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Environmental DNA survey in areas of the Kimberley region at risk of invasion by cane toads

Report

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Front cover photographs. Left: Nyikina Mangala Ranger collecting water samples for eDNA detection at Snake Creek, Nyikina Mangala Country (photo Nyikina Mangala Rangers). Right: Balanggarra Ranger collecting water samples for eDNA detection in the east of Adolphus Island (photo Balanggarra Rangers).

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Acronyms

TropWATER . Centre for Tropical Water and Aquatic Ecosystem Research

NESP..... National Environmental Science Program

Abbreviations

eDNA..... environmental DNA

qPCR..... quantitative real-time polymerase chain reaction

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Executive summary

Over the past 80 years, cane toads have progressively moved north-west from where they were originally introduced in Queensland. The invasion front is now moving through the Kimberley region, Western Australia, including nearshore islands, having reached Adolphus Island in 2021. Cane toads have now been observed as far as Fitzroy Crossing, central Kimberley. Although most of the Kimberley region is arid and only offers seasonal waterbodies for cane toads to breed, each wet season allows them to move further. Refuge sites can offer cane toads the opportunity to survive the arid dry season and keep advancing west. Community groups and Indigenous rangers along with state and local government agencies have monitoring programs in place to detect new incursions of cane toads.

We engaged with three Indigenous ranger groups from different areas in the Kimberley in order to collect water samples from key waterbodies and analyse them for presence of cane toad environmental DNA (eDNA). The Balanggarra Rangers collected water samples from four sites in Adolphus Island (offshore island), the Nyikina Mangala Rangers collected water samples from eight sites in the Fitzroy River area (mid-west Kimberley) and the Paruku Rangers collected water samples from seven sites around the Lake Gregory area (south-east Kimberley) for cane toad eDNA detection. The three ranger groups collected eDNA samples with minimal training and following the 'user-friendly' field methods developed at the TropWATER eDNA laboratory. Field sampling was carried out successfully, and field quality controls confirmed that no contamination was introduced in the samples during collection. No cane toad eDNA was detected at any of the sites. However, at Lake Gregory, Paruku Rangers observed cane toads near the sampling site during field work. We attribute the false negative detections to the small number of sites covered at this large site, as well as the small volume of water sampled. This highlights the importance of using multiple detection methods when carrying out cane toad monitoring. In the future, large water samples should be collected to avoid false negative detections. Continuous sample collection during rangers' programmed activities will complement monitoring programs and target early incursions of cane toads in the area.

1. Introduction

Since their introduction to Australia in the 1930s, cane toads, *Rhinella marina*, have successfully spread and colonised Queensland and the Northern Territory and are now expanding through the Kimberley region, Western Australia. This invasive pest represents a threat to native biodiversity due to its toxicity and it has been recognised as a threat to the northern quoll *(Dasyurus hallucatus*; Woinarski et al., 2011), monitor lizards (*Varanus spp*.; Doody, et al., 2006) and some snakes (Phillips, et al., 2006). By 2017, cane toads had reached the east Kimberley (Pizzatto, et al., 2017). Additionally, the wet season is a major driver of cane toad expansion on offshore islands of the Kimberley, especially those located close to river mouths (Gibson & Mckenzie, 2012). For example, cane toads were present on the Kimberley coast adjacent to Adolphus Island in 2012 (Doughty, et al., 2012) and reached the island through rafting in freshwater plumes and debris after a major flooding event that year. The same situation has been observed in offshore islands of Queensland (Woinarski et al., 2011). Cane toad presence in Adolphus Island is particularly threathening to native biodiversity given that this large island contains endemic species and provides refuge for other species with contracted ranges in mailand (Gibson & Mckenzie, 2012).

Community members formed the Kimberley Toad Busters (KTB) group during the late 1990s to prevent the spread of cane toads into the Kimberley (Boulter et al., 2006). Although the KTB has made progress eradicating some toad populations, the invasion front is advancing and cane toads have now been confirmed to be present at the Fitzroy Crossing township, central Kimberley (http://www.wangki.org.au/news/cane-toads-confirmed-fitzroy-crossing). It has previously been observed that toads leading the invasion fronts have elongated limbs that allow them to travel faster than previous generations (Smith & Phillips, 2006). A recent study revealed that toads at the edge of the invasion at Kununurra (north-east Kimberley) had indeed dispersed further, moved faster and with more directionality than conspecifics that invaded another location in the Northern Territory six years earlier (Pizzatto et al., 2017). Therefore, constant monitoring is key to determining when cane toads arrive into a new location.

Indigenous ranger groups in key areas susceptible to cane toad invasion routinely monitor their presence. However, monitoring capability is limited by the remoteness of the region and the need to conduct surveys at night (Doughty et al., 2012). Environmental DNA (eDNA) offers a perfect alternative to labour-intensive cane toad detection methods. Environmental DNA is the trace genetic material shed by organisms into their environment (i.e. water, soil, etc; Taberlet, et al., 2012). Although cane toads are not strictly aquatic animals, adult individuals need to come in contact with fresh waterbodies daily in order to hydrate (Schwarzkopf & Alford, 2002). This means that they can deposit genetic material into the waterbodies and thus be targeted by eDNA methods. Different studies have proven that cane toad eDNA can be effectively recovered from water (Smart, et al., 2015; Tingley, et al., 2019; Villacorta-Rath et al., 2020). Additionally, eDNA detection has been proposed as a more sensitive technique than traditional methods (Smart et al., 2015). An aquaria-based experiment determined that cane toad eDNA can be detected in water samples as short as five minutes after one toad has come into contact with a small waterbody (Villacorta-Rath et al., 2020). Environmental DNA methods not only represent a sensitive tool for early detection of cane toad incursions but also allows Indigenous ranger groups to conduct the field sampling during their normal monitoring activities. The Centre for Tropical Water and Aquatic

Ecosystem Research (TropWATER) has developed user-friendly field methods that allow non-specialists to collect water samples for eDNA analysis. These field methods have been trialled by Indigenous rangers in the Torres Strait Islands as well as by government agencies in Moreton Island for cane toad detection (Villacorta-Rath & Burrows, 2019, 2020; Villacorta-Rath, et al., 2019; Villacorta-Rath & Burrows, 2020). The present study was conducted in collaboration with three Indigenous ranger groups – Balanggarra Rangers, Nyikina Mangala Rangers and Paruku Rangers, who collected water samples for cane toad eDNA detection at key sites during their cane toad monitoring activities.

2. Methodology

2.1 eDNA sampling

Environmental DNA kits, including materials for eDNA sample collection and a field protocol were sent to each of the three Indigenous ranger groups prior to field collection. Water samples for eDNA analysis were collected and preserved from a total of 19 sites in three different areas in the Kimberley region – Adolphus Island (an island in the Cambridge Gulf), Nyikina Mangala Country (Fitzroy River area, mid-west Kimberley) and Paruku Country (also known as Lake Gregory, south-east Kimberley), by the Balanggarra Rangers, the Nyikina Mangala Rangers and the Paruku Rangers, respectively (Table 2.1, Figure 2.1, Figure 2.2, Figure 2.3).

At each site, five replicated 30 mL samples were collected using a new, clean Falcon tube and decanted into a new, clean Falcon tube containing 10 mL of Longmire's preservative solution (Longmire, Maltbie, & Baker, 1997). At every site, a field blank was also taken to ensure that the process of sample collection did not introduce contamination (Goldberg et al., 2016). The field blank consisted of decanting 30 mL of laboratory-grade water into a Falcon tube containing 10 mL of Longmire's preservative solution. The final volume of all field samples, including controls, and preservative solution was therefore 40 mL.

Table 2.1. Field sites in the Kimberley sampled for cane toad eDNA detection by three different Indigenous ranger groups.

Indigenous ranger group	General sampling area	Site name	Latitude (°)	Longitude (°)	Collection date
Balanggarra	Adolphus Island	Adolphus North waterholes	-15.086626	128.143927	19/02/2021
		Adolphus West Creek	-15.13417	128.1359	19/02/2021
		Adolphus East Creek	-15.14375	128.1582	19/02/2021
		Adole			14/06/2021
Nyikina Mangala	Nyikina Mangala Country	Snake Creek, barrage end	-18.18552	124.49389	31/05/2021
		Noonkanbah Road, Midjidigun	-18.27848	124.54559	31/05/2021
		Stone Creek Billbong	-18.39390	124.59154	01/06/2021
		Quiet Billabong	-18.11438	124.40555	01/06/2021
		Turtle Pools	-18.09753	124.41311	01/06/2021
		17 Mile Dam	-18.05116	124.30868	01/06/2021
		Myroo	-18.06979	124.20977	01/06/2021
		Livering Billabong	-18.03393	124.197979	01/06/2021
Paruku	Paruku Country	Nyarna Pool 1	-19.686775	127.579073	25/05/2021
		Nyarna Pool 2	-19.686550	127.5799	25/05/2021
		Lake Gregory 1	-20.17903	127.5132	26/05/2021
		Lake Gregory 2	-20.18113	127.5135	26/05/2021
		Yunbu P3	-20.20288	127.31304	26/05/2021
		Yunbu P4	-20.203637	127.308524	27/05/2021
		Sturt Creek P5	-20.077678	127.352124	27/05/2021

Figure 2.1. Field sites in Adolphus Island, north Kimberley, sampled for cane toad eDNA detection by the Balanggarra Rangers and Western Australian Parks and Wildlife Service. A total of four sites were sampled duing 2021; however, GPS points for only three sites are available

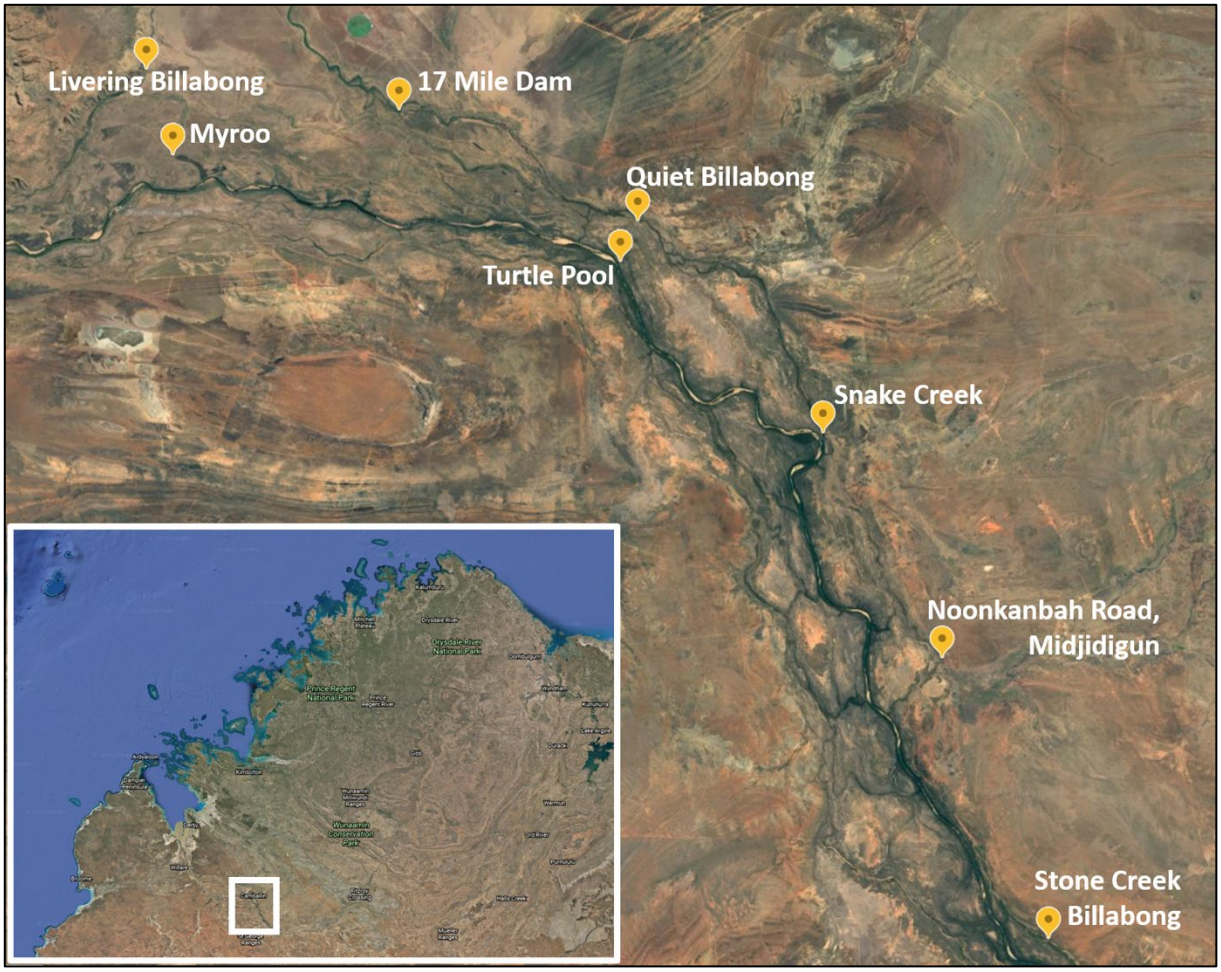

Figure 2.2. Field sites in Nyikina Mangala Country, mid-west Kimberley, sampled for cane toad eDNA detection by the Nyikina Mangala Rangers and the Western Australian Department of Biodiversity, Conservation and Attractions.

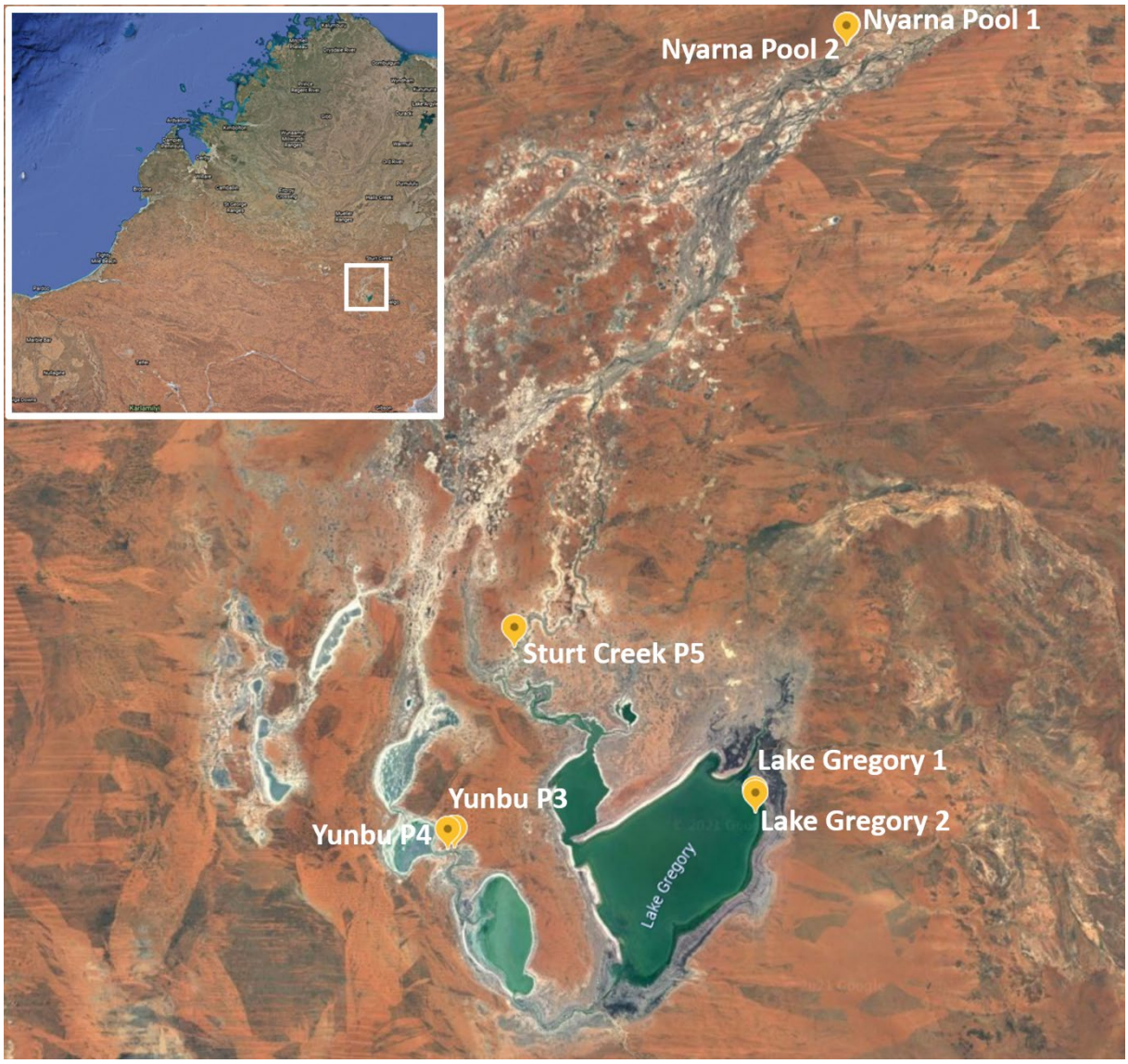

Figure 2.3. Field sites in Paruku Country, south-east Kimberley, sampled for cane toad eDNA detection by the Nyikina Mangala Rangers and the Kimberley Land Council.

2.2 Environmental DNA extractions

Environmental DNA extractions were carried out at the dedicated TropWATER eDNA laboratory, James Cook University, Townsville. Prior to eDNA extraction, bench top surfaces and floor were decontaminated with 10% bleach and subsequently wiped with water and ethanol. Falcon tubes and lids containing the field samples were wiped using the same procedure to avoid cross-contamination during tube handling.

Half of the contents of each Falcon tube (20 mL) were decanted into new, clean Falcon tubes of 50 mL capacity for eDNA extraction. We followed a glycogen-aided isopropanol precipitation protocol developed at TropWATER, as described by Edmunds & Burrows (2020). For all extractions, 20 mL sample aliquots were mixed with 5 µL glycogen (200 mg/mL), 20 mL isopropanol and 5 mL NaCL (5M). Samples were then incubated overnight at 4°C and subsequently centrifuged at 6,750 g for 10 min to form a pellet. The supernatant

was then discarded and pellets were dissolved in 600 µL of lysis buffer (guanidinium hydrochloride and TritonX; pH 10), transferred into a 2 mL tube, and frozen overnight. Environmental DNA present in the samples was lysed at 50°C for five hours and a subsequent precipitation step was carried out by adding 1 µL glycogen and 1,800 µL polyethylene glycol (PEG) buffer to the samples. Samples were centrifuged at 20,000 g for 30 min to form a pellet that was then washed twice using 70% ethanol. After the ethanol washes, the pellet was dried and eDNA was resuspended in 100 µL MilliQ water. Finally, eDNA was purified using the Qiagen DNeasy® PowerClean® Pro Cleanup kit and eluted in 100 µL elution buffer. A negative extraction control was added to each batch of eDNA extractions to ensure that no contamination was introduced during laboratory procedures (Goldberg et al., 2016).

2.3 Detection of species-specific DNA by quantitative polymerase chain reaction (qPCR)

Detection of cane toad eDNA was performed using a aPCR assay developed at TropWATER that targets the *16S* mitochondrial gene of *Rhinella marina* (Edmunds & Burrows, 2019). qPCR assays were run on a QuantStudio™ 5 Real-Time PCR System (Thermo Fisher Scientific Australia Pty Ltd) in a total of four white 384-well plates sealed with optical films (Thermo Fisher Scientific Australia Pty Ltd). We ran 20 µL reactions and each qPCR assay consisted of 6 µL of template DNA and 14 µL of master mix (10 µL PowerUp SYBR Green Master Mix; 1 µL forward primer at 5 µM; 1 µL reverse primer at 5 µM; 2 µL MilliQ® water). Thermal cycling conditions were as follows: initial denaturation and activation at 95°C for 2 min then 60 cycles of 95°C for 15 secs and 65°C for 1 min. A subsequent melt curve analysis was performed to generate dissociation curves by transitioning from 65°C to 95°C at 0.15°C sec^{-1}. In each plate, three positive reference samples containting DNA from the target species were added, as well as three non-template controls (NTC). The NTC samples did not contain the target species DNA and their lack of amplification indicated that no contamination was introduced during plate handling.

2.4 Data analysis

All plates were analysed with a common fluorescence threshold (0.4) using QuantStudio™ Design and Analysis Software (version 1.4.2; Thermo Fisher Scientific Australia Pty Ltd) before export and subsequent analyses in Microsoft Excel. Samples were considered putative positive detections if: (1) the amplification curve crossed the common fluorescence threshold within 55 cycles; (2) dissociation temperature within 99.7% confidence interval of each the species genomic DNA standards (Edmunds & Burrows, 2019); and 3) corresponding equipment controls, field blanks, and extraction blanks were not contaminated

Amplicons from putative positive detections were sequenced at Australian Genome Research Facility (AGRF) to determine if they were true detections. A nucleotide BLAST was performed and amplicon sequences from the samples considered putative positive detections were considered as true detections if there was ≥ 98% pairwise identity with the *16S* gene of the species.

3. Results

Based on the assessment criteria to consider a positive detection of the target species, no presence of cane toad eDNA at any sampling site was detected. All field and extraction control samples were verified to be devoid of the target species eDNA by qPCR.

4. Discussion

Due to the sensitivity of the technique, environmental DNA monitoring allows detection of early incursions of invasive animals (Jerde, Mahon, Chadderton, & Lodge, 2011). In the case of cane toads, eDNA methods have proven to be more sensitive to detection of low number of individuals than traditional trapping (Smart et al., 2015). Because the chances of success of an eradication program are low once invasive species have settled into an area, early detection of incursions is critical (Pluess et al., 2012). Cane toads are currently advancing through the Kimberley and have reached nearshore islands as well as the mid-west region. Annual wet seasons have allowed cane toads to advance, not only into mainland areas but also into nearshore islands, through rafting on debris from river mouths. However, it is uncertain whether cane toad populations could persist in arid areas of the Kimberley that only exhibit seasonal waterbodies (Doughty et al., 2012). The present study was conducted to determine presence of cane toad eDNA at seasonal or permanent waterbodies within three diferent areas of the Kimberley region during the year 2021 – Adolphus Island, Nyikina Mangala Country and Paruku Country. All the Indigenous ranger groups that collected water samples for the present eDNA study conduct continuous monitoring in their area of work. The Balanggarra Rangers carry out continuous cane toad demographic monitoring at Adolphus Island, the Nyikina Mangala Rangers manage the Fitzroy River waters and monitor it for feral animals, among other activities, and the Paruku Rangers manage the wetlands around Lake Gregory.

Indigenous ranger groups showed interest in collecting eDNA sampling during their traditional cane toad monitoring activities. Sampling was conducted by Indigenous ranger groups and state and local government authorities, who required minimal training to carry out the fieldwork. By following the instruction manual, staff were able to successfully collect water samples. Environmental DNA field methods include a filed control to ensure that sample handling in the field did not introduce contamination (Goldberg et al., 2016). Field controls from all field sites tested negative for cane toad eDNA, suggesting that field materials were handled with care and samples were collected appropriately.

At Adolphus Island, the Balanggarra Rangers conduct field work several times per year to monitor the cane toad population on the island. During the first sampling campaign in February 2021, there was one site the rangers were not able to access. Given the ease-of-use of eDNA field methods, the rangers were willing to collect water samples from the additional sampling site during their following visit to the island. The absence of cane toad eDNA in the collected samples was discussed with the Project Coordinator for Invasive Animals of the Parks and Wildlife Service. She confirmed that there have not been any toads recorded since the year 2020 and they hypothesised that drought conditions for a number of years had not allowed the population to persist. Experimental studies have determined that cane toad eDNA can be reliably detected in water for up to four days after animals have been in contact with a waterbody (Cecilia Villacorta-Rath et al., 2020). Therefore, absence of eDNA suggests that cane toads have not been in contact with the water for the past four days.

Despite the fact that we did not find presence of cane toad eDNA, this pest has been observed at the Fitzroy Crossing, in Nyikina Mangala Country, in previous years. No cane toads were recorded during fieldwork at any of the sampling sites. On the other hand, Paruku Rangers found cane toads at Lake Gregory during eDNA sampling. This false negative result

could have been due to not sampling enough sites at Lake Gregory, a large waterbody of approximately 13 km of length and 7 km of width, where samples were collected from only two sites. However, access to the whole perimeter of the lake in remote areas like this one is usually limited. Additionaly, in this case, sampling a small volume of water (30 mL) is not appropriate. This is because eDNA is not evenly distributed in a waterbody and therefore a larger water volume needs to be collected in order to maximise the chances of capturing the available eDNA (Goldberg, et al., 2018). When sampling small waterbodies, such as the billabongs at Nyikina Mangala Country or the creeks on Adolphus Island, collecting a sample volume of 30 mL can provide reliable results (Villacorta-Rath & Burrows, 2020; Villacorta-Rath et al., 2019). However, when dealing with a large waterbody with limited access, a larger water volume should be collected.

5. Recommendations

In the present study, Indigenous rangers collected water samples of small volume (30 mL) at different sites in the Kimberley region. We failed to detect cane toad eDNA at Lake Gregory, although Paruku Rangers found cane toads in the area. The false negative would be avoided by (1) including more sampling sites at large sampling sites and (2) collecting a larger volume of water (i.e. 300 mL). Therefore, in the future, sampling sites and water volume should be planned according to the extension of the target waterbody, rather than using the same sample volume for all sites. Continuous monitoring during ranger activities should be carried out to add another tool in the box for detection of early incursions.

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