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# Monitoring for invasive *Cabomba caroliniana* eDNA in two Darwin River billabongs following eradication attempts

Report

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\*Weed Management Branch, Department of Environment and Natural Resources, Northern Territory Government.

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Front cover: Cabomba eradication boat, Northern Territory Government (photo: Christopher J. Collins)

Back cover: *Cabomba caroliniana* infestation (photo: Leslie J. Mehrhoff)

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## Acronyms

**NESP** ..... National Environmental Science Program

**DENR** ..... Department of Environment and Natural Resources

## Abbreviations

<b>aDNA</b> .....	Artificial deoxyribonucleic acid
<b>CI</b> .....	Confidence interval
<b>eDNA</b> .....	Environmental deoxyribonucleic acid
<b>gDNA</b> .....	Genomic deoxyribonucleic acid
<b>qPCR</b> .....	Quantitative polymerase chain reaction
<b>T<sub>m</sub></b> .....	Melting temperature

## Acknowledgements

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## Executive summary

*Cabomba caroliniana* (cabomba) is an introduced aquatic plant that has become established in many parts of Australia. In the Northern Territory, two billabongs in the Darwin River (Lok Landji and Stan) have been chemically treated as part of an eradication attempt. Follow-up monitoring involved using drop-cameras for underwater visual surveys, benthic raking, and, more recently, the application of eDNA monitoring. This report presents results from an eDNA survey for cabomba in these two billabongs.

Using a previously validated eDNA assay (*C.caroliniana\_matK*; Edmunds and Burrows, 2019) cabomba eDNA was detected in Lok Landji Billabong approximately 2 years after chemical treatment while cabomba eDNA was not detected in Stan's Billabong approximately 10 years after chemical treatment. Within Lok Landji Billabong surface water samples were collected at 42 sites and 10 sub-samples were collected from a conglomerate sample pumped from near the billabong bottom at six sites. Within Stan's Billabong, five surface water samples were collected from two discrete sites. Lok Landji surface samples from three sites contained detectable levels of cabomba *matK* copies (70, 3, and 1), while near-bottom conglomerate sub-samples contained significantly more cabomba *matK* copies ( $152.71 \pm 22.86$ ; average  $\pm$  SEM). No cabomba eDNA was detected in Stan's Billabong surface samples nor in any field ( $n = 3$ ) or extraction ( $n = 5$ ) blanks. Bidirectional Sanger sequencing confirmed that all three surface sample detections and all five representative near-bottom conglomerate sample detections were positive for cabomba.

In light of the potential for legacy DNA to be harboured in Lok Landji sediment following eradication (i.e., eDNA reservoir) the detected cabomba eDNA may or may not have originated from living plants. As such, Lok Landji Billabong should continue to be monitored concurrently using drop-cameras, benthic raking, and eDNA to ensure that living plants, if present, are discovered as soon as possible.



# 1. Introduction

*Cabomba caroliniana* (cabomba) is an introduced aquatic plant from the Americas and has become established in many parts of Australia (Northern Territory, Queensland, Victoria). In the Northern Territory it is only known to occur in the Darwin River. Cabomba management in the NT (i.e., eradication attempts) has included chemical treatment of known populations in the Darwin River with Agricrop Rubber Vine Spray Herbicide (800 g/L of 2,4-D n-butyl ester active ingredient) from 2004 to 2015 and Shark® herbicide (240 g/L of carfentrazone-ethyl active ingredient) from 2015 to 2018 (Cabomba Eradication Program Northern Territory, 2019). Follow-up monitoring is based on visual surveys using camera drops and benthic rake samples but, more recently, eDNA sampling has been introduced to determine if any living cabomba remains in the chemically-treated billabongs.

Lok Landji and Stan's Billabongs, which are discrete sections of the Darwin River, were chemically treated in cabomba eradication attempts in 2004 to 2018 and 2004 to 2006, respectively (Cabomba Eradication Program Northern Territory, 2019). These locations have been routinely monitored since chemical eradication attempts using benthic raking and drop-cameras for visual assessments of billabong benthos. No live cabomba has been detected in Lok Landji Billabong since January 2017 or Stan's Billabong since November 2006 using these visual assessment methods. As an extension to routine visual assessment methods we applied eDNA sampling to determine if detectable levels of cabomba are present in either billabong. More specifically, the following 30 mL water samples were collected to assess cabomba eDNA presence: 1) Lok Landji surface samples ( $n = 42$ ); 2) Lok Landji near-bottom conglomerate sample encompassing six sites ( $n = 10$  sub-samples); 3) Lok Landji blanks ( $n = 3$ ); 4) Stan's surface samples ( $n = 5$ ), and 5) Stan's blanks ( $n = 2$ ).

## 2. Methodology

### 2.1 Field sites

All field sampling sites were located in the Darwin River, which is located approximately 65 km south of Darwin City in the Top End of the Northern Territory, Australia. The Darwin River has a tropical savanna climate with distinct wet and dry seasons and average maximum temperatures of approx. 30°C year round. The river itself stretches for a total of 16 km from the Darwin River Dam wall, draining into Darwin Harbour. Lok Landji and Stan's Billabong are perennial water bodies located in the lower and mid reaches of the river.

Lok Landji is a 2.25 km long billabong with a variable width up to 40 m and depths to 10 m, with average centre depth around 3–4 m. Input from springs during the dry season ( $\geq 200$  L per second) keeps the water fresh, clear and moving. The Lok Landji section of the river is separated from the upper sections by shallow, rocky anabranches with low flow rates following end of wet season.

Stan's Billabong is in the mid section of the Darwin River and is about 50 m long and relatively narrow at approx. 10–15 m wide and an average centre depth of 2 m. This billabong has little-to-no input from spring water. As such, water in Stan's Billabong tends to stagnate towards the end of the dry season as tannins build up and effectively reduce light penetration into the river.

### 2.2 Sample collection

Unfiltered water samples (30 mL) were collected from both the surface and near-bottom in Lok Landji Billabong and from the surface only in Stan's Billabong (Figure 1, Figure 2, Table 1). Surface samples were collected from 42 ( $n = 1$  each) and 2 ( $n = 2$  or 3) discrete sites within Lok Landji and Stan's Billabongs that ranged in depth from 1 to 5.5 m (green dots; Figure 2A and Figure 2B), respectively. 30 mL surface samples were collected from the boat at each site by submerging 50 mL LoBind® tube (Eppendorf Pty Ltd, Australia) just below water surface, decanting down to 30 mL, adding 10 mL Longmire's preservative (Longmire, et al., 1992; Edmunds and Burrows, in review) with Verispensor® 2 liquid dispenser (Eppendorf Pty Ltd, Australia), and inverting five times to mix. Lok Landji near-bottom conglomerate sample contained approximately 5 L pumped from near the billabong bottom into one container at six sites (red dots, Figure 2A). 30 mL sub-samples ( $n = 10$ ) were then collected from 30 L container by pouring 50 mL of conglomerate sample into a new 50 mL LoBind® tube, decanting down to 30 mL (off side of boat), adding 10 mL Longmire's preservative with Verispensor® 2 liquid dispenser, and inverting five times to mix.

Gloves were changed between collection of each surface sample in Lok Landji and Stan's Billabongs given their spatial discretion; however, gloves were not changed between collection of each sub-sample from near-bottom conglomerate given their lack of spatial discretion. Lok Landji and Stan's Billabongs surface sample field blanks ( $n = 1$  each) were collected by adding 30 mL DNA-free water to 10 mL Longmire's and inverting five times to mix prior to commencement of sample collection (i.e., handling and preservative blank). The Lok Landji near-bottom conglomerate sample field blank ( $n = 1$ ) was collected by pumping approx. 5 L clean water through bleach sterilized sump into new 30 L container, pouring 50 mL clean water from sterilized container into new 50 mL LoBind® tube, decanting down to 30

mL, adding 10 mL Longmire's with Verispensor® 2 liquid dispenser, and inverting five time to mix (i.e., sump, bucket, handling, and preservative blank).

All Longmire's preserved samples from Lok Landji and Stan's Billabongs ( $n = 58$ , 40 mL each; Figure 1, Figure 2, Table 1) were stored in the dark at ambient temperature from collection on 12 and 13 December, respectively, until arrival at James Cook University (Townsville, Queensland Australia) on 22 February 2019. Samples remained at room temperature throughout batch-wise eDNA extractions (24 March–18 May 2019).



Figure 1. Location of Lok Landji and Stan's Billabongs on the Darwin River.





Figure 2. Maps of Lok Landji (A) and Stan's (B) Billabongs wherein 30mL surface water samples were collected (green dots). Subsequent near-bottom conglomerate sample collection was conducted in Lok Landji Billabong (A) on same day at six locations in the western half of the billabong (red dots). Red boxes in panel A indicate sites within Lok Landji Billabong where surface water samples contained *C. caroliniana* eDNA. Note that Lok Landji near-bottom conglomerate sub-samples ( $n = 10$ ) are not spatially discrete (see Section 2.2).

## 2.3 eDNA extraction

Inherent eDNA was extracted from 40 mL unfiltered water samples using the PPLPP workflow with 5 µL and 1 µL in-house glycogen added to initial and terminal precipitations, respectively (Edmunds and Burrows, in review). In order to accommodate 40 mL samples the PPLPP workflow was modified as follows: 1) half of each sample (20 mL) was transferred into a new 50 mL LoBind® tube prior to initial isopropanol precipitation, 2) pellets derived from the sample halves were resuspended in 300µL lysis buffer (50% of total), and 3) resuspended sample halves were recombined into one 2 mL LoBind® tube (600µL lysis buffer total).

Samples were processed across five extraction batches with final elution volume of 200 µL for batches 1 and 2 or 100 µL for batches 3–5. Following extraction, 100 µL elution (i.e., 50% or 100% of final elution for batches 1–2 or 3–5, respectively) was purified using DNeasy PowerClean Pro Clean Up Kit (Qiagen Pty Ltd, Australia; Edmunds and Burrows, in review). Each extraction batch ( $n = 5$ ) included one extraction control (30 mL DNA-free water mixed with 10 mL Longmire's) that was processed and analyzed concurrently (i.e., same reagents, equipment, inhibitor purification, and assay). Extraction batches 1–3 concurrently extracted and analyzed field blanks 1–3 (Table 1).

Table 1. Summary of *C. caroliniana* eDNA sample collection dates, ID, types, locations, and detections in two Darwin, NT billabongs. Coordinates provided for first six NBC samples refer to six sites within Lok Landji where near-bottom conglomerate samples were collected (see Figure 2). For samples with positive eDNA detections the number of positive assay replicates ( $n$ ) and corresponding matK copy number are provided. \*Sample type: S = Surface sample; B = Blank sample; NBC = Near-bottom conglomerate sub-sample.

Sample ID	Sample type*	Latitude	Longitude	Location	eDNA detected
B3	B (S)	Car	Car	Stans	N
STAN1	S	-12.775063	130.985074	Stans	N
STAN3	S	-12.775063	130.985074	Stans	N
STAN5	S	-12.775063	130.985074	Stans	N
STAN2	S	-12.775556	130.98484	Stans	N
STAN4	S	-12.775556	130.98484	Stans	N
B2	B (NBC)			Shed	N
B1	B (S)			Shed	N
325	S	-12.746413	130.987671	Lok Landji	N
322	S	-12.746409	130.987393	Lok Landji	N
349	S	-12.746494	130.98703	Lok Landji	N
313	S	-12.746408	130.98657	Lok Landji	N
257	S	-12.746055	130.986104	Lok Landji	N
215	S	-12.745782	130.98574	Lok Landji	N
133	S	-12.745338	130.985269	Lok Landji	N
102	S	-12.745161	130.984808	Lok Landji	N
97	S	-12.745172	130.984348	Lok Landji	N
65	S	-12.744978	130.983883	Lok Landji	N
61	S	-12.744997	130.983521	Lok Landji	N
49	S	-12.744901	130.983332	Lok Landji	N



27	S	-12.744722	130.983055	Lok Landji	N
23	S	-12.744721	130.982688	Lok Landji	N
6	S	-12.744633	130.982227	Lok Landji	N
57	S	-12.745001	130.981952	Lok Landji	N
106	S	-12.745275	130.981586	Lok Landji	N
146	S	-12.745455	130.981494	Lok Landji	N
197	S	-12.745732	130.981131	Lok Landji	N
227	S	-12.745912	130.981038	Lok Landji	N
265	S	-12.746185	130.980764	Lok Landji	N
287	S	-12.746367	130.980582	Lok Landji	Y ( <i>n</i> = 1; 70 copies)
342	S	-12.746549	130.980399	Lok Landji	N
388	S	-12.746729	130.979943	Lok Landji	N
430	S	-12.746913	130.979941	Lok Landji	N
408	S	-12.746826	130.979664	Lok Landji	N
441	S	-12.747001	130.979575	Lok Landji	N
405	S	-12.746828	130.979388	Lok Landji	N
422	S	-12.74692	130.979205	Lok Landji	N
378	S	-12.74674	130.979019	Lok Landji	N
335	S	-12.74656	130.97865	Lok Landji	N
271	S	-12.746293	130.978463	Lok Landji	N
250	S	-12.746113	130.97837	Lok Landji	Y ( <i>n</i> = 1; 3 copies)
236	S	-12.746024	130.978185	Lok Landji	N
234	S	-12.746022	130.978005	Lok Landji	N
205	S	-12.745856	130.977728	Lok Landji	N
159	S	-12.745577	130.977445	Lok Landji	Y ( <i>n</i> = 1; 1 copy)
157	S	-12.745579	130.977261	Lok Landji	N
138	S	-12.74549	130.977076	Lok Landji	N
153	S	-12.745587	130.976901	Lok Landji	N
<hr/>					
NBC1	NBC	-12.744732	130.982136	Lok Landji	Y ( <i>n</i> = 1; 106 copies)
NBC2	NBC	-12.74558	130.977077	Lok Landji	Y ( <i>n</i> = 3; 81-236 copies)
NBC3	NBC	-12.745829	130.977638	Lok Landji	Y ( <i>n</i> = 4; 106-347 copies)
NBC4	NBC	-12.745913	130.980947	Lok Landji	Y ( <i>n</i> = 1; 138 copies)
NBC5	NBC	-12.746989	130.979929	Lok Landji	Y ( <i>n</i> = 2; 1-71 copies)
NBC6	NBC	-12.747067	130.979665	Lok Landji	Y ( <i>n</i> = 2; 124-347 copies)
NBC7	NBC			Lok Landji	Y ( <i>n</i> = 2; 127-163 copies)
NBC8	NBC			Lok Landji	N
NBC9	NBC			Lok Landji	N
NBC10	NBC			Lok Landji	Y ( <i>n</i> = 3; 91-300 copies)

## 2.4 eDNA detection

Presence of cabomba eDNA was determined using a previously developed quantitative polymerase chain reaction (qPCR) assay (*C.caroliniana\_matK*; Edmunds and Burrows, 2019). Each 10 or 20 µL reaction (*n* = 6 technical replicates per field sample) contained 5 or 10 µL PowerUP® Sybr Green Master Mix (Thermo Fisher Scientific Pty Ltd, Australia), 0.9 or

1.8 µL forward primer (900 nM final), 0.9 or 1.8 µL reverse primer (900 nM final), 3 or 6 µL extracted eDNA (see above), and 0.2 or 0.4 µL DNA-free water. Room temperature master mix (7 or 14 µL) was loaded into white 96-well plate (Life Technologies Inc., Australia) with Multipette® Xstream electronic dispensing pipette (Eppendorf Pty Ltd, Australia) fitted with 500 µL CombiTip® (Eppendorf Pty Ltd, Australia) in a UV-sterilized PCR cabinet (Esco Pty Ltd, Australia) in a dedicated eDNA laboratory at James Cook University (Townsville, Queensland Australia). Plates were sealed with optical adhesive film (Life Technologies Australia Ltd Pty), briefly vortexed (10 sec), pulse spun (10 sec), loaded into an opened tray of QuantStudio3™ Real-Time PCR System (Life Technologies Inc., Australia), and wiped thoroughly with nonabrasive Kimwipe® to remove smudges or dust (i.e., ensure a complete optical seal transparency) before closing QuantStudio3™ tray and commencing qPCR run.

All assays were run under the following cycling conditions: initial UDG incubation at 50°C for 2 min then initial denaturation at 95°C for 2 min followed by 45 cycles of 95°C for 15 secs and 65°C for 1 min (ramp rate = 2.7°C/sec) before terminal dissociation curve generation (65°C–95°C, ramp rate 0.15°C/sec). QuantStudio™ Design and Analysis Software (version 1.4.2; Life Technologies, Australia) was used to set threshold fluorescence (0.2), import *C.caroliniana*\_matk aDNA standard curve (Edmunds and Burrows, 2019), and export data to Microsoft Excel.

Batch 1 samples were run once using 10 µL reactions (18% or 9% screened of Qiagen purified or total elution, respectively). Batch 2 samples were run three times (twice using 10 µL and once using 20 µL reactions for 100% or 50% screened of Qiagen purified or total elution, respectively). Batch 3 samples were run twice (once using 10 µL and once using 20 µL for 100% screened of Qiagen purified total elution). Batch 4 and 5 samples were each run twice using 20 µL reactions (72% screened of Qiagen purified total elution).

Cabomba eDNA detections that exhibited  $\Delta T_m$  within 99.7% confidence interval (CI) of gDNA standards (see Edmunds and Burrows, 2019) were considered putatively positive and representatives for each  $\Delta T_m$  value were Sanger Sequenced bidirectionally for confirmation (Trujillo-Gonzalez, et al., 2019).

### 3. Results

Across all Lok Landji surface samples ( $n = 42$ ) cabomba eDNA was detected at three sites (#287, #250, and #159) in low abundance (70, 3, and 1 copies), respectively, following  $\geq 50\%$  elution screening (Figure 2A; Table 1). Across ten sub-samples of the Lok Landji near-bottom conglomerate sample cabomba eDNA was detected in significantly higher abundance, despite lower elution screening (9%), than was observed across surface sample detections ( $152.71 \pm 22.86$  vs.  $24.59 \pm 22.85$  copies; average  $\pm$  SEM; Welch-corrected two-tailed T-test:  $P=0.0095$ ; Figure 3). Of note is that two bottom conglomerate sub-samples (NBC8 and NBC9; Table 1) yielded no cabomba eDNA detections in the 9% elution screened.

In terms of overall detection rate, bottom conglomerate sub-samples averaged 30% (18/60 assays) across 9% elution screened while surface samples averaged 0.543% (3/552 assays) across  $\geq 50\%$  elution screened. On a sample-by-sample basis, detection rate ranged from 16.7% (1/6 assays) to 66.7% (4/6 assays) for bottom conglomerate sub-samples containing eDNA ( $n = 8$ ; Table 1) while detection rate was 5.6% (1/18 assays) for each surface sample containing eDNA. ( $n = 3$ ; Figure 2A).

No cabomba eDNA was detected in Stan's Billabong surface water samples (Figure 2B; Table 1). Similarly, no cabomba eDNA was detected in field and extraction blanks ( $n = 3$  and 5), respectively.

Sanger sequences of representative putative positive detections ( $n = 8$ ) exhibited  $\geq 96.8\%$  pairwise identity with targeted cabomba *matK* gene region within chloroplast genome (GenBank accession AF543729) thus confirming all detections as true positives. Of note is that poor sequence quality due to limited template available for Lok Landji surface detections at sites #250 and #159 (3 and 1 copies) the confirmation pairwise alignment to *C. caroliniana matK* (GenBank accession AF543729) is based on 27 bp region (6% and 12%) with 100% match, respectively.



### ***C. caroliniana* eDNA Detection in Lok Landji Billabong**

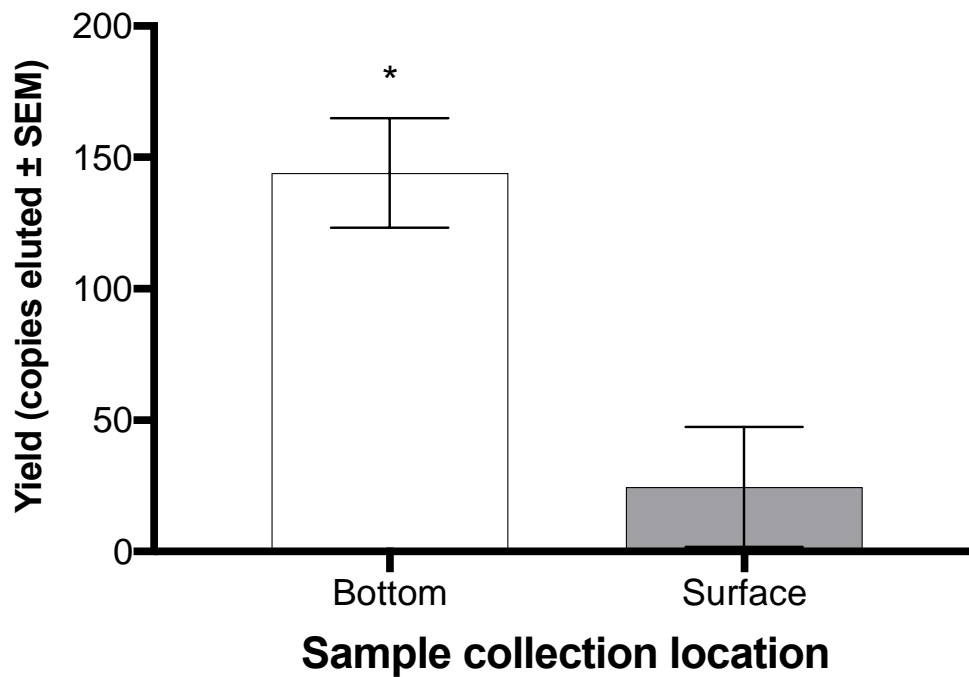


Figure 3. Average number of *C. caroliniana* eDNA copies ( $\pm$  SEM) recovered in final elution of extracted 30 mL water samples as determined by extrapolation from aDNA standard curve (Edmunds and Burrows, 2019). The bottom conglomerate sub-samples contained significantly more *C. caroliniana* eDNA than surface samples (\* $P=0.0095$ ; see Section 3). Note that *C. caroliniana* eDNA detections in surface and near-bottom conglomerate sub-samples can and cannot be spatially resolved, respectively (see Section 2.2).

## 4. Discussion

This constitutes the first application of eDNA monitoring to invasive aquatic plants following chemical eradication treatments, to our knowledge; however, eDNA monitoring has been applied to the detection of invasive fish following eradication attempts (Banks, et al., 2016; Dunker, et al., 2016; Davison, et al., 2017; Davison, et al., 2019). No cabomba eDNA detections in Stan's Billabong confirms the years of visual surveys and currently held notion that cabomba is no longer extant in this waterbody; however, eDNA detections in Lok Landji Billabong suggest that cabomba might still be present.

In light of the knowledge gap regarding cabomba decay rate following chemical eradication treatment with Agricrop Rubber Vine Spray Herbicide or Shark® herbicide (Cabomba Eradication Program Northern Territory, 2019), especially under Northern Territory environmental conditions (e.g., temperature, UV, pH, microbial community), it is difficult to discern whether detected eDNA originated from living plants or legacy DNA present in the sediment surface layer (i.e., remnant DNA from recently decayed or still decaying plant material). The significant increase in cabomba eDNA copies obtained from Lok Landji bottom samples versus surface samples (Figure 3) does not help resolve this ambiguity in that more eDNA would be expected in bottom samples regardless of living plant or legacy DNA origin.

Little is known about the longevity of eDNA detection following the chemical eradication of invasive species, with Dunker et al. (2016) providing the only demonstration to date of eDNA detectability following removal of all living individuals of target species. More specifically, eDNA of northern pike (*Esox Lucius*) was detected in four ponds stocked with fish carcasses (5,410–7,219 g/Ha-m) for approximately 230 days after eradication of all living individuals (Dunker, et al., 2016). As such, detected cabomba eDNA in Lok Landji Billabong approximately 750 days post-eradication could have plausibly originated from legacy DNA given larger pre-eradication biomass (10,000–15,000 g/Ha-m; Christopher Collins personal communication). Aquatic plants can require  $\geq 300$  days to fully decay *in situ* (Burkholder and Bornside, 1953) depending on environmental conditions and plant physiology (Jewell, W., 1971), with stem viability increased if under mounds of saturated substrate (e.g., billabong bottom following herbicide-mediated die-off; Dugdale, et al., 2013). Moreover, cabomba does not typically propagate through seed dispersal (Dugdale, et al., 2013); however, seed-based propagation has been observed in northern Australia (Schooler, et al., 2009). In light of this potential for cabomba seed presence within Lok Landji prior to chemical eradication, slow seed decay could provide an additional eDNA reservoir to slowing decaying stems (Burkholder and Bornside, 1953; Jewell, W., 1971) or lead to recolonization along with any small fragments that still contain leaf node (Schooler, et al., 2006; Jacobs and Macisaac, 2009).

On the other hand, detected cabomba eDNA could have plausibly originated from living plants given that current surveillance techniques (e.g., benthic raking and drop-cameras) do not provide absolute confidence in living plant detection. Regardless of origin, positive detections in surface samples (Figure 2, Figure 3; Table 1) demonstrate the sensitivity and utility of eDNA for detection of living plants or legacy DNA present at bottom of targeted waterbody.

## 5. Recommendations and conclusions

Failure to detect cabomba eDNA in all other Lok Landji surface samples ( $n = 38$ ; Table 1) or in any of the Stan's Billabong surface samples ( $n = 5$ ; Table 1) cannot be interpreted as conclusive evidence of cabomba absence from these billabongs (Roussel, et al., 2015).

To ensure earliest possible detection of cabomba recolonization within chemically treated eradication zone(s), concurrent eDNA and visual monitoring should be undertaken regularly until both approaches yield no detections. Future eDNA monitoring efforts should aim to determine if detections originate from living plants or legacy DNA.

Preference should be given to spatially discrete near-bottom samples over spatially discrete surface given the higher detection likelihood of eDNA shed from living plants (if present). However, if logistical challenges prevent this (e.g., equipment decontamination between sites), then 30 mL surface samples can be utilized. Of note is that surface samples require screening a higher percentage of extraction elution ( $\geq 50\%$ ) than bottom samples (9%) to ensure low-copy eDNA is detected if present and that this additional screening incurs higher analytical costs.

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