of eDNA in a small number of samples. Secondly, officers require training to interpret eDNA-based testing results and determine appropriate contingencies in the event of false positives. False-positive detections due to cross-contamination during collection in this study indicates that officers must readily assess when eDNA-based testing in the field would be unsuitable and sample collection unreliable.

Field-based molecular testing would be another tool available to biosecurity officers to increase the likelihood of detection and to rapidly assess the presence or absence of a pest. However, detections would still require verification through further inspections and laboratory-based methods. Deploying portable molecular technologies for pest detection in biosecurity requires the coordination of scientific, regulatory, and operational authorities to better determine the boundaries in which implementing this technology would ultimately be suitable. There is also an overarching need for an international collaborative framework aimed at unifying molecular sampling and analysis methodologies to facilitate the development of standards and encourage uptake of these techniques.

The operational use of laboratory and field-based eDNA molecular technologies requires the implantation of biosecurity standard operating procedures and legislation. Collection of eDNA in this study was subject to the priority of the biosecurity response, which was to clean and fumigate the contaminated area. For this reason, and in the absence of any policy on how and when to use eDNA-based methods, sampling in both responses was only undertaken after officers completed an initial examination at each site. Officers proceeded to clean the area prior to fumigation, as per biosecurity response requirements, aiming primarily to clean the area rather than collect samples for eDNA-based testing. The response in Fyshwick also highlighted the importance of using clean equipment to collect eDNA samples, as contamination of the vacuum cleaner that had been used in a previous biosecurity response with confirmed khapra beetle infestations was confirmed using the Franklin™ Thermocycler.

This study demonstrates the critical need for standard protocols in sample collection and for controls in the eDNA workflow to indicate where potential sources of error are proliferating in biosecurity applications. In doing so, the results of this study were used to inform DAFF, which funded further avenues of research to improve and develop standardised eDNA sampling methods for biosecurity applications. Ongoing research aims to detect *T. granarium* eDNA and environmental RNA in shipping containers to inform officers on the active presence of pest species. This research also aims to develop standard operating procedures to collect, store, extract and analyse environmental samples for the presence of pest species and implements rigorous collection methods to minimise sample cross-contamination within and between biosecurity responses.

CASE STUDY B: USING PORTABLE TECHNOLOGIES FOR MULTI-SPECIES DETECTION

SUMMARY

- Environmental DNA samples collected from an international aquarium-trade wholesaler were sequenced and analysed using the Illumina MiSeq and the Oxford Nanopore MinION portable sequencer.
- High-quality sequencing was achieved for 12s, 16s and cytochrome oxidase I gene (COI) regions; however, sequencing of cytochrome b data failed to produce high-quality and reliable results.
- The Illumina MiSeq benchtop sequencer produced a total of 3,034,707 reads for 12s, 2,526,954 reads for 16s, and 9,581,228 reads for COI. In comparison, the portable MinION sequencer produced a total of 152,438 reads for 12s, 17,438 reads for 16s, and 6,830 reads for COI.
- Although Illumina MiSeq total reads were significantly larger than the MinION, both platforms reliably identified fish diversity present at the Q.P.S. International Aquatics (Thailand) facility, demonstrating operational capacity from portable technologies in identifying ornamental fish diversity.
- For species identification, this project recommends using and optimising the 12s and 16s gene regions of ornamental fish species, and field testing sequencing technologies for operational optimisation.

BACKGROUND

The Oxford Nanopore MinION sequencer enables real-time DNA and RNA sequencing and analysis without the need for a specialised laboratory. This has many advantages in providing *in situ* use by sequencing at the sampling location, eliminating the need for transportation of samples back to a laboratory. MinION devices have the potential to offer a simple, affordable, and portable approach to DNA sequencing which would be highly applicable to biosecurity operations.

For portable sequencing technologies to be adopted, there is a need to ensure they provide comparable results to standard laboratory sequencing technologies. In this case we trialled the Oxford Nanopore MinION sequencer.

RESULTS

PORTABLE NEXT GENERATION SEQUENCER (NGS) PCR INHIBITION-PILOT STUDY

A total of 172,574 sequence reads with a mean quality score of 10.26 were obtained when sequencing the Lambda control (Figure 2A). In comparison, a total of 918 sequences with a mean quality score of 7.5 were obtained when sequencing undiluted DNA extracts from water samples (Figure 2B), while 8,762 sequences were obtained when sequencing undiluted, PCR amplicons (Figure 2C). Importantly, the sequencing run from sampled water had a total of 44,360 sequences with a mean quality of 3.5 (Figure 4). This suggests that inhibitors significantly affected the sequencing quality of the MinION. PCR inhibitors in environmental samples can limit eDNA PCR-based molecular analyses following collection of samples from wild ecosystems where the presence of a species is unknown (McKee et al. 2015). This pilot study suggests that nanopore sequencing platforms may struggle to sequence eDNA samples with a high concentration of inhibitors. Most importantly, this pilot study highlights the need to purify or amplify eDNA prior to sequencing using nanopore kits to achieve higher resolution.

Sequencing eDNA using the Illumina MiSeq platform

High-quality sequencing was achieved for 12s, 16s and COI gene regions using the Illumina MiSeq sequencer (Figure 3). Over 75% of sequence reads for 12s and 16s were above the 30 QC standard

percentile with an error rate of 1.77 (Figure 3A), while 84.65% of total reads produced for COI were above the 30 QC standard percentile with an error rate of 0.88 (Figure 3B). A total of 3,034,707 high-quality sequence reads were obtained for 12s, 2,526,954 for 16s, and 9,581,228 for COI. Amplicons for cytochrome b with a size of 1,222 bp did not have the necessary quality to be sequenced using Illumina-based platforms. This issue was also observed when sequencing genomic DNA from non-permitted fish species in Phase 2, where primers designed to amplify the cytochrome b gene region did not consistently produce high-quality amplicons for Sanger sequencing.

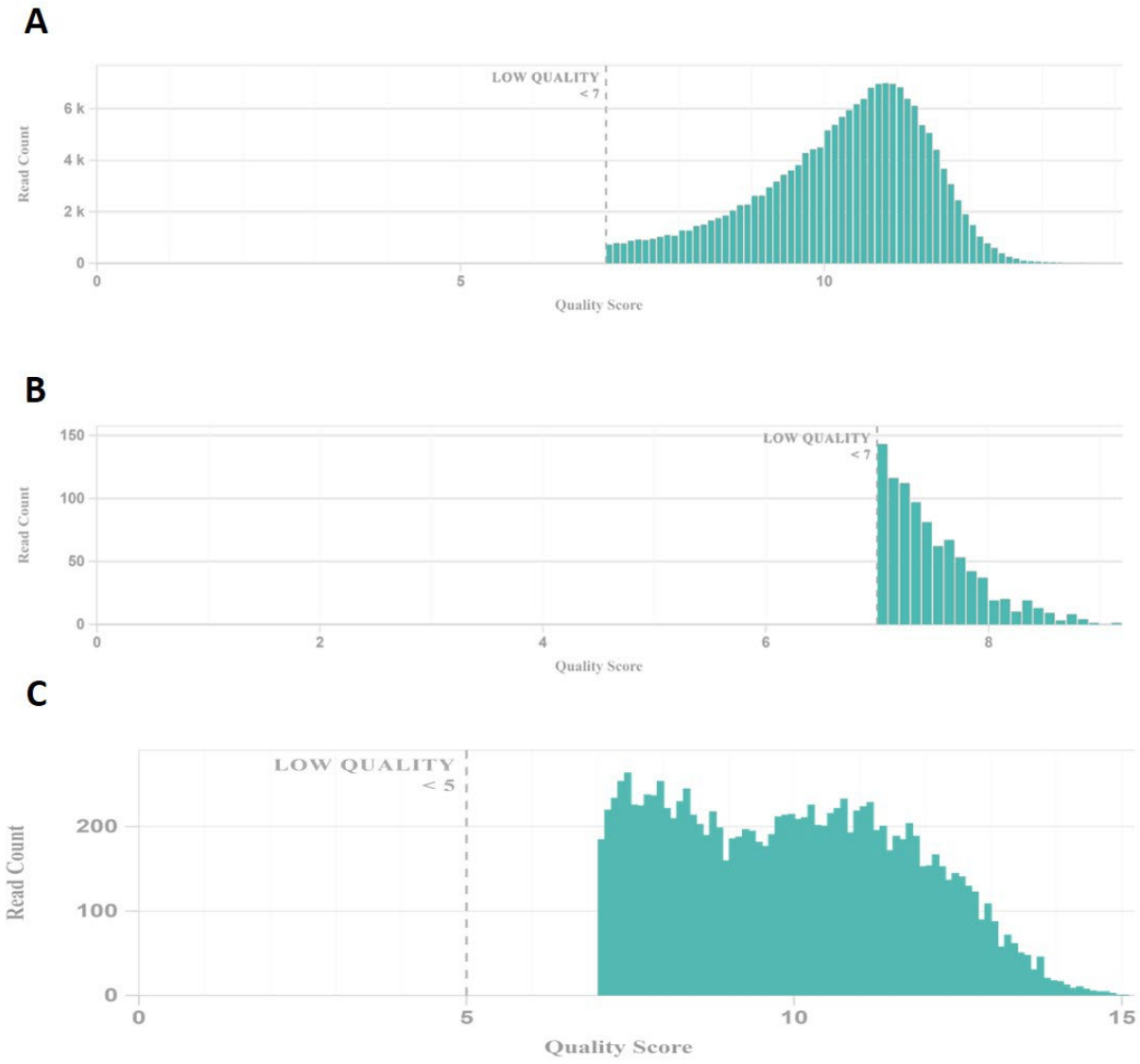


Figure 2. Sequence read count with and overall quality for the purified Lambda control (A), raw aquarium samples sequenced with a Rapid Barcoding Kit (B) and PCR amplicons sequenced using a Rapid Barcoding Kit (C)

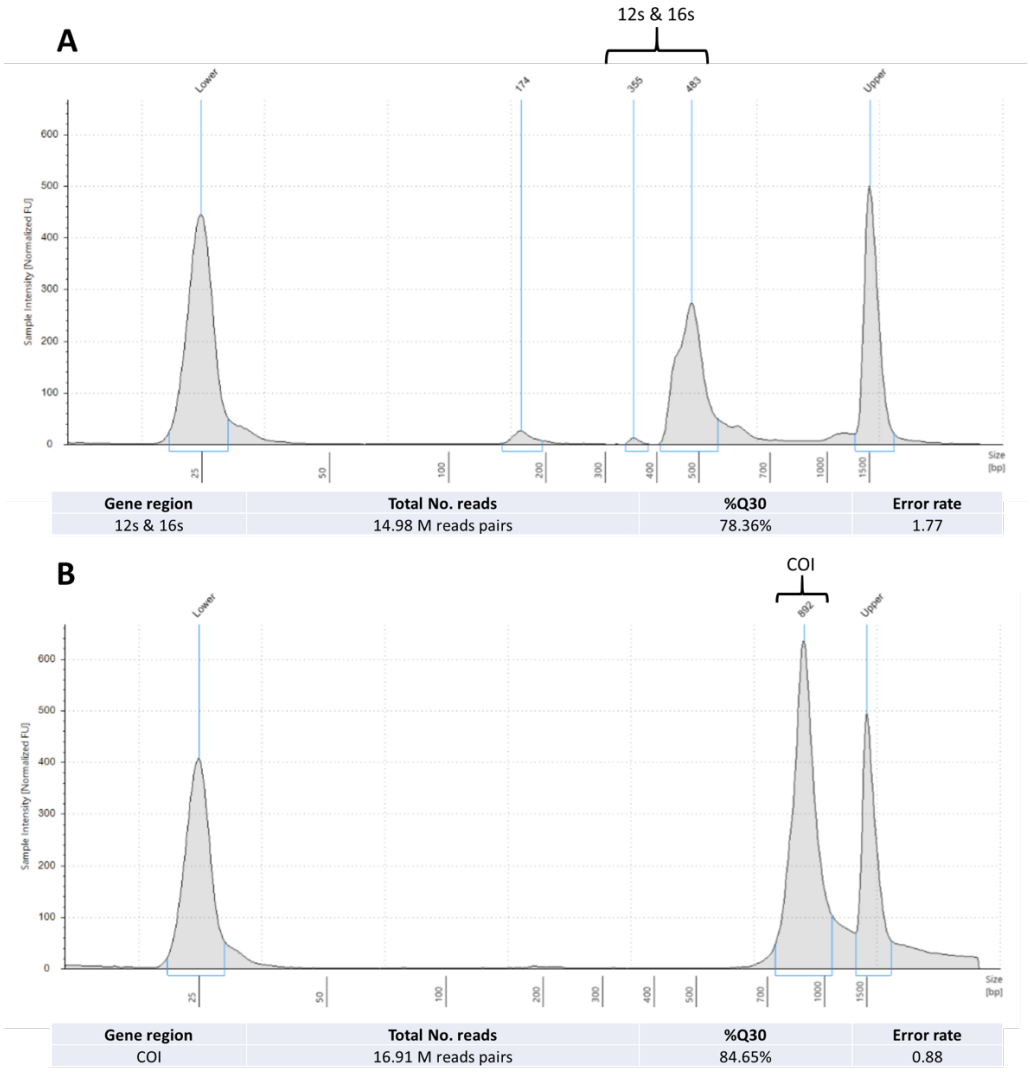


Figure 3. Amplicon size and sequencing quality for 12 s, 16 s (A) and Cytochrome Oxidase I (B) obtained with the Illumina MiSeq sequencer at the Ramaciotti Centre for Genomics

SEQUENCING EDNA USING THE OXFORD NANOPORE TECH. MINION SEQUENCER

Oxford Nanopore Technologies (ONT) developed a new generation of DNA/RNA sequencing technology. This tool offers real-time analysis in fully scalable formats that can analyse native DNA or RNA and sequence any length of fragment to achieve short to ultra-long read lengths. However, nanopore sequencing is novel compared to other standard sequencing platforms, and because of this, new methods, chemistries and protocols are continuously tested and changed by its developers in real time. So far, this project has completed a total of 27 separate sequencing runs using water samples collected in the facilities of Q.P.S. International Aquatics. These test runs involved using different kits, purifying protocols and PCR assays. Given the changing nature of nanopore sequencing methods and kits, this project continues to test suitable methods. Among all tested kits, there have been two different methods that produced high-quality and suitable results: the Rapid Barcoding Sequencing method (Kit: SQK-RBK004) and the ligation method (Kit SQK-LSK109) using native barcodes (EXP-NBD104). Here we discuss representative results to showcase the suitability of portable sequencing technologies for biosecurity applications.

The ONT MinION sequencer was used to sequence multiple samples simultaneously through metabarcoding or single samples to optimise sequence read outputs. In the case of metabarcoding applications, the ONT MinION successfully sequenced eDNA raw samples using purified eDNA extracts including the ONT ligation sequencing and native barcoding kits (141,805 sequence reads, QC = 8.8; Figure 4A). In addition, the MinION previously amplified PCR amplicons sequenced using

ligation and native barcoding kits (152,438 sequence reads, QC = 8.8; Figure 4B). Single samples could be amplified alone to improve sequencing output using previously amplified PCR products, ligation kits and rapid sequencing kits (7,580 sequence reads, QC = 10.31: Figure 4C). In comparison to sequencing PCR amplicons using ligation and native barcode kits, the rapid barcoding sequencing kits produced much lower sequence read outputs (Figure 5).

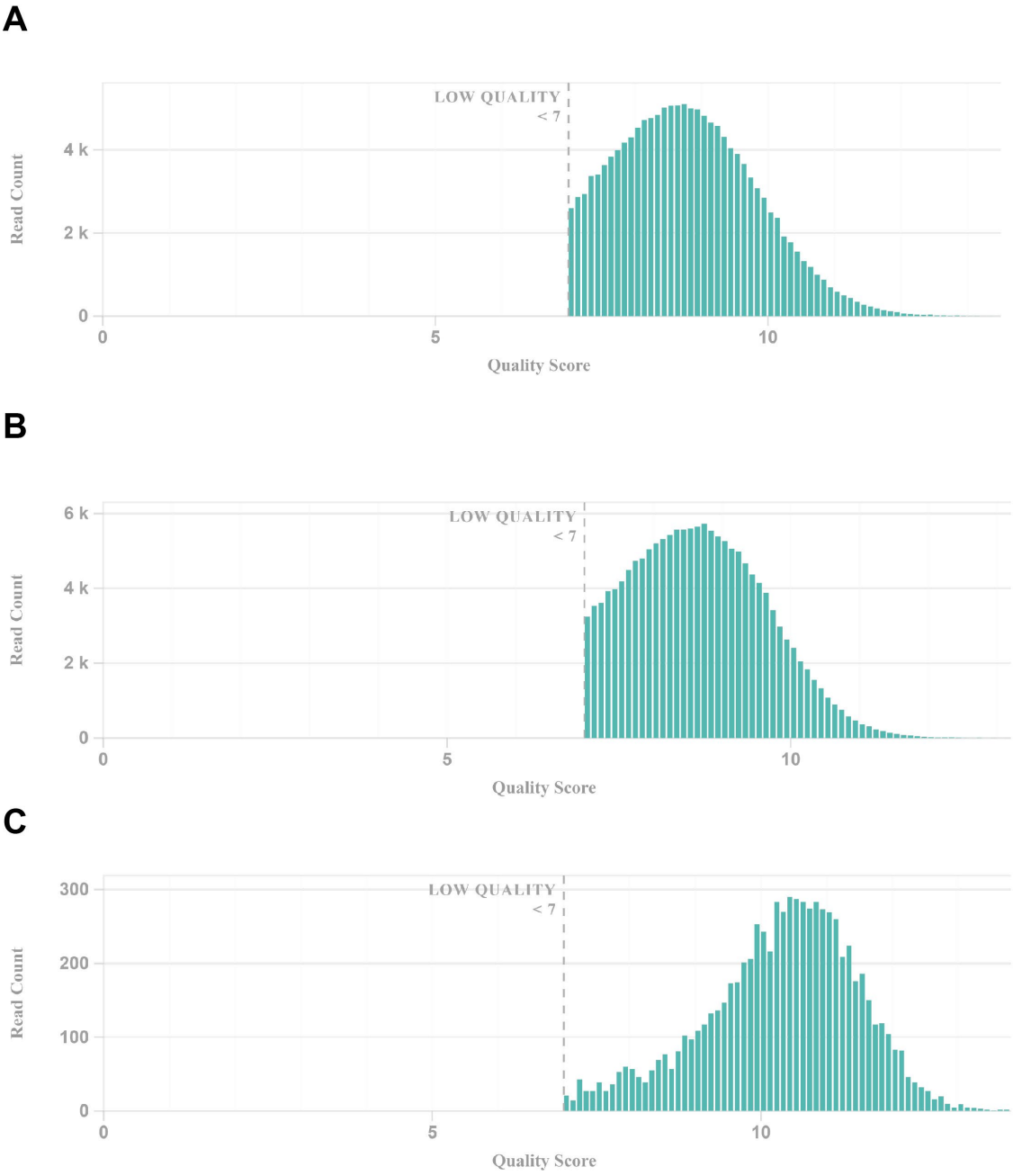


Figure 4. Sequence read count and overall quality of purified samples sequenced with the ONT MinION portable sequencer. Graphs represent sequencing of purified eDNA extracts with ONT ligation sequencing (SQK-LSK109) and native barcoding kits (EXP-NBD104) (A), purified PCR amplicons (12 S gene region) sequenced with ONT ligation sequencing and Rapid Barcoding Kits (B), and purified PCR amplicons from one sample (Q.P.S.-Australian quarantine) using the ONT ligation sequencing and rapid sequencing kits (C).

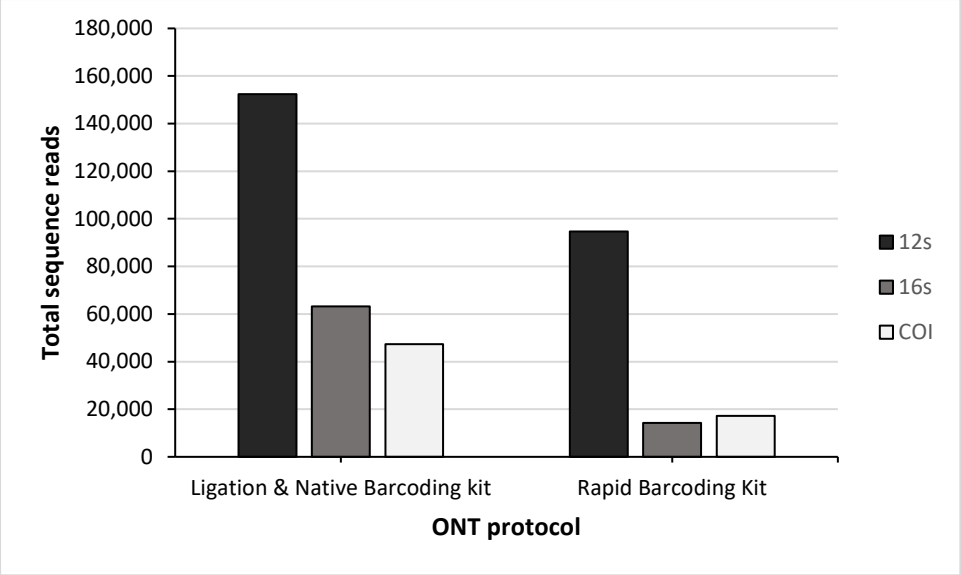


Figure 5. Total read counts achieved using ONT ligation sequencing (SQK-LSK109) and native barcoding kits (EXP-NBD104), and Rapid Barcoding Kits (SQK-RBK004) used to sequence purified PCR amplicons for 12 s, 16 s and cytochrome oxidase I

Both Illumina and nanopore sequencing provided high-quality sequencing results suitable for bioinformatic analysis. Illumina produced a significantly high number of total reads compared to the MinION sequencer. This result was expected, as the Illumina MiSeq can produce up to 25 million sequences compared to the MinION, which so far has been reported to produce approximately five million sequences with high-quality DNA. Results from this milestone indicate that Illumina sequencing provided superior quality and more sequencing reads than nanopore sequencing.

Nanopore sequencing protocols significantly affected the number of reads that could be produced by the MinION. Sequencing raw eDNA produced an unreliably low number of reads, indicating that using the faster sequencing protocols provided by ONT would produce unreliable results within the framework of Australian biosecurity. This project recommends further testing with two different nanopore chemistries and protocols: the ONT ligation sequencing (SQK-LSK109) and native barcoding kits (EXP-NBD104), and the Rapid Barcoding Kits (SQK-RBK004).

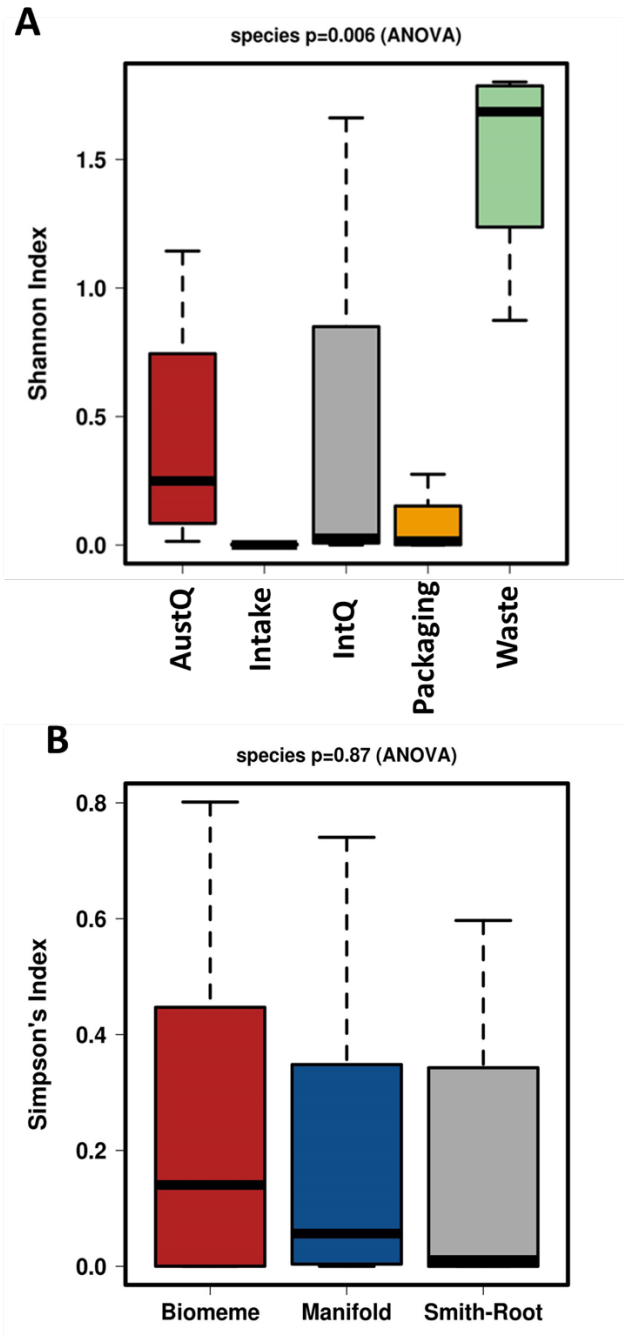


Figure 6. Species-level diversity achieved through Illumina MiSeq sequencing between sample locations in Q.P.S. International Aquatics (A) and between collection protocols (B). Diversity was calculated using the Shannon Diversity Index to account for evenness between locations and the Simpson's Diversity Index to account for proportionality between sampling methods.

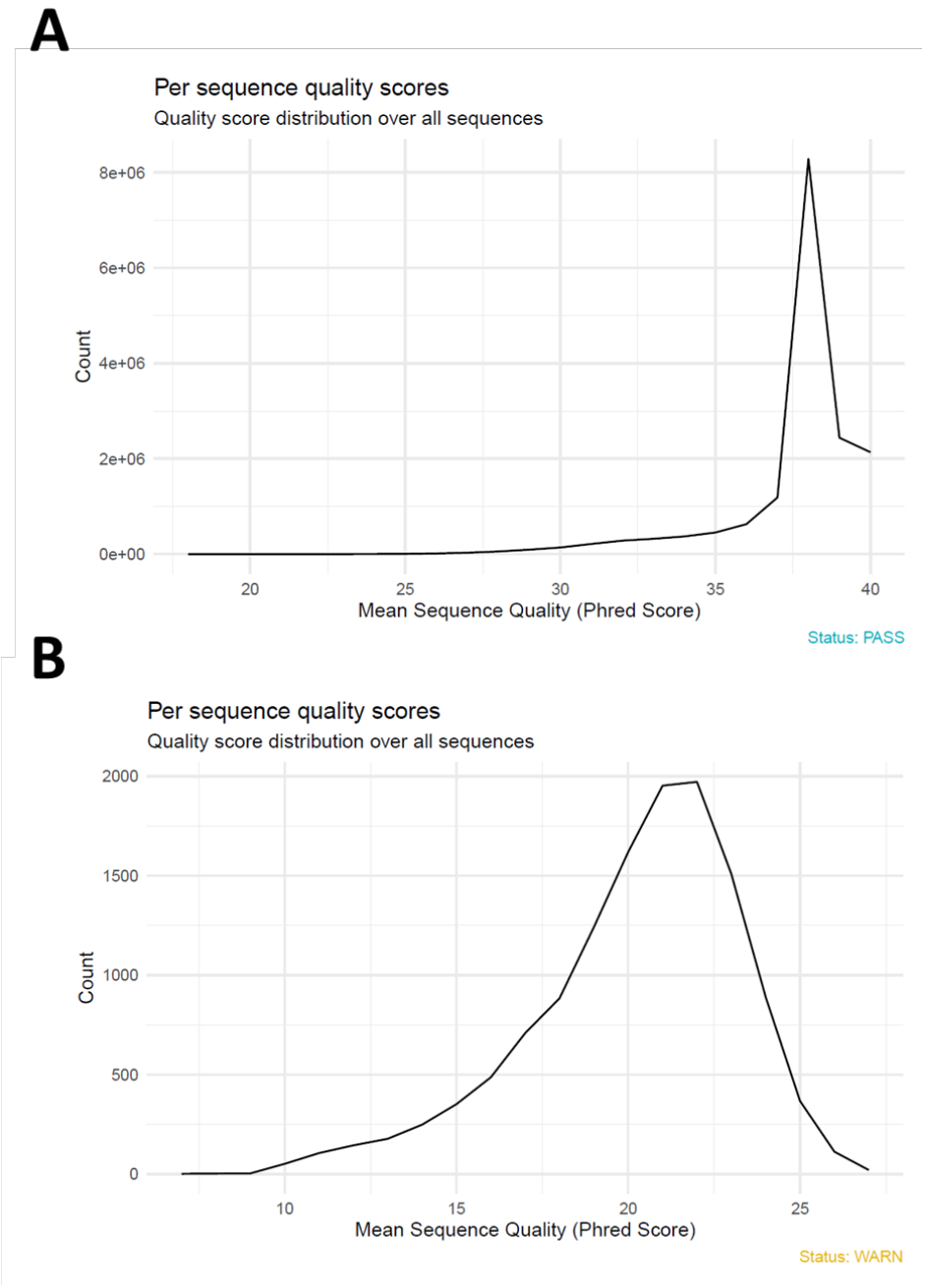


Figure 7. Mean sequence quality (Phred Score) of Illumina (A) and Nanopore (B) sequencing platforms

Sequence reads identified as *Epalzeorhynchos bicolor* (NCBI AP011204) were the most abundant in the Australian quarantine intake source (Figure 8). This result is expected, as the sampled quarantine tank had more than 50 *E. bicolor* individuals being quarantined for export to Australia. Similarly, sequence reads identified as *Carassius auratus* (goldfish; KM659025) were the most abundant in the international quarantine water source (Figure 8), which had > 150 *C. auratus* individuals inside the sampled tank. Interestingly, the MinION sequencer also reported the presence of *Cyprinus carpio* (carp; KJ511883), a species which was not present in the international quarantine water source at the time of sampling. It is possible that sequence reads identified as *C. carpio* are a result of incorrect basecalling during sequencing of the MinION, resulting in many potential *C. auratus* sequences being identified as *C. carpio*. Given that *C. auratus* and *C. carpio* are closely related cyprinid species, with hybrids reported in prior studies, small genetic differences due to basecalling errors in the MinION sequencer could then result in misidentification of sequence reads.

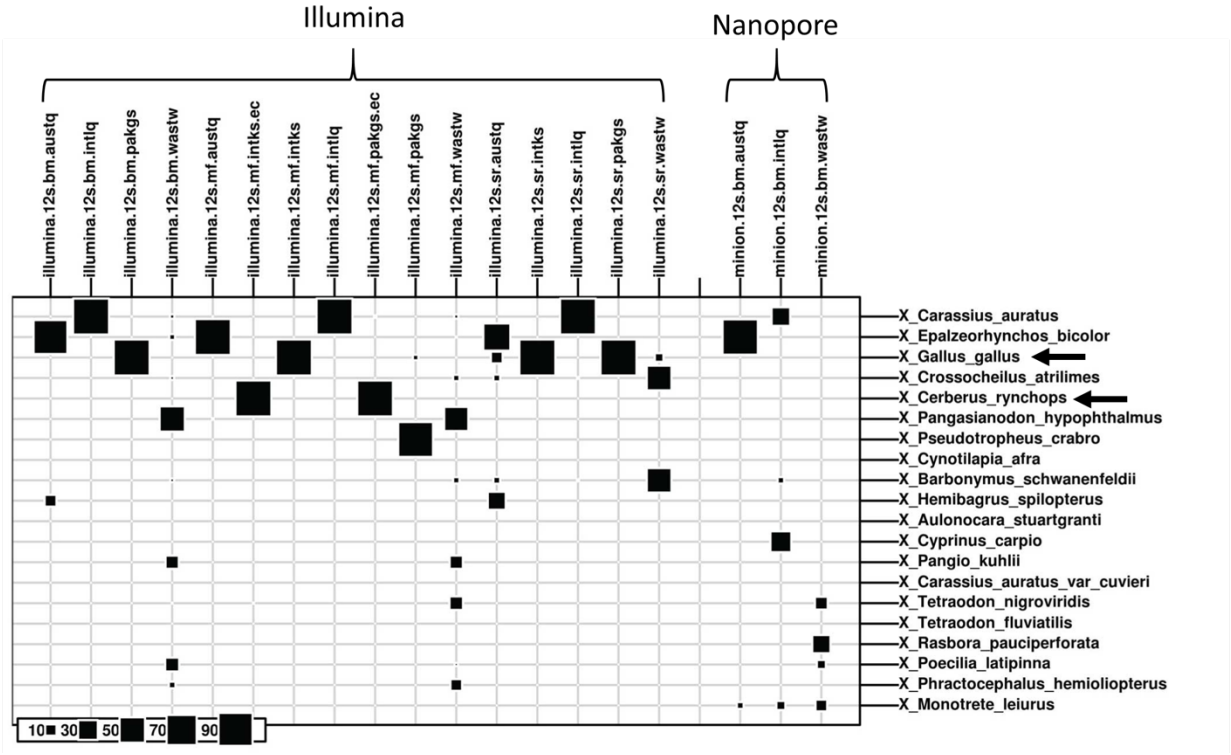


Figure 8. Relative abundance of sequence reads identified from environmental samples collected from five different water sources in Q.P.S. International Aquatics. Operational taxonomic units obtained for the 12s gene region are included in the figure. Arrows highlight high abundances of non-fish DNA being amplified by the 12s primers of this study identified individually using NCBI BLAST.

A low abundance of sequence reads was detected from 11 other fish species, all of which are endemic to the Malay Peninsula. All detected species are sold by Q.P.S. International Aquatics. Detection of residual DNA is common in eDNA-based detection (Trujillo-González et al. 2019; 2020). Given that quarantine facilities at Q.P.S. International Aquatics consist of rows of tanks connected to a main recirculating system with biological filters and skimmers, it is possible for DNA to be detected from other species being quarantined in close proximity to sampled tanks.

A high abundance of non-fish DNA was still amplified by all primers selected for testing in this project (Figure 8; arrows). Although using a rigurous reference database would eliminate detection of these sequence reads, amplification would still occur preferentially to highly abundant DNA, such as highly common organisms present in the study site (e.g. *Gallus gallus* and *Cerberus rynchops*; Figure 8). Given the conserved nature of gene regions such as 12s, 16s and COI, highly optimised primer pairs can still amplify DNA from non-target vertebrate groups. This may not be a complication with benchtop sequencers such as the Illumina MiSeq, but it certainly puts a strain on a portable sequencer such as the MinION, where the number of reads through sequencing is much lower.

DISCUSSION

Overall, both platforms successfully detected targeted species in the Australian quarantine water source and in the international quarantine water source. Both platforms detected a high diversity of fish eDNA from the wastewater source, and did not detect fish DNA present in the intake and packaging water sources. This suggests that sequencing technology would be suitable to ensure compliance of imported ornamental fish consignments (i.e. all fish consignments must be shipped using clean water), and determine compliance of exporting companies in following biosecurity conditions for Australia (i.e. fish must be quarantined in isolation for a minimum period of seven days prior to export). This milestone also highlights that a significant amount of sequencing was occupied by non-fish, non-target eDNA present in the water. Therefore, it is recommended that exporters provide samples of packaging water with every shipped fish consignment as a negative control for eDNA testing by biosecurity officers.

OBJECTIVE 2: DEVELOP NEW TESTS FOR TARGETED HIGH-RISK INVASIVE ANIMALS

SUMMARY

- Two previously developed assays for red-eared slider turtles (REST) were tested for specificity and sensitivity in Australian conditions.
- Both assays were found to be reasonably sensitive, making them well-suited to eDNA applications, but one assay showed increased efficiency and was able to reliably detect lower eDNA concentrations.
- The more sensitive assay was further tested on water samples collected from a mesocosm (ecosystem confined to a small area), achieving 100% amplification success in all eDNA samples even one week after turtles had been removed.
- This highlights the ability of the eDNA assay to detect the species in high densities, even when individuals are no longer present.
- eDNA surveys conducted in this study at field sites also confirmed the ability of the assay to detect REST (*T.s. elegans)*, although detection rates were low.
- The failure to reliably detect the highly invasive *T.s. elegans* from urban pond sites despite concurrent confirmation of their presence suggests that eDNA surveys conducted through analysis of water samples may add little value to current monitoring efforts for this invasive species.
- We showed the utility of enhancing collection of eDNA using modification of artificial haul out sites, which is suggested to be the most efficient approach and is recommended for any ongoing operations using eDNA for this species.

DETECTION TOOLS FOR RED-EARED SLIDER TURTLES

This is a modified and condensed version of the manuscript:

Rojahn J, Trujillo-González A, Gleeson D, Cutter N and Furlan E (submitted) 'Does mesocosm validation of environmental DNA methods translate to natural environment monitoring applications? A case study detecting a high-profile invader; the red eared slider turtle, *Trachemys scripta elegans*, in Australia'.

BACKGROUND

The red-eared slider turtle (REST) (*Trachemys scripta elegans)* is a high-risk invasive species that have been detected across a number of jurisdictions. Since 2000, 70 REST have been detected in Victoria (Vic), with 20 of these found in or adjacent to Victoria rivers and lakes. The source of each animal is exclusively illegal trade, suspected to be either directly from overseas or domestically bred and distributed. Without ongoing intervention this species will increasingly be detected in the wild in Victoria and could establish a breeding population like those in New South Wales and Queensland. Given this species is illegally brought into Australia from South-East Asia and poses associated exotic animal disease risk, a tool that increases the efficiency and effectiveness of surveillance is required. Current detection efforts for *T.s. elegans* are time- and resource-intensive and include: scent detector dogs, visual observation and reporting, opportunistic hand capture, and trapping and removal. eDNA surveillance has the potential to inform the distribution of *T.s. elegans*, improving management efforts for this invasive species by providing a highly sensitive and cost-effective detection tool (Kakuda et al. 2019).

We aimed to develop eDNA methods to detect *T.s. elegans* in Australia. Two previously published marker sets have been designed to detect *T.s. elegans* and validated for use in eDNA surveys in their respective countries: PCR primers were developed by Davy et al. (2015) to detect the invader in Canada, and a qPCR assay was developed and tested for use in Japan by Kakuda et al. (2019). Rather than create yet another *T.s. elegans* marker set, we aim to extend the utility of these existing markers by evaluating their potential to provide highly specific and sensitive eDNA detection of *T.s. elegans* in Australia. We first validate the specificity of these markers for use Australia-wide. Following the development of a probe to compliment the Davy et al. (2015) primer set, we directly compare the sensitivity of detection of these two assays. We then selected the most optimal assay for application to eDNA detection surveys of *T.s. elegans* from mesocosm and field sites where traditional detection methods are being employed. We discuss the utility of eDNA sampling methods for monitoring of *T.s. elegans* in Australia and considerations for detection of turtles more broadly through eDNA analysis of water samples. This data will be useful to inform current and future conservation and management strategies of turtles using eDNA-based approaches.

METHODS

ASSAY SELECTION

Two previously designed eDNA assays were selected for cross-validation and testing for Australian eDNA applications. At the beginning of this research, both assays had been validated for use in their respective countries and were therefore potentially viable for Australian ecosystems. PCR primers were developed by Davy et al. (2015) targeting *T. s elegans* in Canada, and for use in this study a TaqMan™ probe was developed for quantitative PCR analysis (qPCR). A qPCR assay developed for use in Japan (Kakuda et al. (2019) was also selected. Both assays were ordered as ready-made TaqMan™ assays (Applied Biosystems, Vic, Australia) for use in qPCR analysis.

SENSITIVITY AND SPECIFICITY TESTING

In-silico testing was performed on both assays with molecular interactions of the primers assessed using OligoAnalyzer ® software (IDT, Iowa, United States), Primer3 and Primer3 Plus (Version 4.1.0) (Untergasser et al. 2012). The specificity of the assays was tested against the National Centre for Biotechnology Information (NCBI) BLASTn database (full details available in supplementary material). MAAFT (Version 1.4.0) alignments were constructed in Geneious (Version 10.2.6) to assess nucleotide mismatches of primer sequences against Australian native species. The qPCR sensitivity of each assay was assessed by obtaining the limit of quantification (LoQ) and limit of detection (LoD) for a synthetic gBlock of each amplicon region (IDT), tissue-derived DNA extract, and eDNA extract (see supplementary material). Assays were also tested for specificity on DNA extracted from *T.s elegans* tissues (11 samples) and Australian native species (20 samples across seven genera) distributed throughout Australia. qPCR reactions used throughout assay testing and eDNA analysis were made up in 20 µL qPCR reactions consisting of 10 µL of TaqPath ProAmp Master Mix (Applied Biosystems, Vic, Australia), 1 µL of either TaqMan™ assay (Applied Biosystems, Vic, Australia), 4 µL of RNase/DNase free water (Invitrogen, CA, United States) and 5 µL of DNA, except for sensitivity testing using gBlocks, where 1 µL of copy number oligonucleotide was added to the reaction and made up to a final volume of 20 µL with RNase/DNase-free water. Quantitative PCR was performed using a ViiA™ 7 Real-Time PCR System (Applied Biosystems, Vic, Australia) with cycling conditions set at 50 °C (2 minutes), 95 °C (5 minutes), followed by 55 cycles of 95 °C (15 seconds) and 30°C (30 seconds) with a final extension for two minutes at 60 °C. The most optimal assay was then selected for subsequent testing on eDNA samples based on the previously given description.

RED-EARED SLIDER TURTLE SURVEYS

CONVENTIONAL MONITORING

In the Greater Sydney area, management efforts for red-eared slider turtles include use of scent detector dogs, visual observation and reporting, opportunistic hand capture, and trapping and removal. Conventional monitoring was conducted by officers of the New South Wales Department of Primary Industries using a variety of methods (e.g. cathedral and basking traps) and eDNA sites were selected for sampling based on previous and current conventional monitoring efforts (Table 2 and 3). Confirmed sighting and trapping data was then summarised to reflect current and potential occupancy at each site in relation to eDNA sampling events (Table 3).

ENVIRONMENTAL DNA MONITORING

Environmental DNA monitoring was conducted from October 2018 to December 2020 at seven sites in Sydney, NSW (Table 2 and 3). Sites were selected based on their previous or current occupancy for REST. Sites ranged in size and scale, while the density of turtles varied at sites according to conventional monitoring data (i.e. visual observation, reporting from the general public and trapping data) (Table 3). Water sampling was conducted after first inspecting the site to assess the number of eDNA samples to collect and after noting any visual observation of turtles. Samples collected from smaller sites (e.g. Webbs Dam and Wiley Park) were collected from approximately even intervals around the perimeter of the water. Samples collected from larger urban sites (e.g. Randwick Pond) were collected from areas where previous or current conventional monitoring efforts were conducted. Filtering was performed either onsite or at at a laboratory at the University of Canberra (see Rojahn et al. 2018 for a full description of the eDNA sample processing). Equipment controls, which consisted of 1 L of ultraviolet-sterilised water, were collected prior to filtration to check for contamination of the equipment and were run in qPCR analyses with all eDNA samples. Field controls were processed for all sampling events which consisted of a 1 L bottle filled with ultraviolet-sterilised water that was filtered alongside each DNA sample batch using the methods described for the corresponding sampling occasion.

A control site was also selected to test the validated assay on eDNA samples. An enclosure was chosen to house surrendered and/or trapped REST recovered during management operations. The control site shares characteristics with some urban ponds and water bodies which REST inhabit (e.g. limited flow, vegetation, construction) but reduced in scale. In total, 10 red-eared slider turtles were placed inside the enclosure in the month prior to eDNA sampling but were restricted to a smaller section of the enclosure. Three sampling events took place over a five-week period, with six eDNA samples collected on each occasion using the eDNA sampler. Samples were collected at approximately even intervals around the enclosure to monitor eDNA concentration and assess the spatial heterogeneity of eDNA. Turtles were removed from the enclosure immediately following the second sampling occasion enabling samples collected on the final sampling event to monitor eDNA degradation (Table 4).

EDNA EXTRACTION AND PCR

All eDNA extractions were performed in the University of Canberra trace DNA laboratory, which is designed as a PCR-free environment to limit contamination. A modified Qiagen DNeasy Kit protocol was used to extract genetic material from filter papers with a final elution of 100 µL to maximise potential yield. These protocols were described in Renshaw et al. (2015) and Hinlo et al. (2017). Self-preserving filter papers were first removed from their housings and placed in new tubes for extraction using sterilised forceps (see Thomas et al. 2019). Quantitative PCR was then used to determine the presence/absence of target DNA in eDNA samples following set-up and PCR conditions described in assay testing. Triplicate reactions were performed for each extract and each qPCR set-up also included a five-point standard curve of a gBlock fragment in concentrations ranging from 1,000,000 to 100 copies/µL. Additionally, a SYBR® Green universal fish assay (UniFish) was added to qPCR set-ups to assess inhibition in the reaction (Furlan and Gleeson 2017). Finally, non-template controls (NTC) were included during each set-up to assess potential cross-contamination as well as a single positive control of tissue-derived *T.s. elegans* DNA. Approximately 10% of positive eDNA amplicons

from the control site and all positive amplicons from field sites were sent to the Australian National University's Biomolecular Resources Facility (ANU-BRF) for Sanger sequencing.

STATISTICAL ANALYSES

For qPCR data, results were visually inspected, and reactions were only considered positive if a clear exponential amplification curve could be observed over a common threshold in any replicate with a Ct value below 45. While the universal fish assay was included to account for inhibition, some sites may not be occupied by fish (i.e. the control site). As a result, failed amplification was assessed on a site-by-site basis. A failed amplification for both the species-specific and universal fish assay in all samples indicated improper sample processing or the presence of PCR inhibitors. Successful PCR replicates for each eDNA sample were averaged, and the standard deviation was calculated. qPCR data was then summarised and compared with conventional monitoring datasets.

RESULTS

ASSAY DESIGN AND VALIDATION

Analysis of the *in-silico* data revealed that both assays analysed in this study are specific to *T.s. elegans* and dissimilar to non-target species in Australia. The minimum number of nucleotide mismataches across primer bindings sites between target and non-target species was 10 for the Davy et al. (2015) assay, and 11 for the Kakuda et al. (2019) assay. *In-vitro* testing successfully amplified in all replicates of target species' DNA and replicates did not amplify in any non-target species genera, cross-validating assays for eDNA applications throughout Australia. Sensitivity testing showed that both assays can amplify low quantities of target molecules. However, *in-silico* analysis of the Davy et al. (2015) assay revealed a higher potential for dimerisation, and for this reason the Kakuda et al. (2019) assay was selected for use in subsequent experiments.

CONTROL SITE

The Kakuda et al. (2019) assay successfully amplified eDNA from *T.s. elegans* in all water samples and replicates from the control site (Table 4) while the universal fish assay amplified in 27 of 54 replicates (Table 2). During the first two sampling events when turtles were present, the mean Ct ± SD values representing eDNA concentration varied depending on the sample collection point in the enclosure. The values ranged between 25.639 ± 1.112 to 28.136 ± 0.520 closest to the turtles (A and B, Table 4), to values of 31.293 ± 0.225 to 32.275 ± 0.343 in samples collected the farthest from the turtles (Table 4). Environmental DNA was still detected in samples collected a week after turtles were removed (Ct = 27.865 ± 0.034 to 35.112 ± 1.272). However, eDNA concentration from these samples did not reveal any correlation with the eDNA sample location within the enclosure, suggesting both degradation and homogenisation of the eDNA molecules in the water occurred over time (Table 4).

FIELD SAMPLING AND CONVENTIONAL MONITORING

Analysis of eDNA samples collected from field sites returned positive results at two sites, De Fraites Wetland and Wiley Park 01 (Table 2), and these results were validated with conventional monitoring data (Table 2 and 3). However, amplification did not occur at the five remaining sites, even though sites were potentially inhabited by turtles, or turtles were trapped or sighted at these sites (Table 2 and 3). While occupancy was not able to be confirmed at sites when all eDNA surveys were conducted, on two sampling occasions (Webbs Dam and Wiley Park (WP02); Table 3), sightings were reported on the day of sample collection – yet eDNA surveys did not return positive detections. The universal fish assay amplified at all sites, ranging from 26.67% to 100% success in replicates and in 83% or more of replicates at five of the seven field sampling sites. Standard curves for the Kakuda et al. (2019) assay indicated an average PCR efficiency of 98.308% (± 95% CI = 0.796) and an r^2 value of 0.992 (± 0.006). All controls – NTC, equipment, extraction, positive and field controls – performed as expected.

IMPROVING DETECTION FOR REST

Turtles regularly haul out of their water habitat to bask to control their internal body temperature. The creation of basking pontoons has been used by Queensland Department of Agriculture and Fisheries as a way of monitoring REST in locations where they are suspected. Given pontoons are a known location for turtles to aggregate, this provides an opportunity to enhance eDNA collection and improve detection of REST using swabbing methods from pontoon surface substrate. Although camera monitoring was in place at these sites, problems occurred around the security of equipment given the suburban nature of these locations. The use of eDNA could complement camera surveillance and could yield DNA that would enable information regarding individual identity and attribution of provenance, which could also provide further valuable assistance in an incursion.

DISCUSSION

In this work, we cross-validated a previously designed qPCR assay for use in eDNA surveillance of the invasive red-eared slider turtle, *T.s. elegans,* in Australia. We tested the validated assay on water samples collected from a control site housing *T.s. elegans*, achieving 100% successful amplification in all eDNA samples, even one week after turtles had been removed. However, we failed to detect *T.s. elegans* DNA in five of seven field sites that turtles are known to inhabit, and no detection was recorded at two sites with concurrent visual detection. At sites where conventional monitoring and eDNA both recorded positive detections, amplification occurred in only three out of 15 replicates at one site (two out of five samples) and two out of 27 replicates (one out of nine samples) at the other. Furthermore, even though we implemented robust and validated eDNA water-sampling approaches that produced improved detectability compared to traditional methods for other taxa (Hinlo et al. 2017), we were not able to establish this finding for detection of the invasive red-eared slider turtle from field sites.

Detection probabilities using eDNA are strongly influenced by the physiology and traits of species (Stewart 2019). Even though species-specific detection of eDNA from water samples often provides valuable presence information to help inform species management, characteristics of certain taxa may render these eDNA surveys a less appropriate option. Evaluating which species are particularly challenging for eDNA-based approaches is important, because failure to reliably detect certain species facilitates uncertainty and therefore potential for misguided management action (Roussel et al. 2015; Darling et al. 2020; Jerde 2021). Previous studies have successfully detected turtle eDNA from water samples in controlled conditions (Davy et al. 2015; Adams et al. 2019), mesocosms (Kelly et al. 2014), wetlands (Tarof et al. 2021; Fyson and Blouin-Demers 2021) and marine environments (Harper et al. 2020). However, while studies have demonstrated the capacity of eDNA surveillance through water sampling to monitor turtle populations across a range of environmental conditions (Kakuda et al. 2019; Feng et al. 2020; Tarof et al. 2021), these taxa have also posed challenges to such eDNA approaches (Raemy and Ursenbacher 2018; Adams et al. 2019; Tarof et al. 2021). Turtles shed eDNA in a different way to taxa such as fish and amphibians (Raemy and Ursenbacher 2018; Adams et al. 2019; Harper et al. 2020). The ability of turtles to shed material may be compromised by their lack of a mucous layer, and hard keratinised skin (Adams et al. 2019). Turtle excretions contribute less to eDNA than those from fish and amphibians (Akre et al. 2019; Harper et al. 2020).

However, given current survey methods for red-eared slider turtles can be invasive as well as time- and resource-intensive (García-Díaz et al. 2017), the efficient sampling and non-invasive nature of eDNA-based approaches could still prove extremely beneficial to surveliance efforts if eDNA detectability for this invasive species could be improved. Proximity to the shedding source can affect eDNA detection (Pilliod et al. 2014; Deiner and Altermatt 2014; Dunker et al. 2016). Findings from our control site show that the eDNA concentration varied noticeably between sample locations because turtles were limited to a smaller area of the enclosure and flow within the site was minimal. Therefore, comparative sampling approaches at larger lentic bodies with lower densities of turtles could facilitate poor detection probabilities because turtles have a greater area to deposit eDNA, are able to move freely, and the likelihood of sampling near a turtle is greatly reduced. Furthermore, lentic systems such as urban ponds could present further challenges to water-based eDNA approaches for turtles because the spatial configuration of eDNA in such systems is potentially patchy and unevenly

distributed (Lacoursière-Roussel et al. 2016; Harper et al. 2019). Hence, even with optimised workflows that consider the physiological and behavioural traits of turtles, the inconsistent and low eDNA production rates of turtles combined with the spatial configuration of available eDNA in lentic systems could explain a proportion of the eDNA detection errors (false negatives) observed in previous research and within our study.

Overall, environmental DNA analysis for the purpose of determining the onset of invasive species incursions require clarity of limitations prior to implementation (Morisette et al. 2021; Pilliod et al. 2014). In the context of management of REST in Australia, densities of this priority pest species could be extremely low at incursion sites, and therefore sensitive tools are needed to generate data. Furthermore, because of invasive pressures associated with the pet trade and other avenues, incursions could occur at all times of year, requiring effective and robust approaches regardless of factors such as seasonality and behavioural traits. Even with optimised eDNA workflows, typically an onerous sampling effort is required to detect species incursions in such low density (Furlan et al. 2019). Physiological traits of turtles could further compound the effort required to obtain accurate occupancy data and reduce the potential value of such eDNA surveillance strategies to management.

Our failure to reliably detect the highly invasive REST from urban pond sites – including some sites with known occupancy – suggests that eDNA surveys conducted through analysis of water samples may add little value to some monitoring efforts for this invasive species in Australia. This is particularly noteworthy when other methods provide similar types of data – for example, determining presence/absence through visual observation and trapping. Consequently, with rare and elusive species already requiring larger survey effort, the practicality of water-based eDNA approaches for certain taxa may be voided, especially considering the scale of the sampling ecosystem in which eDNA surveys are conducted (Burian et al. 2021). Hence, resources devoted to turtle surveillance may be better used elsewhere or by using different eDNA approaches such as through the collection of soil or swab samples, which have been effective for other reptile taxa (Matthias et al. 2021). With finite resources available for management efforts, establishing which detection survey approaches are to be implemented and how resources can be effectively used is essential to the overall conservation of biodiversity (Morisette et al. 2021). A preliminary study undertaken using pontoons and swabbing of these from locations in Brisbane showed enhanced detection in a pond where turtles were known to be present from camera surveillance. We also detected REST DNA from pontoon swabs in a nearby pond that had not shown presence with camera surveillance, so this shows promise as an alternative method to enhance eDNA collection and detection rates.

*Table 3. Details of the sampling sites, sampling effort and detection results for the environmental DNA-based monitoring survey for red-eared slider turtles in Sydney, NSW. Detection results are given as the total number of PCR replicates performed, the number of amplifications for the universal fish assay, and positive PCR replicates for each site. *Indicates samples analysed from the control site.*

Location	Latitude	Longitude	Month	Year	No. samples	Method	No. PCRs		
							Total	UniFish	Positive
Bonnyrigg Wetland (Clear Paddock Basin)	–34.194049	149.738498	November	2020	6	eDNA sampler	18	15	0
Busby's Pond (Centennial Parklands)	–33.899914	151.233218	June	2019	10	Manual filtration + self-preserving filter paper	30	15	0
Control site*	–	–	November	2020	6	eDNA sampler	54	27	54
De Fraites Wetland	–33.87346	150.96111	November	2020	5	eDNA sampler	15	15	3
Randwick Pond (Centennial Parklands)	–33.901254	151.233623	November	2018	8	Manual filtration	24	22	0
Webbs Dam (Evatt Park)	–33.980808	151.042842	August	2019	10	Manual filtration + self-preserving filter paper	30	8	0
Wiley Park 01 (Upper and Lower)	–33.926584	151.073329	October	2018	9	Manual filtration	27	26	2
Wiley Park 02 (Upper)	–33.926305	151.074475	November	2020	6	eDNA sampler	18	18	0

Table 4. Details of the conventional sampling monitoring efforts performed at sites and results from efforts from November 2018 – December 2020. Control site enclosure not given (see Table 3).

Location	October 2018 – November 2020		Additional comments	eDNA detection
	Trapped	Confirmed sightings		
Bonnyrigg Wetland (Clear Paddock Basin)	2	1	Two trapped in month of eDNA sampling	Not detected
Busby's Pond (Centennial Parklands)	1	1	Confirmed sightings in nearby ponds	Not detected
De Fraites Wetland	10	2		Positive
Randwick Pond (Centennial Parklands)	2	-	Confirmed sightings in nearby ponds	Not detected
Webb's Dam (Evatt Park)	2	1	Additional 2x individual sighted on day of sampling	Not detected
Wiley Park 01 (Upper and Lower)	1	1	Additional 1x individual sighted pre- and post-sampling	Positive
Wiley Park 02 (Upper)	1	1	Additional 2x individuals sighted on day of eDNA sampling	Not detected

Table 5. Ct values and standards deviations (±) for eDNA samples collected from the positive control site enclosure across three sampling events. FN03 samples were collected one week after the 10 turtles were removed from the enclosure. Letters indicate location of sample collection at the site (i.e. F is farthest from turtles while A, B and D were most proximal to the turtles in the enclosure). A detailed figure showing the sampling approach is available in the supplementary material.

	Sample location at experimental control site					
Sample event	A	B	C	D	E	F
First	25.639 ± 1.112	26.145 ± 0.659	29.054 ± 0.204	26.772 ± 0.072	32.997 ± 0.790	31.293 ± 0.225
Second	25.719 ± 0.039	28.136 ± 0.520	26.218 ± 0.025	25.945 ± 0.975	26.647 ± 0.004	32.275 ± 0.343
Third	35.112 ± 1.272	31.627 ± 0.699	31.913 ± 0.385	30.085 ± 0.216	27.865 ± 0.034	32.431 ± 0.129

DETECTION TOOLS FOR THE ASIAN BLACK-SPINED TOAD IN AUSTRALIA

BACKGROUND

The Asian black-spined toad (ABST), *Duttaphrynus melanostictus,* is considered to be a high-risk invasive species. The most likely pathway of introduction is as a stowaway from South-East Asia. There have been a growing number of ABST detections pre- and post-border across Australia. The risks posed by this species are well documented. The growing numbers of detections of ABST means agencies increasingly need to be operationally prepared to respond to incursions. eDNA is the most promising technology as a cost-effective active and passive surveillance tool and to increase the efficiency of delimitation and post-control.

Since 2000, Australia has recorded over 100 interceptions of *D. melanostictus* at ports (Massam 2010; Mo 2017; Tingley et al. 2017), among other incidences of detections elsewhere in the country (Henderson et al. 2011). As such, the risk that viable populations could establish in Australia makes planning for quarantine and surveillance vitally important (Massam 2010; Shine 2010), as reinforced by a 2016 risk report released by the Queensland Department of Agriculture and Fisheries (Csurhes 2016). If an established population should arise, then containment becomes a priority (Epanchin-Niell and Hastings 2010) because concerted efforts early in the invasion process have the highest likelihood of eradication success (Simberloff 2003), as has been demonstrated with small groups of cane toads (Tingley et al. 2017). Once established, only a few populations of invasive amphibians have been eradicated (Kraus 2009; Beachy et al. 2011; Wingate 2011). Furthermore, a feasibility study conducted on *D. melanostictus* in Madagascar one year after initial detection indicated that the likelihood of a successful eradication was low (McClelland et al. 2015), emphasising the importance of detecting early incursions before viable populations can establish (Reardon et al. 2018).

The aim of this project was to develop a validated eDNA method for the detection of *D. melanostictus* using water samples. We tested a hydrolysis-probe-based qPCR assay targeting a species-specific region of the mitochondrial DNA of *D. melanostictus*. This assay was shown to be capable of detecting trace DNA present in water samples, as a complement to methods of detection currently existing in surveillance and monitoring. In addition, we also developed a triple-plex probe assay that is both species-specific to *D. melanostictus,* and able to differentiate between individuals of the predominant haplotypes of Indonesia and other regions represented in the National Center for Biotechnology Information (NCBI) database, primarily South-East Asia. This assay will enable eDNA testing on water samples to complement routine surveillance efforts to detect the presence of *D. melanostictus,* as well as determining the provenance of captured specimens prior to their entry into Australia.

METHODS

A total of 1,017 accessioned mitochondrial sequences for *D. melanostictus* were downloaded from the NCBI on 12 November 2020, of which 384 aligned to the COX3–ND3 region of the mitochondrion genome (AY458592). The assay was designed to amplify 258bp of the COX3–ND3 region, with no mismatches to any *D. melanostictus* sequences. A TaqMan assay was designed to target a region which was common within *D. melanostictus*, as well as a separate region where haplotype-specific probes could be designed to target Indonesian and non-Indonesian populations (Table 6). The probes were designed to be added in multiplex to identify the target species as well as the provenance of Indonesian or non-Indonesian individuals. There were no mismatches to any *D. melanostictus* sequences. The closest related species to *D. melanostictus* in Australia, *Rhinella marina*, had six total mismatches to the primers, and an additional two to the species-specific assay, and nine and seven mismatches to the Indonesian and non-Indonesian haplotype-specific probes, respectively. All other species examined displayed greater mismatches to the assay.

Specificity of the assay was tested against samples of tissue-derived DNA from five specimens of *D. melanostictus,* and non-target native and invasive anurans (Table 7). This included another invasive toad, *Rhinella marina*, which could be expected to co-occur at incursion sites and in the natural environment. Alignment of the assay components to the sequences of *R. marina* showed six total mismatches to the primers, and an additional two to the probe. The *D. melanostictus* specimens were from different populations in Laos, Singapore, and Indonesia. Tissue samples from non-target species were supplied by the Australian National Wildlife Collection (AWNC), and *D. melanostictus* tissue samples were provided by the Australian Biological Tissue Collection (ABTC).

The assay sensitivity was tested using genomic DNA and a gBlock artificial oligonucleotide (IDT) designed on the targeted 258bp fragment, with a sequence inversion enabling the detection of cross-contamination (Table 8). Each qPCR reaction included 12.5 µL of TaqMan Environmental Master Mix 2.0 (Applied Biosystems), 1 µL of each 10 µM primer, 1 µL of 5 µM probe, 5 µL of sample DNA, and DNAse/RNAse free water added to 25 µL. Samples were analysed using a ViiA 7 Real-Time PCR System (Applied Biosystems) with reaction conditions of 95 °C for 10 minutes, followed by 50 cycles of 95 °C for 15 seconds and annealing at 58 °C for 1 minute. Analysis was carried out using QuantStudio Real-Time PCR Software v1.6.1 (Applied Biosystems). A tenfold dilution series of artificial oligonucleotide was made for final reaction concentrations of 10^0–10^7 copies per reaction. This was repeated with genomic DNA, resulting concentrations of 0.001 pg – 1 ng per reaction. Both dilution series had 12 replicates. The limit of detection (LoD) was the lowest concentration of template DNA at which at least 95% of replicates amplified, and the limit of quantification (LoQ) was the lowest concentration where the reactions produced a coefficient of variation (CV) of under 35% (Klymus et al. 2020).

To further test the assay, mesocosm experiments were performed at the Institut Pertanian Bogor (IPB), Indonesia. These were intended to resemble natural or anthropogenic water sources that would be typical habitats for *D. melanostictus*, including the volume of water and typical duration of toad presence. Three experimental mesocosms with toads of mass 27 g, 49 g, and 86 g were set up, with an additional non-toad control mesocosm. Each mesocosm involved a container which was filled with 20 L of tap water. Once the water had been left to stand for 48 hours, another control sample was taken before a toad was placed in the water. The toad had been handled for three minutes to stimulate it to urinate before being placed in the water (Villacorta-Rath et al. 2020). Water was then sampled from the edge of the container opposite to the toad at 5, 30 and 60 minutes. Individual samples were taken due to the presence of the same time and logistical constraints typically encountered in field sampling and surveillance. After the 60-minute sample was taken, the toad was removed from the water, and the water was sampled every 24 hours thereafter for six days. Samples of 200 mL were also taken from three evenly distributed points around the circumference of a stagnant ornamental pond of approximately 200 L on IPB campus, which was known to have contained *D. melanostictus* individuals from recent visual observations. Samples were shipped at ambient temperature to the University of Canberra for analysis.

DNA was extracted from the filters using DNeasy Blood and Tissue kits (Qiagen), with in-house modifications to the initial steps of kit extraction protocol to accommodate extraction from water filters.

First, after sterilisation of the external filter capsule by wiping with five per cent bleach then 80% ethanol, the preservative buffer was expelled using a sterile 5 mL syringe. The extraction buffer was a mix of 720 µL ATL and 80 µL Proteinase K from the DNeasy kit components. The filter cap from the Wilderlab kit was re-attached, and a new 1 mL syringe containing the extraction buffer was connected to the Luer Lock adapter and used to force the extraction buffer into the filter capsule, saturating the filter membrane. The filter and connected syringe were incubated at 56 °C for 40 minutes with agitation. Post-incubation, the cap was removed and the syringe was used to force all of the fluid through the filter and collected in a separate tube. This filtrate was used in subsequent steps as per the DNeasy kit instructions. Final eDNA elution was in 100 µL of DNase/RNase-free water. qPCR analysis was conducted as described previously, using three technical replicates. To address potential PCR inhibition, neat, 10- and 100-fold dilutions of each sample were tested to determine the dilution that yielded the lowest Ct values for each sample.

The acronym MGB,NFQ represents Minor Groove Binder Non-Fluorescent Quencher (Thermo Fisher). Amplicon 258 nt. mismatches to *Rhinella marina* are underlined.

Table 6. Primers and probes developed to detect the COX3-ND3 region of Duttaphrynus melanostictus

Forward primer	TCTTATATGTCTCAATTTACTGAT
Reverse primer	CCGTATGGAATAKGGGAG
Species-specific probe	FAM-AATKAGTACYAATGACTTCCAATC-MGB,NFQ
Indonesian haplotype-specific probe	NED-TTTTGTTCTCCTCGCCAC-MGB,NFQ
Non-Indonesian haplotype-specific probe	VIC-ATTTGTCCTCCTCACCATAAC-MGB,NFQ

Table 7. Tissue samples of native and invasive anurans used to test specificity of the assay. Specimens from the Australian National Wildlife Collection (AWNC) and Australian Biological Tissue Collection (ABTC)

Scientific name	Common name	AWNC specimen
Adelotus brevis	Tusked frog	AWNC A01787
Assa darlingtonia	Marsupial frog	AWNC A01987
Crinia signifera	Common Eastern froglet	AWNC A01706
Cyclorana novaehollandiae	New Holland frog	AWNC A01849
Litoria bella	Cape York graceful tree frog	AWNC A03487
Litoria inermis	Peter's frog	AWNC A01830
Pseudophryne bibronii	Bibron's toadlet	AWNC A02307
Rana daemeli	Water frog	AWNC A01742
Uperoleia fusca	Dusky toadlet	AWNC A01995
Crinia signifera	Common eastern froglet	AWNC A01706
Litoria verreauxii alpina	Whistling tree frog	AWNC A02315
Pseudophryne corroboree	Southern corroboree frog	AWNC A01861
Papurana daemeli	Water frog	AWNC A01742
Litoria verreauxii alpina	Whistling tree frog	AWNC R15565
Pseudophryne dendyi	Dendy's toadlet	AWNC 34597
Pseudophryne pengilleyi	Northern corroboree frog	AWNC A070221
Rhinella marina	Cane toad	AWNC A02866 AWNC A02521
Duttaphyrnus melanostictus	Asian black-spined toad	ABTC 28279 ABTC 48007 ABTC 48042 ABTC 105927 ABTC 149825

Table 8. Artificial oligonucleotide used to test sensitivity of the assay

TCTTATATGTCTCAATTTACTGATGAGGCTCATATTTTCTTAGTATAATTAGTACTAATGACTTCCA ATCATTAAGTCTTGGTTAAACACCAGGAGAAAATAATGCCCCTTTTTGTTCTCCTCGCCACGGCAA TCGTATTTATCCT**CTAGGATTGCCCAGTTTCGGCGTGAGCATACCACTCTCAAAAAGACTCAGT CCCGACTACCAACCATTAGTCTTTGACTCTCACCGG**TGCCCGACTCCCATATTCCATACGG

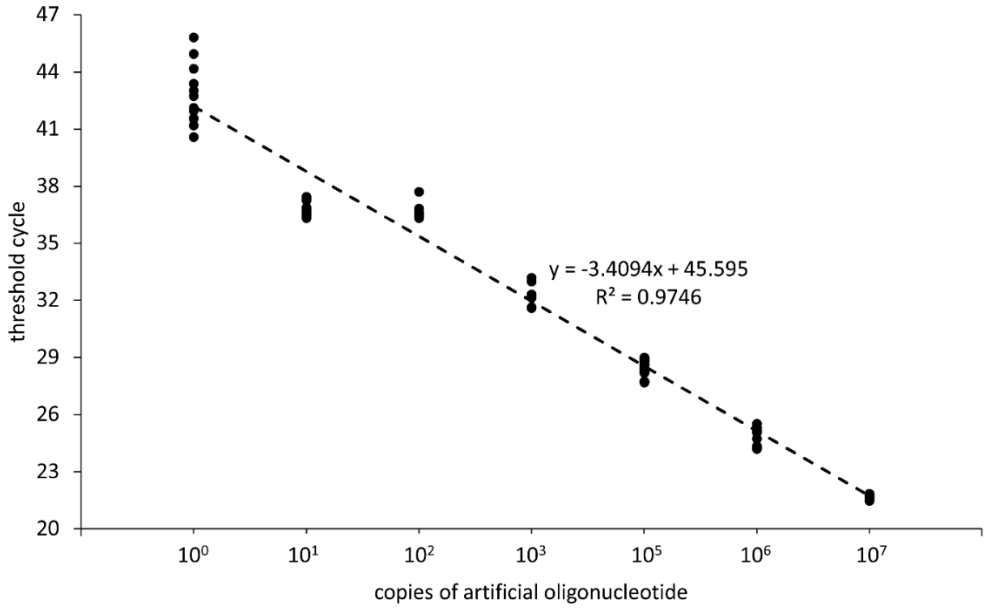
RESULTS

Quantitative PCR on *D. melanostictus* DNA extracted from tissue produced 95.25% efficiency (R^2 = 0.99) with the species-specific probe, 91.13% efficiency (R^2 = 0.97) with the probe specific to the non-Indonesian haplotype, and 102.75% efficiency (R^2 = 0.95) with the probe specific to the Indonesian-haplotype. When conducted with artificial oligonucleotides, this produced 69.11% efficiency (R^2 = 0.99) with the species-specific probe, 62.08% efficiency (R^2 = 0.98) with the probe specific to the non-Indonesian haplotype, and 72.73% efficiency (R^2 = 0.99) with the probe specific to the Indonesian-haplotype.

Positive detections were made for the respective probes as determined by the origin of the individual specimen (Table 9). The species-specific probe was near-identical in performance (considering efficiency and Ct values) when compared to either of the other multiplexed probes. Amplification with artificial-DNA indicated an ability to detect individual copies of the target sequence using each probe (Figure 11). However, only two of six replicates amplified in these reactions, so the limit of detection (LoD) was determined to be one copy, while the limit of quantification was considered as where at least five replicates amplified, which was in reactions containing 10 copies, which were the next lowest concentration tested. Tissue samples could yield positive detections from 1 pg of genomic DNA. There were no positive detections in non-targeted species or negative controls.

From the environmental samples, *D. melanostictus* were detected at six of the 11 sites where presence was unknown. Twenty to 50% of the samples in each site where toads were detected tested positive (10/44 eDNA samples; Table 10). No environmental samples tested positive for the non-Indonesian haplotype, and no positive detections were made in the negative controls. The minimum Ct of positive detections was 28.62, and the maximum was 39.22. Comparing these values to the standard curve run on the same plate, these indicate an approximate minimum concentration of genomic toad DNA in the original eDNA extractions of 0.13 pg, and a maximum of 742.81 pg (Figure 9). Three environmental samples produced amplicons able to be Sanger sequenced: accessions MZ399716, MZ399717 and MZ399718.

A



B

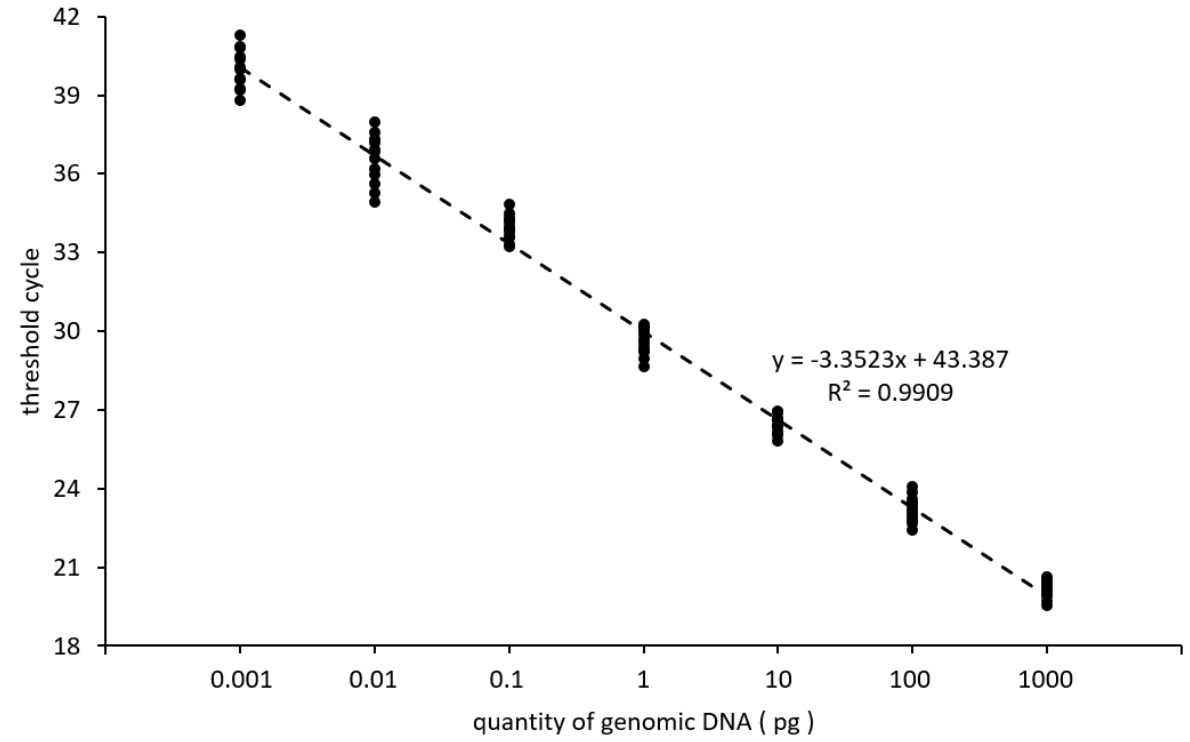


Figure 9. Ct values of the assay performed on different quantities of genomic DNA derived from artificial oligonucleotide (A) and tissue (B)

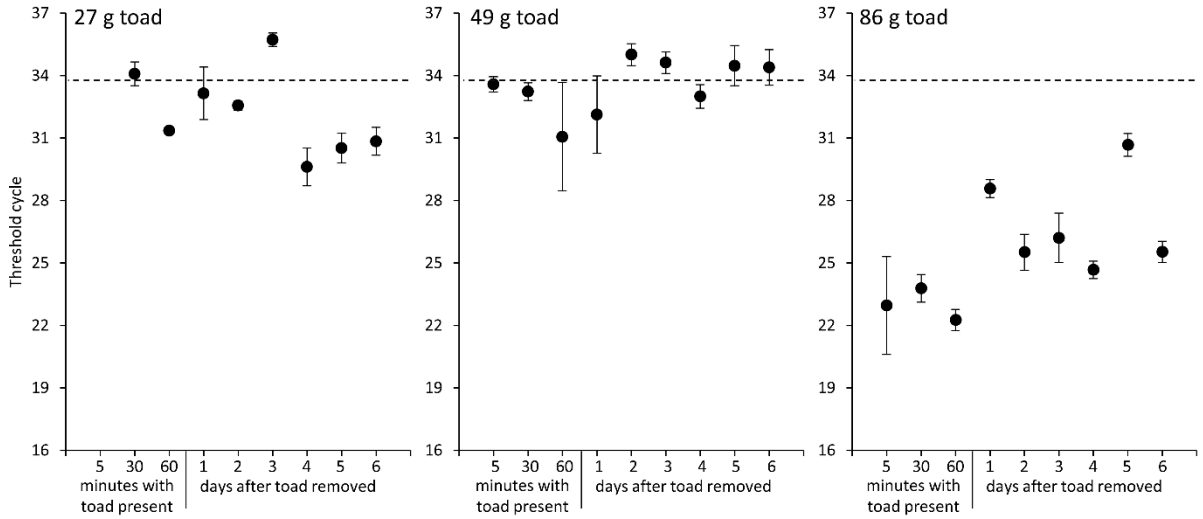


Figure 10. Ct values of the assay performed on samples from mesocosm experiments with toads of different masses (dotted line represents the limit of quantification. Bars represent standard deviation).

Table 9. Assay detection of Duttaphrynus melanostictus–corresponding haplotypes and populations

ABTC: Australian Biological Tissue Collection

Species-specific probe (FAM)	Non-Indonesian haplotype probe (VIC)	Indonesian haplotype probe (NED)	Sample origin	ABTC
+	+	-	Singapore	28279
+	-	+	Indonesia	48007
+	-	+	Indonesia	48042
+	+	-	Laos	105927
+	-	+	Indonesia	149825

Table 10. Sites and absolute counts of detections for each sample and sample type

	Longitude	Latitude	Positive detections	Negative detections
eDNA, toads known to be present	106.72649	–6.562123	2	1
eDNA, toad presence unknown	106.72274	–6.559001	3	3
	106.71959	–6.551604	2	3
	106.72653	–6.552627	1	4
	106.72594	–6.551963	1	3
	106.72079	–6.548557	1	3
	106.72661	–6.559394		7
	106.72837	–6.560735		3
	106.72139	–6.550620		3
	106.72723	–6.560584		2
	106.72291	–6.559046		2
Individual toads in buckets	106.72668	–6.560562	1	2
Negative control	106.72668	–6.560562		3

Note: Samples are 1 in 10 dilutions.

DISCUSSION

We designed an assay capable of specifically identifying *Duttaphrynus melanostictus* for the purpose of Australian biosecurity surveillance and monitoring. The assay can detect DNA from trace amounts of material in aquatic mesocosm experiments which emulated the volume and typical duration of stay of the organism in small water sources.

Our experiments suggest that five minutes of the toad being present in 20 L of water was insufficient for detection when sampling the opposite edge of the container from where the toad was located, but by 30 minutes the toads in all experiments were detected in this way. In experiments with smaller toads, the amount of material that the toad had initially shed into the water could yield detectable *but not necessarily quantifiable* amounts of DNA, which did not degrade to the point of non-detection over the six days. All reactions from the mesocosm containing the 86 g toad had quantifiable amounts of DNA present, and while it may be intuitive that larger toads could shed more material into the water, we cannot exclude that in one or more of the experiments the toad excreted waste into the water or were more active while in the container, thereby shedding more material or increasing dispersal.

There were differences of up to 100-fold in the amount of quantifiable DNA measured in some mesocosm samples taken one day to the next. Possible reasons include the decomposition, continued dispersal, and changing distribution of cellular material shed by the toad. This was reflected in the results from the samples from the environmental site with previously confirmed toad presence, which returned positive detection for only two of the three samples. While consistent volumes of 200 mL were able to be filtered in these samples, this could not be anticipated across different environmental sites. Some sites included suspended particulates in the water sources, meaning that different volumes of water are able to be filtered, thereby affecting the probability positive detections. In addition, there are other factors that must be accommodated in sampling for eDNA and analysis of the extracted DNA, such as mitigating the impact of co-extracted PCR inhibitors that interfere with amplification of target DNA. This was performed in this study by testing dilutions of each sample to determine the optimum dilution. Other environmental factors such as UV exposure and microbial activity can degrade target DNA in freshwater environments over time spans ranging from a single day to four weeks (Dejean et al. 2012; Thomsen et al. 2012; Pilliod et al. 2014; Goldberg et al. 2015).

We were unable to collect additional environmental samples to include a wider range of variables, such as water sources in anthropogenic habitats relevant to biosecurity and border control where drains, gutters, and other standing water are routinely present. We also were not able to evaluate the wide range of environmental variables that could impact detectability, including the presence of PCR inhibition. We were unable to compare the performance of this assay to traditional sampling methods such as physical trapping, visual identification or call identification. An inherent consideration for the application of eDNA surveillance is that positive detections can confirm that the target DNA is present in the environment, whereas no positive detections cannot exclude *that the organism is present but not detected* through the use of the particular sampling methodology. A calculable amount of sampling must be undertaken to give a theoretical threshold of confidence to negative detections within the scenario (Furlan et al. 2016), yet this quantity of sampling is often impractical and not financially viable.

The multiplex nature of the assay also provides a means of understanding the provenance of an incursion, being able to suggest if the point of departure was likely from within Indonesia or from other ports where *D. melanostictus* is known to exist. This could inform risk management both in Australia and at the point of departure. Using the species-specific probe individually could reduce cost of the assay where provenance data is not important. However, the species-specific probe was designed to target a separate region of the mitochondrial genome to the haplotype-specific probes, and can provide additional specificity to the assay.

OBJECTIVE 3: DEVELOPING A MULTI-SPECIES DETECTION FRAMEWORK

BACKGROUND

The alternative to a single-species-specific detection approach – that is, analysing eDNA with high-throughput sequencing – allows for multiple taxa identification simultaneously from a single sample using metabarcoding. Metabarcoding is especially useful for discerning non-target or unanticipated taxa, and has the capacity to inform end users of the presence of all taxa present in a sample and to analyse multiple samples simultaneously. At the commencement of this project, the approach had shown promise; however, a clear standardised framework has not been established or validated as is the case with single-species detection. For metabarcoding to be consistently applied to uses such as surveillance and border-detection, challenges regarding the workflow need to be overcome. Obstacles include identifying discriminatory DNA markers and understanding likely bias from PCR and sequencing.

Despite the many advantages of eDNA metabarcoding, the technique is prone to errors which can arise at various stages in the workflow. Errors can be classified into two major sources: (i) contamination, which occurs when foreign DNA enters the workflow; and (ii) misidentification, which arises from errors generated as a result of the workflow, such as the creation of a novel amplicon during DNA amplification, the creation of a novel sequence read during sequencing, or assigning a sequence read to the incorrect taxon. Contamination can lead to false-positive detections (i.e. apparent detection of a taxon when it is absent from the environment), while misidentification can lead to either false-positive or false-negative detections (i.e. failing to detect a taxon when it is present in the environment). This results in altered estimates of taxonomic richness and diversity.

This objective's work was carried out in two stages. The first was to build on our previous work (Bylemans et al. 2019) and define workflow principles for the use of metabarcoding. The second was facilitated through an additional contract from the Department of Agriculture, Fisheries and Forestry (DAFF) to develop a national set of guidelines through a consultative and collaborative process involving all key practitioners in Australia and New Zealand.

METHODS AND RESULTS

IDENTIFYING ERROR AND ACCURATELY INTERPRETING METABARCODING RESULTS

The methods and case study used to develop principles for identifying error and accurately interpreting results are found in this publication:

Furlan EM, Davis J and Duncan RP (2020) 'Identifying error and accurately interpreting environmental DNA metabarcoding results: A case study to detect vertebrates at arid zone waterholes', *Molecular Ecology Resources*, 20(5):1259–1276.

The key results are summarised in Figures 11 and 12.

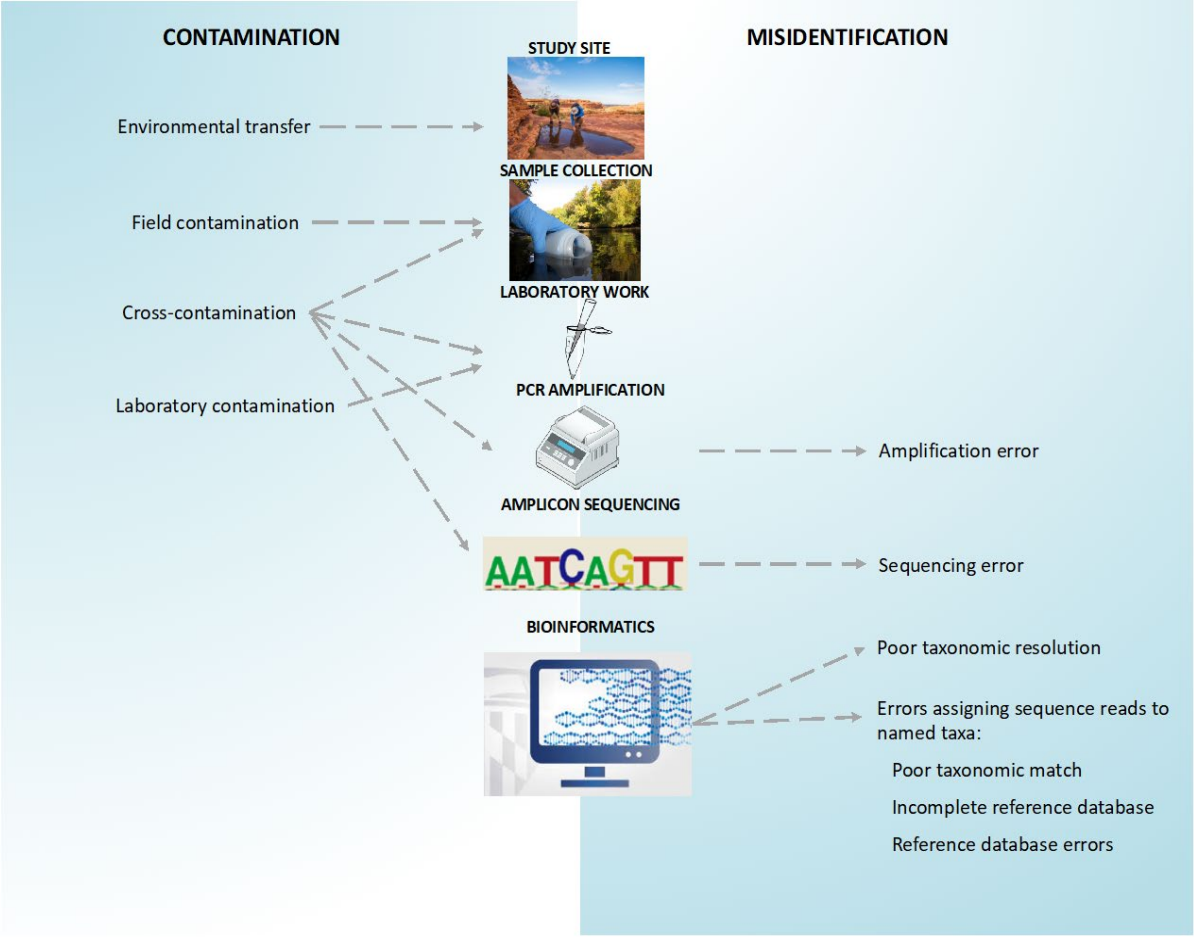


Figure 11. Metabarcoding workflow and potential avenues for contamination or misidentification error in an environmental DNA study

While all surveys are faced with the challenge of false-negative errors, false-positive errors remain a somewhat unique feature of eDNA surveys. When using eDNA methods it is important to address the issues of false-positive detections and acknowledge that a trade-off exists between decreasing the false-positive rate and increasing the false-negative rate.

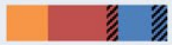
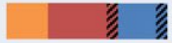
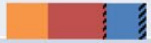
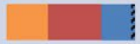
Step	Reasoning	Example from this study	Total taxa identified in this study
1. Sequencing error removed	SNPs, chimeras, or other artefacts can arise during sequencing	Reads filtered based on quality (average quality score <Q30) and size (<80 bp). Chimeras and singletons removed.	
2. Amplification error removed	SNPs can arise during PCR	Rare sequence reads combined with more abundant reads if they vary by ≤2 SNPs	
3. Poor taxonomic resolution accounted for	The gene region targeted may be unable to differentiate closely related taxa	Taxonomies assigned to incorporate all taxa with bit-scores within 4% (or ~1 SNP) of the best match	
4. Imperfect taxonomic matches accounted for	The closest match in the database may be a congeneric or more distant relative	Ensure bit-score of alignments ≥130. Assign to species if alignments show ≥99% identity, genus if ≥97% identity, family if ≥95% identity, and order if ≥90% identity.	
5. Reference database expanded and omissions accounted for	Reference databases are likely to be incomplete causing sequence reads to be misassigned to related taxa	Identify local taxa absent from reference database. Re-assign reads to a higher taxonomic level if matched to a congeneric or confamilial species.	
6. Reference database errors corrected or removed	Reference databases may contain sequence errors or incorrect/outdated nomenclature	Database matches manually reviewed for error and corrected or excluded.	
7. Negative control contaminants removed	Contaminants identified in controls have similar likelihood of arising in experimental samples	Sequence reads detected in negative control samples are excluded from corresponding experimental samples	
8. Unrepeatable results removed	True detections should be repeatable, given DNA concentrations are sufficient	Suspect samples re-run and inconsistent results excluded	
9. Possible environmental transfer contaminants identified	(e)DNA can be transferred through environmental processes (like flowing water) or via human- or animal-mediated transfer	Possible contaminants from environmental transfer manually identified and exclude from dataset	
10. Possible field contaminants identified	(e)DNA contaminants can be introduced by researchers while sampling	Possible field contaminants manually identified and exclude from dataset	
11. Possible cross-contaminants identified	(e)DNA or PCR product from alternate samples or projects can lead to contamination	Possible (e)DNA and PCR contaminants manually identified and exclude from dataset	
12. Possible laboratory contaminants identified	Reagents or labware can contain contaminants	Possible laboratory contaminants manually identified and exclude from dataset	

Figure 12. Stepwise process to identify and remove error in metabarcoding studies and appropriately interpret results. Technical examples of how to implement each step, as applied in the study, are provided. The final column indicates the total number of end-point taxa detected in the case study (expected and unexpected) following each error-removal step.

In summary, knowing where and how to invest (often small) survey budgets for biodiversity monitoring is challenging. While eDNA metabarcoding surveys have been shown to provide value in terms of cost savings and sheer volume of data collection, ensuring the reliability of results is critical. We show that errors can be encountered at any stage of the eDNA metabarcoding workflow due to various contamination and misidentification issues. We advocate a three-pronged approach to address errors in eDNA metabarcoding studies: (a) mitigate error, (b) identify and remove errors, and (c) appropriately interpret the results. While we provide a comprehensive review of potential error sources, the opportunity for errors at each stage will differ between studies. We encourage researchers to tailor their approaches to error-removal according to their particular study, taking into consideration their study scope, the environmental substrate sampled, the taxonomic group targeted and gene region amplified. Increasing accuracy around the results and interpretation of eDNA metabarcoding surveys will greatly benefit researchers and practitioners alike as this field continues to expand in popularity and application.

DEVELOPING NATIONAL EDNA TEST VALIDATION AND BIOMONITORING GUIDELINES

The National eDNA Test Validation Guidelines and Environmental DNA Protocol Development Guide for Biomonitoring were two key outputs from this objective. The test validation guidelines were proposed to follow currently accepted criteria for assay development and validation outlined by the World Health Organization for Animal Health (OIE) (OIE 2019). The OIE guidelines provide a robust framework to develop and validate diagnostic assays of infectious diseases of terrestrial and marine organisms, and provide a high-quality standard baseline for assay developed within the context of Australian biosecurity.

A series of consultation workshops were commenced in August 2021 involving academics, eDNA experts, government scientists, policy officers and officials. These were undertaken both online and in-person where possible (depending on COVID-19 restrictions). A final workshop was undertaken at the Biosecurity Symposium held on the Gold Coast, 3–5 May 2022. The final drafts were submitted to DAFF on 30 June 2022, with publication of the guideline documents in August 2022.

Both guidelines are available for download: https://www.ecodna.org.au/environmental-dna-protocol-and-test-development-guidelines-for-biomonitoring/

Figure 13. Cover pages of two eDNA guidelines published and available for download

OBJECTIVE 4: OPERATIONALISE EDNA THROUGH BUILDING CAPABILITY FOR ROUTINE APPLICATION BY END USERS AND PARTNER AGENCIES

BACKGROUND

At the commencement of this project, one of the key limitations identified in the uptake and application of eDNA was the ability of agencies to access these tools as part of their mainstream surveillance operations. One of the necessary steps in providing eDNA tests to the highest standard is to initiate the process of accreditation under ISO 17025, which is an internationally recognised accreditation for testing laboratories and administered by the National Association of Testing Authorities (NATA).

The 17025 standard evaluates technical expertise and systems so that clients, regulatory authorities and the courts have confidence in test results. As eDNA becomes more widely used, the credibility of the results will become more essential. This will be critical for enforcement and prosecution of Australia's biosecurity policies. Therefore, it was identified that at least one facility in Australia will require this level of competence. The EcoDNA team at University of Canberra was chosen to work closely with relevant government parties and NATA in developing a schedule and framework for accreditation, both as a facility and for specific tests.

The other requirement for operationalising eDNA was to work closely with DAFF to create national eDNA testing capability. The aim was to develop a network of eDNA practitioners and providers, with one central facility having oversight of the quality assurance, controls and standardisation.

SUMMARY OF OUTCOMES

ACCREDITATION AND PROFICIENCY TESTING SCHEMES

ACCREDITATION

In April 2021, the EcoDNA lab contracted the services of MAS Management Systems, an external consultancy specialising in NATA accreditation. The key deliverables were the:

- development of a quality manual and supporting documentation that meets NATA accreditation requirements
- delivery of training in NATA accreditation requirements, internal auditing and measurement uncertainty to all the EcoDNA lab members to sustainably resource its quality management system and meet NATA requirements in the future.

As of September 2022, the supporting documentation and draft quality manual has been collated. Meetings were undertaken with NATA in August 2022 to provide informal feedback on documents and progress. The aim is for the final application to be submitted and for the first formal NATA accreditation inspection to take place in Q1 2023. The reason for this timing is due to a new facility being refurbished at the University of Canberra, primarily to accommodate the increased requirements of the National eDNA Reference Centre.

PROFICIENCY TESTING

This was addressed through a collaborative publication with eDNA practitioners and DAFF:

Trujillo-González A, Villacorta-Rath C, White NE, Furlan EM, Sykes M, Grossel G, Divi UK and Gleeson D (2021) 'Considerations for future environmental DNA accreditation and proficiency testing schemes', *Environmental DNA*, 3(6):1049–1058, https://doi.org/10.1002/edn3.243.

In summary, environmental DNA proficiency testing schemes need context, standards, and an understanding of how eDNA-based assessments will enable reproducible eDNA methods in the future.

We outlined the importance of quality control to assess inhibition and degradation in eDNA samples and how proficiency testing schemes could be designed to assess these measures, as well as laboratory proficiency in reliably detecting eDNA. A flowchart demonstrating how a proficiency testing scheme might operate is presented in Figure 14.

Proficient eDNA service providers could give private and governmental entities confidence in eDNA methods, allowing regulating entities to routinely ensure providers use technically feasible, precise, and repeatable eDNA standard methods.

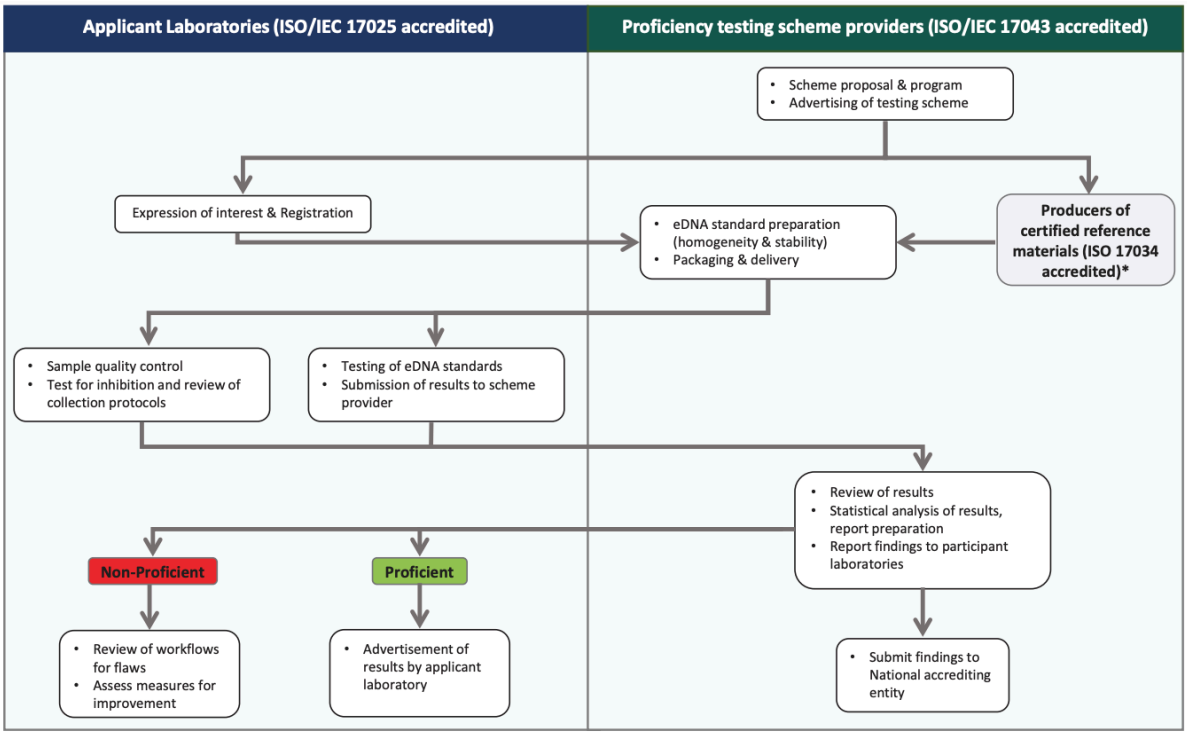


*Figure 14. Proficiency testing scheme flowchart. *Proficiency testing scheme providers may alternatively develop in-house standardised genomic material as part of their proficiency testing schemes*

NATIONAL EDNA REFERENCE CENTRE AND EDNA COLLABORATION CENTRE APPOINTMENT, APPROVAL AND REVIEW PROCESS

The Department of Agriculture, Water and the Environment has appointed the EcoDNA laboratory at the University of Canberra as the National eDNA Reference Centre for a defined term. The Department will annually review the ability of the designated National eDNA Reference Centre to meet the specified criteria.

The National eDNA Reference Centre will accept all submissions from laboratories and other organisations for approval and establishment of an Australian state- or territory-based eDNA Collaboration Centre. The National eDNA Reference Centre will assess each of the submissions on merit, and approve and publish eDNA Collaboration Centre contact details as appropriate.

The National eDNA Reference Centre will annually review the ability of the designated eDNA Collaboration Centres to meet each of the role and responsibility criteria of an Australian state- or territory-based eDNA Collaboration Centre.

In the event of a significant change in the ability of an eDNA Collaboration Centre to meet its requirements – for example, the loss of accreditation or a designated expert – the laboratory is

required to immediately advise the National eDNA Reference Centre of its intentions to retain or relinquish eDNA Collaboration Centre status.

REPORTING REQUIREMENTS

The National eDNA Reference Centre will submit an annual report to DAFF of its activities and a consolidated annual report of the activities of the eDNA Collaboration Centres.

Each eDNA Collaboration Centre will submit an annual report of activities to the National eDNA Reference Centre.

Reports will comprise information against the criteria for endorsement, and include confirmation of expertise, data on testing levels, a summary of research including publications, a record of training activities, and any improvements made as part of a continual improvement process to improve products, services, or processes.

When an eDNA Collaboration Centre fails to meet the requirements or relinquishes its status, the National eDNA Reference Centre will require a statement justifying retention of eDNA Collaboration Centre status.

ACCESS TO NATIONAL EDNA REFERENCE CENTRE AND COLLABORATION CENTRE SERVICES

The National eDNA Reference Centre and the Australian & New Zealand (ANZ) eDNA Network of eDNA Collaboration Centres provide expertise and services to the Australian Government, international, Australian and New Zealand laboratories and other private/public organisations on a cost-recovery basis deemed appropriate by that laboratory for the purpose of sustaining high-quality service and the Department's National eDNA Testing Program.

NATIONAL EDNA REFERENCE CENTRE CONTACTS

Professor Dianne Gleeson Dianne.Gleeson@canberra.edu.au

Dr Alejandro Trujillo-González Alejandro.Trujillogonzalez@canberra.edu.au

EDNA COLLABORATION CENTRE CONTACTS

Approved laboratories 2022	Contact
TropWater (Townsville, Qld) James Cook University	Cecilia Villacorta Rath cecilia.villacortarath@jcu.edu.au
EnviroDNA (Melbourne, Vic)	Helen Barclay hbarclay@envirodna.com
EcoGenetics Lab (Melbourne, Vic) Deakin University	Craig Sherman craig.sherman@deakin.edu.au
TReND (Perth, WA) Curtin University	Nicole White Nicole.White@curtin.edu.au
eDNA Frontiers (Perth, WA)	Shane Herbert shane.herbert@curtin.edu.au
SARDI Diagnostics Laboratory – Aquatic (SA)	Marty Deveney marty.deveney@sa.gov.au Kathryn Wiltshire Kathryn.wiltshire@sa.gov.au
SARDI Diagnostics Laboratory – Soil (SA)	Danièle Giblot-Ducray Daniele.Giblot-Ducray@sa.gov.au Alan McKay alan.mckay@sa.gov.au

One of the functions of the NRC will be the establishment and administration of a network of public and private eDNA Collaboration Centres in Australian states and territories and in New Zealand.

Laboratories within the ANZ eDNA Network expressing an interest in becoming an eDNA Collaboration Centre will be required to comply with the following criteria:

- seek professional affiliation with the Southern eDNA Society, ANZ eDNA Network
- employ scientific staff recognised as experts in the specified target species and have an available range of eDNA/eRNA sampling techniques and tests for the target species
- conduct research to develop new eDNA/eRNA sampling techniques, tests and protocols for specified target species, and encourage collaborative studies with other laboratories and organisations
- prepare data for NRC evaluation, submitted in support of the review, updating and approval process for the validation of eDNA tests in accordance with the Environmental DNA Test Validation Guidelines for the specified target species
- prepare data for NRC evaluation, submitted in support of the review, updating and approval process of Environmental eDNA Test Protocols (ETPs) for the specified target species
- gather, process, analyse and disseminate data relevant to the in-field use and laboratory aspects of the specified target species
- where appropriate, be able to sequence, accession and supply biological reference products used in laboratory testing for the specified target species
- participate in NRC Proficiency Testing Schemes for eDNA
- have appropriate accreditation in place including, accreditation for Schedule 5 of the Memorandum of Understanding for NATA Accredited Facilities between the Department of Agriculture and Water Resources (now the Department of Agriculture, Water and the Environment) and the National Association of Testing Authorities. Schedule 5: Laboratories conducting sampling and/or testing of DNA/RNA from environmental samples (eDNA/eRNA). **NB: Only laboratories providing eDNA testing services for the Department that are applied for the purposes of environmental and biosecurity risk management (*EPBC Act 1999, Biosecurity Act 2015, Export Control Act 2020)* are required to have Schedule 5 accreditation.**
- have capability to provide operational support as specified by the Department. Operational support and other services will be supplied at a reasonable cost agreed to by the department and the NRC in consultation with eDNA Collaboration Centres and reviewed annually.
- have capability to provide confirmatory testing and troubleshooting services.

NATIONAL EDNA REFERENCE CENTRE POLICY AND PRINCIPLES FOR THE APPOINTMENT AND GOVERNANCE OF EDNA COLLABORATION CENTRES

ROLES AND RESPONSIBILITIES OF THE NATIONAL EDNA REFERENCE CENTRE

National eDNA Reference Centre (NRC): Provide functionality at least equivalent to any Australian premier centre of expertise and employ scientific staff recognised as experts in eDNA sampling and testing for both research and laboratory services (Reference: DAWE-NRC Partnering Arrangement, Schedule 2, ratified 20/01/2022).

eDNA Collaboration Centres: Establish and administer a network of Australian state and territory-based eDNA Collaboration Centres across the private/public laboratory sectors.

Accreditation: Have appropriate accreditation in place including, accreditation for Schedule 5 of the *Memorandum of Understanding for NATA Accredited Facilities between the Department of Agriculture and Water Resources* (now the Department of Agriculture, Fisheries and Forestry) *and the National Association of Testing Authorities. Schedule 5: Laboratories conducting sampling and/or testing of DNA/RNA from environmental samples (eDNA/eRNA).*

Capability and Preparedness: Have an available range of eDNA/eRNA sampling protocols and tests for target species, including those not ordinarily available at other Australian laboratories.

Standards: Facilitate standardisation of eDNA sampling and testing techniques relevant to the specified target species. Including maintenance of the, a) *Environmental DNA Test Validation Guidelines*, and b) *Environmental DNA Test Protocols (ETP) Guide for Authors*.

Research and Development: Coordinate, conduct and administer research to develop, evaluate and validate new eDNA/eRNA sampling protocols and tests for specified target species and encourage multi-institutional collaborative studies with other laboratories and organisations.

Test Validation: Evaluate, update, approve and publish reports for the validation of eDNA tests in accordance with the *Environmental DNA Test Validation Guidelines* for the specified target species.

Environmental DNA Test Protocols (ETP): Evaluate, update, approve and publish Standard Operating Procedures and ETPs for the sampling, identification, control and exclusion testing of the specified target species.

Biological Reference Material: Provide and update guidelines on the collection, design, storage and use of biological reference material uses as controls and any other reagents used in the identification of the specified target species. Where appropriate, sequence, accession and supply biological reference products and any other reagents used in laboratory testing for the specified target species.

Reporting and Data Analysis: Gather, process, analyse and disseminate data relevant to the specified target species via a laboratory reporting system integrated with the Department's data and analysis platforms.

Operational Support: Provide operational support including implementation, training, sampling, testing and confirmatory testing services to private and public stakeholders and laboratory personnel in Australian and international partner laboratories, within the National eDNA Testing Program, as required. Operational support and other services will be supplied at a reasonable cost agreed to by the Department and the National eDNA Reference Centre in consultation with eDNA Collaboration Centres and reviewed annually.

Proficiency Testing: Design, update and administer an Environmental DNA Proficiency Testing Scheme for specified target species.

ROLES AND RESPONSIBILITIES OF AN EDNA COLLABORATION CENTRE

To carry out the role requirements, an eDNA Collaboration Centre is required to comply with these criteria:

- To be eligible as an eDNA Collaboration Centre, the laboratory must have a professional affiliation with the Southern eDNA Society, ANZ eDNA Network.
- Employ scientific staff recognised as experts in the specified target species and have an available range of eDNA/eRNA sampling techniques and tests for the target species.
- Conduct research to develop new eDNA/eRNA sampling techniques, tests and protocols for specified target species and encourage collaborative studies with other laboratories and organisations.
- Prepare data for National eDNA Reference Centre evaluation, submitted in support of the review, updating and approval process for the validation of eDNA tests in accordance with the *Environmental DNA Test Validation Guidelines* for the specified target species.
- Prepare data for National eDNA Reference Centre evaluation, submitted in support of the review, updating and approval process of ETPs for the specified target species.
- Gather, process, analyse and disseminate data relevant to the in-field use and laboratory aspects of the specified target species.

- Where appropriate, sequence, access and supply biological reference products and any other reagents used in laboratory testing for the specified target species.
- Participate in National eDNA Reference Centre Proficiency Testing Schemes for eDNA.
- Have appropriate accreditation in place including accreditation for Schedule 5 of the Memorandum of Understanding for NATA Accredited Facilities between the Department of Agriculture and Water Resources (now the Department of Agriculture, Fisheries, and Forestry) and the National Association of Testing Authorities. Schedule 5: Laboratories conducting sampling and/or testing of DNA/RNA from environmental samples (eDNA/eRNA).
- Have capability to provide operational support as specified by the Department of Agriculture, Fisheries, and Forestry. Operational support and other services will be supplied at a reasonable cost agreed to by the Department and the National eDNA Reference Centre in consultation with eDNA Collaboration Centres and reviewed annually.
- Have capability to provide confirmatory testing and troubleshooting services.

ACKNOWLEDGMENTS

We acknowledge the Ngunnawal people as the Traditional Owners of the land where we live and work, and the custodians of the Canberra region. We respect and value their past, present, and ongoing connection to the land and cultural beliefs.

We thank present and past members of the Centre for Invasive Species Solutions for their guidance and commitment to our research, including Andreas Glanznig, Richard Price, Ian McDonald, Yvette Cazabon, Aaron Pobjie and Frank Exon. David Ramsey, Peter Caley, and Phill Cassey greatly assisted in building support within the Incursions Domain framework space. John Virtue was a highly valued mentor and adviser. Brad Page provided necessary encouragement.

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*Note: bold references indicate project outputs. Other CISS and IA CRC publications are marked with an **

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