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Environmental DNA survey of Eureka Creek, Upper Mitchell and Walsh River for two invasive tilapia species

Report

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Cover photographs:

Front cover: eDNA sampling in the Walsh River (photo Heather Robson).

Back cover: Walsh River and Eureka Creek junction (photo Madalyn Cooper).

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Acronyms

TropWATER . Centre for Tropical Water and Aquatic Ecosystem Research

BLAST..... Basic Local Alignment Search Tool

NESP National Environmental Science Program

Abbreviations

eDNA..... Environmental deoxyribonucleic acid

MEEL..... Molecular Ecology and Evolution Laboratory

NCBI..... National Center for Biotechnology Information

qPCR..... Quantitative polymerase chain reaction

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Executive summary

Mozambique tilapia (*Oreochromis mossambicus*) and spotted tilapia (*Tilapia mariae*) were introduced to Australia in the 1970s through the aquarium trade and have since spread. In 2008 an invasion front of *O. mossambicus* was discovered in Eureka Creek and effectively eradicated by rotenone. Since that eradication attempt, there has only been one account of an individual *T. mariae* found approximately 100 km downstream of Eureka Creek in the Walsh River.

This report presents the result of an environmental DNA (eDNA) survey conducted along Eureka Creek, the Walsh River catchment, and the Upper Mitchell River catchment that are historically free of *O. mossambicus* and/or *T. mariae*. Water from 14 sites was filtered and eDNA was captured in a 20 µm nylon net filter, which was then preserved in ethanol. eDNA was extracted and screened for presence of *O. mossambicus* and *T. mariae* using a previously validated eDNA assay that targets both tilapia species. *T. mariae* eDNA was detected at only one site, Eureka Creek, and in very low amount. The lack of positive detections at other surveyed sites suggests that tilapia are either absent or present in numbers below current detection probability.

In light of invasion fronts being made up of only a few individuals, tilapia-free waterways should be monitored regularly for tilapia eDNA in order to detect any increase in abundance over time. Future eDNA field surveys should either filter water through smaller pore sizes ($\leq 5 \mu\text{m}$) or capture eDNA by precipitation from intact water samples.

1. Introduction

Mozambique tilapia (*Oreochromis mossambicus*) and spotted tilapia (*Tilapia mariae*) were introduced to Australia in the 1970s through the aquarium trade and have since spread. In 2008 an invasion front of *O. mossambicus* was discovered in Eureka Creek and effectively eradicated by rotenone, thus effectively preventing any further invasion downstream into the Walsh River and onward into the Gulf of Carpentaria (Pearce, et al., 2009; Russell, et al., 2012). Since the 2008 eradication there has only been one account of an individual *T. mariae* found approximately 100 km downstream of Eureka Creek in the Walsh River (reported 23 November 2017; www.daf.qld.gov.au).

Environmental DNA (eDNA) is species-specific DNA released into the local environment as a consequence of biological processes (e.g., mucous, faeces, skin cells, gametes, decay, etc.). It can be captured from environmental samples (e.g., water, soil, sediment, air, etc.) and used to detect species of interest with routine molecular genetic methods (reviewed by Thomsen and Willerslev, 2015; Goldberg, et al., 2016; Lear, et al., 2018). One approach to eDNA sampling is to capture eDNA on a porous membrane by forcing water through the membrane with a portable peristaltic pump and subsequently storing filters in a preservative until transport back to dedicated laboratory and eDNA extraction (Goldberg, et al., 2016). Following extraction each samples is screened for the presence of eDNA from target species using quantitative real-time polymerase chain reaction (qPCR) assays designed to amplify only target species (e.g., *O. mossambicus* and *T. mariae*) by excluding all other co-occurring species (Edmunds and Burrows, 2019a). This eDNA approach has been successfully used for the detection of various rare tropical species (Simpfendorfer, et al., 2016, Bakker, et al., 2017, Ishige, et al., 2017).

The objective of this eDNA-based survey was to assess three Northern Queensland waterways (Eureka Creek, Walsh River, and Upper Mitchell) that are historically free of *O. mossambicus* and/or *T. mariae*. Here we report the results from the collaborative eDNA-based tilapia invasion front survey conducted on 23 and 24 November 2017 by TropWATER and Biosecurity Queensland.

2. Methodology

eDNA was captured by filtering the maximum volume of water that could go through a 20 μm nylon net filter using a hand-held eDNA pump.

2.1 Pre-departure decontamination

The hand-held eDNA pump, associated hoses (10 mm clear vinyl tubing), 3D-printed filter cartridges (Figure 1), scissors and forceps were all decontaminated by soaking in 10% v/v bleach for 10 minutes before thoroughly rinsing with RO water. Filter cartridges were subsequently UV sterilized in a dedicated eDNA fume hood along with all microcentrifuge tubes (2 mL; Axygen Inc.) where filters were preserved and stored after eDNA capture. To reduce the risk of cross-contamination from handling in the field, all DNA-free filter cartridges were pre-loaded with 20 μm nylon net filters (47 mm diameter; Merck Millipore) and all DNA-free microcentrifuge tubes were pre-loaded with 1.5 mL of 95% ethanol.

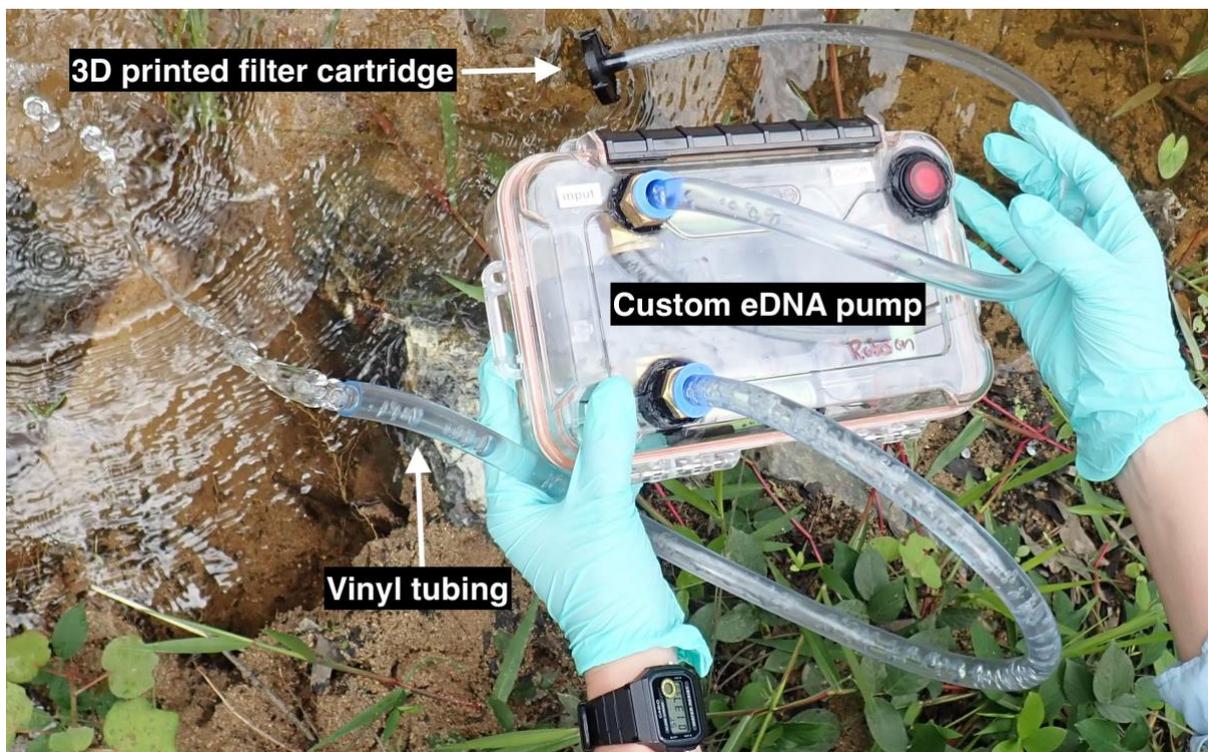


Figure 1. Hand-held eDNA pump and associated 3D-printed filter cartridge and vinyl tubing.

2.2 Field collection of eDNA

eDNA sampling conducted at 14 different sites in the Walsh and Mitchell River catchments (Table 1, Figure 2). At each site, three replicated samples were collected by directly filtering water through a separate DNA-free cartridge containing a 20 μm nylon net filter. Following water filtration, filters were rolled up using forceps, cut in half, and each transferred into a separate DNA-free microcentrifuge tube pre-loaded with preservative (Figure 3).

Table 1. Sampling sites surveyed for a tilapia invasion front using eDNA.

Day	Site	Site name	Latitude (°)	Longitude (°)	Collection	Extraction	qPCR
1	1	DSITI "Walsh Purple"	-17.120914	145.269732	23/11/17	25/11/17	29/8/18
1	2	Collin's Weir (Walsh)	-17.257366	145.294035	23/11/17	25/11/17	29/8/18
1	3	Walsh Red Trib 1	-17.145402	144.981085	23/11/17	25/11/17	29/8/18
1	5	Eureka Creek Crossing	-17.254417	145.084206	23/11/17	25/11/17	29/8/18
1	6	DSITI "Walsh Red Trib 2"	-17.332839	144.949127	23/11/17	25/11/17	29/8/18
2	8	DSITI "Walsh Red Down"	-16.989238	144.305184	24/11/17	30/11/17	29/8/18
2	9	DSITI "Walsh Yellow Trib 4"	-16.670105	144.022696	24/11/17	30/11/17	29/8/18
2	10	DSITI "Walsh Green"	-16.546000	143.785550	24/11/17	30/11/17	29/8/18
2	11	DSITI UW Footage Mitchell 2	-16.518750	143.637378	24/11/17	30/11/17	29/8/18
2	13	Wetlands / Pickford Rd. Mitchell	-16.916161	145.384994	24/11/17	25/11/17	29/8/18
2	14	Rifle Creek	-16.664922	145.326916	24/11/17	25/11/17	29/8/18
2	15	May River / Mitchell Purple Trib 4	-16.588228	145.183114	24/11/17	25/11/17	29/8/18
2	16	McLeod River/Mitchell Purple Trib 3	-16.505323	145.000703	24/11/17	25/11/17	29/8/18
2	17	Mitchell Purple	-16.566100	144.773319	24/11/17	25/11/17	29/8/18

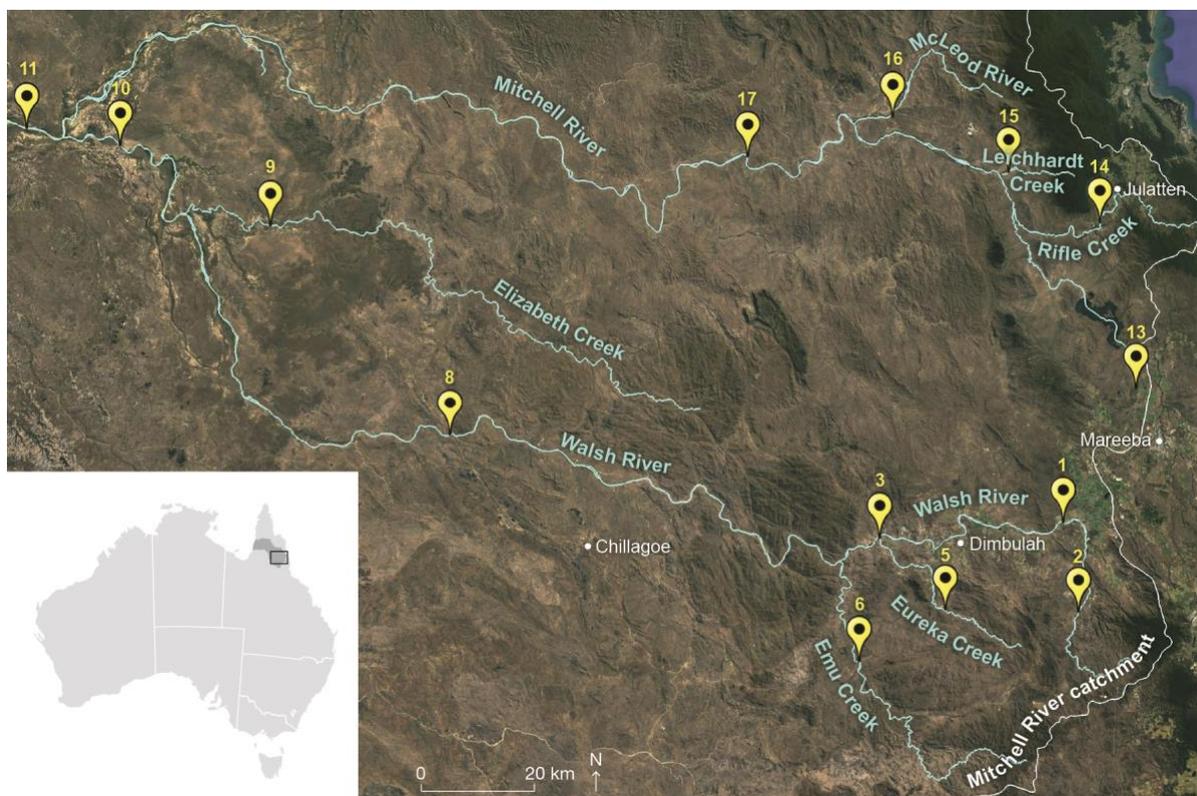


Figure 2. Location of sampling sites in the upper Mitchell River.



Figure 3. Collection of filters containing eDNA sample.

To ensure pre-loaded cartridges used for sample collection were not contaminated before their use in the field, an equipment control was collected at each site prior to collection of the environmental samples. This consisted of filtering 250 mL of DNA-free water through the three sampling cartridges by stacking them in series onto a pre-loaded equipment control cartridge. This way, the equipment control filters captured any contamination present in the ~83 mL passed through each of the three sampling filters; Figure 4). Equipment control filters were then rolled up, cut in half and transferred into preservative as described above.



Figure 4. Collection of equipment and field blank samples at each location.

2.3 Extraction of eDNA from ethanol-preserved filters

Before extraction commenced, bench top surfaces and floor in dedicated eDNA laboratory were decontaminated with 4% and 10% v/v bleach, respectively, as per standard operating procedure. Following lab decontamination, samples were removed from -20°C freezer and allowed to come to room temperature ($\approx 22^{\circ}\text{C}$). One half of the ethanol-preserved filter paper containing captured eDNA was extracted using the ISOLATE II Genomic DNA Kit (Bioline Australia Pty Ltd, Alexandria NSW) following manufacturer's instructions. To remove ethanol before commencement of kit-based extraction, samples were spun down (30 min, $11,000 \times g$), decanted, and air dried in laminar flow fume hood (30-45 min). 180 μL Lysis Buffer GL and 25 μL proteinase K solution (20 mg/mL; New England Biolabs Inc., Ipswich, Massachusetts USA) were then added to each sample followed by brief vortex (10 sec), agitated incubation (56°C , ≥ 1 hr), pulse spin, addition of 200 μL Lysis Buffer G3, brief vortex (10 sec), agitated incubation (70°C , 10 min), addition of 210 μL ethanol (96-100%), and final pulse spin. Total sample volume (600 μL) was then added to a spin-column, spun down to bind eDNA to column (1 min, $11,000 \times g$), washed (500 μL Wash Buffer GW1 followed by 1

min at 11,000 × g), washed again (600 µL Wash Buffer GW2 followed by 1 min at 11,000 × g), and cleared of residual ethanol with final spin (1 min, 11,000 × g). 70 µL of preheated elution buffer (70°C) was added directly to each column, briefly incubated (5 min), and eluted into pre-labelled DNA-free tubes by centrifugation (2 min, 11,000 × g). Eluted eDNA was further purified with the OneStep™ PCR Inhibitor Removal Kit (Zymo Research Corp, Irvine, California USA) following manufacturer's instructions. Samples were stored at -20°C until quantitative real-time PCR (qPCR) detection of tilapia-specific eDNA.

2.4 Detection of tilapia eDNA by qPCR

Detection of tilapia-specific eDNA by quantitative real-time PCR (qPCR) involved two steps. The first step tested each sample for any detectable tilapia-specific eDNA using a qPCR assay designed to detect both *O. mossambicus* and *T. mariae* (Edmunds and Burrows, 2019a). The second step checked for any detectable tilapia eDNA contamination in corresponding equipment controls (ECs) for all samples that tested putatively positive for tilapia using the same tilapia-specific qPCR assay (Edmunds and Burrows, 2019a).

All extracted eDNA samples were diluted 1:2 with molecular grade water (MilliQ®; Thermo Fisher Scientific Australia Pty Ltd, Scoresby VIC) prior to qPCR analysis, which consisted of four technical replicates per sample. All qPCR assays utilised the same volume (10 µL), chemistry (5 µL of 2 x PowerUP SYBR Green Master Mix, Thermo Fisher Scientific, Scoresby VIC ; 0.5µL each primer (500 nM final), 1µL MilliQ® water, and 3 µL 1:2 diluted eDNA), and thermal cycling conditions (UDG incubation at 50°C for 2min, initial denaturation and activation at 95°C for 2 min then 50 cycles of 95°C for 15 secs and 60°C for 1 min then generation of dissociation curves by transitioning from 60°C to 95°C at 0.15°C•sec⁻¹).

All qPCR assays were run on a QuantStudio™ 3 Real-Time PCR System (Thermo Fisher Scientific Australia Pty Ltd, Scoresby VIC) in white 96 well plates sealed with optical films (Thermo Fisher Scientific Australia Pty Ltd, Scoresby VIC) and contained triplicated no template controls (NTCs). All plates were analysed using a common fluorescence threshold (0.2) in QuantStudio™ Design and Analysis Software (version 1.4.2; Thermo Fisher Scientific Australia Pty Ltd, Scoresby VIC) before export and subsequent analyses in Microsoft Excel (version 15.41).

A field site was considered to be putatively positive for tilapia detection if any of the twelve technical qPCR replicates for that site met the following criteria: 1) amplification curve crossed fluorescence threshold within 50 cycles, 2) dissociation temperature within 99.7% confidence interval of tilapia genomic DNA standards, and 3) corresponding equipment controls, field blanks, and extraction blanks did not show amplification. All putative positive detections were diluted 1:3 (10 µL qPCR assay into 20 µL MilliQ water) before sending to Australian Genome Research Facility (AGRF; Brisbane QLD) for purification and bidirectional Sanger sequencing for confirmation of tilapia-specific eDNA amplification.

3. Results

Water at all sites was flowing and contained visible suspended sediment (Figure 5), which caused filters to clog at different rates (Figure 6). Despite the use of larger filters (20 μm), the volume of water filtered through each replicate filter at each location ranged from 2–15 L due to site-specific suspended sediment levels.



Figure 5. Visible suspended sediment in the sampling sites.



Figure 6. 20 μm nylon net filters in 3-D-printed cartridges clogged with suspended sediment.

Based on the above assessment criteria Eureka Creek Crossing (Day 1 Site 5; Table 1) exhibited five putative positive detections (Table 2). These five detections contained 1–3 copies per assay ($C_t = 35.148 - 33.293$) of tilapia eDNA as extrapolated using artificial DNA standard curve (see Edmunds and Burrows, 2019a). Subsequent Sanger sequencing and NCBI BLAST searches confirmed that all five putative positive detections were positive for *T. mariae* eDNA given their $\geq 99.5\%$ nucleotide sequence match to GenBank accession [GQ168026](#). All equipment controls, field controls, and extraction blanks failed to amplify.

Table 2. Summary of tilapia eDNA detection and validation.

Sampling locations		Water samples collected			qPCR analysis			Validation				
Site	Name	Samples (n)	Putative positives (n)	Putative positives (%)	qPCR replicates (n)	Putative positives (n)	Putative positives (%)	Amplicons sequenced (n)	Confirmed positive (n)	Species detected	Nucleotide homology (%)	GenBank Reference Accession
1	DSITI "Walsh Purple"	3	0	0	12	0	0					
2	Collin's Weir (Walsh)	3	0	0	12	0	0					
3	Walsh Red Trib 1 and Walsh Red Up	3	0	0	12	0	0					
5	Eureka Creek Crossing	3	2	66.6	12	7	58.3	7	7	<i>T. mariae</i>	≥ 99.5	GQ168026
6	DSITI "Walsh Red Trib 2"	3	0	0	12	0	0					
8	DSITI "Walsh Red Down"	3	0	0	12	0	0					
9	DSITI "Walsh Yellow Trib 4"	3	0	0	12	0	0					
10	DSITI "Walsh Green"	3	0	0	12	0	0					
11	DSITI UW Footage Mitchell 2	3	0	0	12	0	0					
13	Wetlands / Pickford Rd. Mitchell	3	0	0	12	0	0					
14	Rifle Creek	3	0	0	12	0	0					
15	May river / Mitchell Purple Trib 4	3	0	0	12	0	0					
16	McLeod River/Mitchell Purple Trib 3	3	0	0	12	0	0					
17	Mitchell Purple	3	0	0	12	0	0					

4. Discussion

Eureka Creek, Walsh River, and Upper Mitchell were surveyed for the presence of eDNA from invasive tilapia species *O. mossambicus* and *T. mariae* using tilapia-specific qPCR assay. All equipment, field, and extraction control (“blank”) samples were verified to be devoid of tilapia eDNA by qPCR. All five putative positive detections for Eureka Creek Crossing (Day 1 Site 5) were verified positive for *T. mariae* eDNA by Sanger sequencing and NCBI BLAST searches.

4.1 Quality control

The lack of eDNA detection in all field, equipment, and extraction control samples confirms that no contamination occurred at any stage (e.g., preparation, field work, lab extractions, qPCR setup) and, thus, all qPCR assays are accurate reflections of collected site-specific tilapia eDNA.

4.2 Tilapia detections

Sanger sequencing verified that all five detections at Eureka Creek Crossing (Day 1 Site 5) were true positives for *T. mariae* despite the presence of low eDNA copies. Given the low number of *T. mariae* copies detected at Eureka Creek Crossing and that all other sites were expected to contain few (if any) tilapia, the lack of positive detections at other surveyed sites suggests that tilapia are either absent or present in numbers below current detection probability. For example, Robson, et al. (2015) demonstrated ~15% or 100% eDNA detection probability for 1 or 64 *O. mossambicus* after 4 or 2 days in 400,000 L static water, respectively, when 20 µm nylon net filters were used to screen 2L tank water filtrate. Given the high flow rate of seasonal Northern Queensland waterways, detectability of tilapia eDNA could be even lower for these lotic systems (Shogren, et al., 2017).

Reduced filtrate volume and larger membrane pore size used to overcome rapid clogging could have both contributed to a reduction in overall tilapia eDNA detectability and lack of positive detections at all sites except Eureka Creek Crossing because filter membranes > 10 µm capture less cell-bound eDNA than filter membranes ≤ 10 µm (Turner, et al., 2014).

5. Recommendations

In light of invasion fronts being made up of only a few individuals, tilapia-free waterways should be monitored regularly for tilapia eDNA in order to catch any increase in abundance over time. Future eDNA field surveys targeting low abundance species in tropical waterways with high suspended sediment should attempt pre-filtration to remove sediment $\geq 20 \mu\text{m}$ so that larger water volumes (e.g., $\geq 25 \text{ L}$) can be filtered through smaller pore sizes ($\leq 5 \mu\text{m}$). This could maximize capture of low abundance eDNA, which is critical for invasion front detection. Ongoing development of an effective method for pre-filtration in the field has proven challenging because tropical rivers often have suspended sediments that range from 4–16 μm in diameter (Bainbridge, et al., 2014).

One viable alternative to eDNA capture by filtration is capture by precipitation from intact water samples (reviewed by Thomsen and Willerslev, 2015; Goldberg, et al., 2016; Lear, et al., 2018). Despite the water volumes used for eDNA precipitation being smaller than eDNA filtration (e.g., 15mL vs 2-100L), eDNA precipitation retains inherent eDNA of all sizes (i.e., cell-free and cell-bound). For example, precipitation of intact water samples has been successfully employed for the detection of an elusive and invasive reptilian species in sub-tropical USA (Piaggio, et al., 2014) as well as ab invasive fish (*O. mossambicus*), toad (*Rhinella marina*), and aquatic plant (*Cabomba caroliniana*) in sub-tropical Australia (Edmunds and Burrows, 2019a; Edmunds and Burrows 2019b; and Edmunds and Burrows, 2019c).

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