Pilot Study - Investigating the presence of Giant Burrowing Frog *(Heleioporus australiacus)* using environmental DNA

Wednesday, 24 July 2024	
Project number:	ED_2312CR1
Client:	NSW Natural Resources Commission
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Assay(s):	Giant Burrowing Frog *(Heleioporus australiacus)*
Filter used:	1.2 µm manual disc filter

Highlights

- A targeted species qPCR assay was optimised and validated for Giant Burrowing Frog *(Heleioporus australiacus)*.
- eDNA sampling was undertaken on two occasions (first – pools disconnect, second – pools connected). Between 3 and 5 sites were sampled on each occasion, with 5 samples collected at each site.
- Giant Burrowing Frog *(Heleioporus australiacus)* eDNA was positively detected through qPCR at 4 out of 5 sites sampled during the first sampling event (pools disconnected) and 2 out of 3 sites sampled during the second sampling event (pools connected).
- Site occupancy modelling indicates that three water samples collected at each site or two qPCR technical replicates per sample will achieve a cumulative detection probability of >= 0.95 or a cumulative detection probability of >= 0.95, respectively.
- No contamination was detected in laboratory controls

Background

The Coastal Integrated Forestry Operations Approval (Coastal IFOA), sets out the rules for native timber harvesting in NSW coastal state forests and establishes environmental outcomes that must be achieved. As part of the Coastal IFOA, a monitoring program has been developed at multiple landscape scales by the Natural Resources Commission and includes supporting species management plans designed to manage and protect priority fauna and flora species. There are currently five species management plans supporting the Coastal IFOA, with the Giant Burrowing Frog *(Heleioporus australiacus)* species management plan being one of the five. Exploration of alternative survey methods was recommended as part of the annual review process for the species management plan.

Environmental DNA (eDNA) methods are being used routinely to monitor aquatic animals including fish, amphibians and mammals across waterways, estuaries and wetlands throughout Australian catchments. This method was proposed as a potential alternative to current survey methods for assessing the presence of the Giant burrowing frog, given it has shown to be more sensitive and cost effective for other species. Here we undertake qPCR targeted species assay optimisation for Giant Burrowing Frog *(Heleioporus australiacus)* and utilise it to screen eDNA samples collected from 7 waterway sites in known Giant Burrowing Frog habitat in Broadwater Forest, Eden NSW.

Methods

Sampling

Water samples were collected on two occasions, the first targeted normal conditions where *Heleioporus australiacus* habitat was disconnected pools and the second targeting higher flow conditions at the same or similar sites where the pools were connected and waterway flowing. These two occasions provided a drier and wetter hydrological condition scenario to determine any influence on eDNA detectability.

The first sampling occasions was from the 26th to 27th April 2023 and 25 water samples were collected from 5 sites by Forestry Corporation of NSW staff following sampling protocols developed by EnviroDNA. The second sample occasion was on 8th April 2024 and 25 water samples were collected from 3 waterway sites (2 sites revisited from first sampling occasion). Site location details are provided in Figure 3 and Figure 4 and in the spreadsheet accompanying this report (ED_2312CR1_NRC_GBF Pilot_eDNA Data_Combined.xlsx). At each site, 5 replicate samples were collected by passing up to 2,000 mL of water (mean = 958 mL) through a 1.2 μm manual disc filter. Filtration was undertaken on-site using Smith Root self-preserving filters to reduce DNA degradation during transport of water samples. Filters were stored out of sunlight and at ambient temperature before being transported to the laboratory for processing.

Assay optimisation – Giant Burrowing Frog

For the optimisation of the *Heleioporus australiacus* qPCR assay, 10 isolate sequences in NCBI (www.ncbi.nlm.nih.gov) were used, together with tissue samples from an East Gippsland, Victoria population provided to EnviroDNA by Snowline Ecology and 10 swab samples from *Heleioporus*

australiacus individuals in the Broadwater study area collected by Rohan Bilney (Forestry Corporation of NSW).

A real-time quantitative Polymerase Chain Reaction (qPCR) assay for *Heleioporus australiacus* was optimised by EnviroDNA targeting a portion of the ND4 gene. The assay comprised of forward primer 5'-3' "TGGCCGGTACCCTCCTTAAA", a hybridization probe 5'-3' "ACGCATACCTGCTCTCATTCA" and a reverse primer 5'-3' "GGGGTGGTTAGCGAATGCAG", and produced a product of 88 bp.

Serial dilutions of DNA were used to assess assay efficiency and the limit of detection (LOD), with the assay detecting 0.0001 ng/µl (or 1e-04) input of genomic DNA. We tested the specificity of the assay on normalised DNA extracted from tissue sample and swab samples provided from the target species and individuals for non-target species known from the study region. Species for off-target testing, which indicated no off-target species amplification, included *Crinia signifera, Litoria aurea, Limnodynastes tasmaniensis, Litoria peronii, Adelotus brevis, Litoria kroombitensis, Mixophyes fasciolatus, Limnodynastes peronii* and *Pseudophryne raveni*. The assay appears effective at detecting *Heleioporus australiacus* in the East Gippsland, Victoria and Eden, NSW region and likely throughout the species' southern range but may not be applicable for the populations north of Sydney, NSW. The assay should therefore be verified using tissue samples before using in any other known population areas.

Site Occupancy Modelling – Giant Burrowing Frog qPCR assay

We used site occupancy detection models fitted in a Bayesian framework with a logit link to investigate probabilities of eDNA capture (θ) and qPCR detection (p) using the *Heleioporus australiacus* assay developed. For this analysis, field samples from the same site were considered site replicates, whereas replicate qPCRs conducted on each sample were treated as technical replicates. This replication enabled us to estimate the probability of capturing eDNA in a sample (i.e., availability probability) as well as the probability of detecting eDNA when it is present via qPCR. We assumed multivariate standard normal distributions as priors for each model parameter. We ran models for 10,000 iterations, with 4,000 iterations discarded as burn-in. All analyses were conducted in R.

Posterior estimates of parameters of the site occupancy detection model enabled us to estimate how many samples would be required to be confident that a species was absent from a site if it was undetected, given estimated detection probabilities. To tackle this aim, we used the general formula $\theta^* = 1-(1-\theta)^k$, where θ is the estimated availability probability and k is the number of replicate samples collected at a site. Similarly, we estimated qPCR detection probabilities as $p^* = 1-(1-p)^k$, where p is the estimated (qPCR) detection probability.

Figure 1 below shows the distribution of the number of positive qPCR detections among samples. From this figure we can see that all three qPCR replicates were positive for twelve samples (10 samples in the first sampling occasion and two samples in the second) in which *Heleioporus australiacus* eDNA was detected. Only two samples over the two sampling occasions had a single eDNA detection.

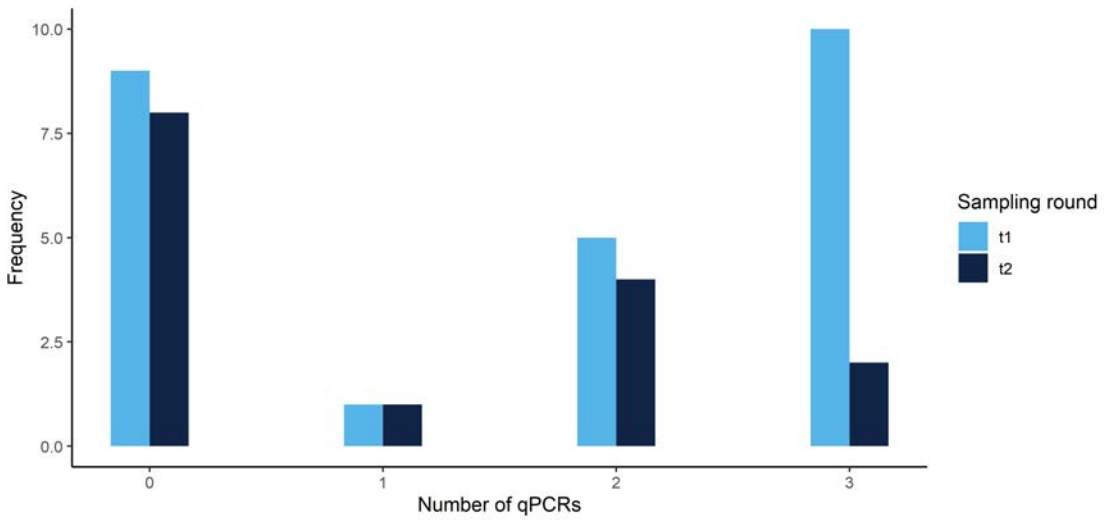

Figure 1. Histogram showing the number of positive qPCR detections per sample for each sampling round (t1 - pools disconnected = light blue, t2 – pools connected = dark blue), where 0 represents no positive qPCR replicates in the sample (e.g., negative sample) and 3 is a total of 3 positive qPCR replicates in the sample.

Figure 2 indicates that the cumulative probability of capturing *Heleioporus australiacus* eDNA in a water sample increases as the number of samples collected at a site also increases (A). This analysis suggests that, regardless of sampling round, 3 water samples collected at each site could achieve a cumulative availability probability >= 0.95. Similarly (B), two qPCR technical replicates per sample results in a cumulative detection probability >= 0.95. Because qPCR detection probability is largely a function of assay sensitivity and laboratory procedures, this parameter is assumed constant between sampling rounds (hence only a single curve is shown in B).

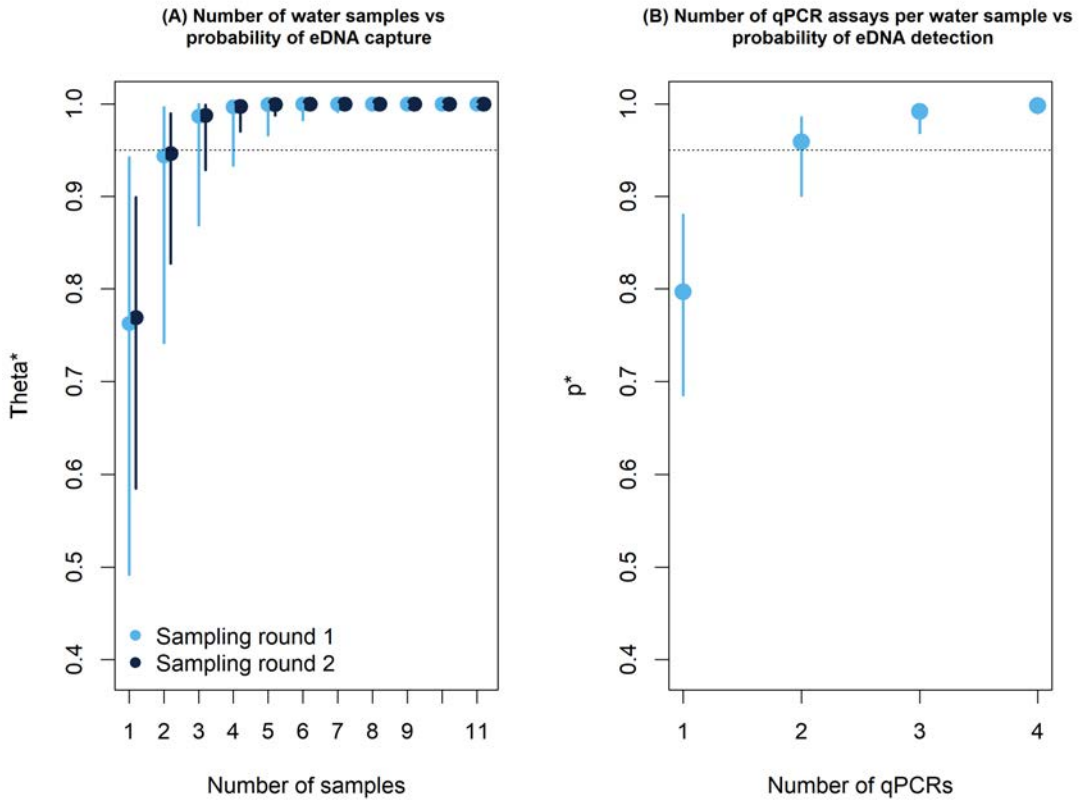

Figure 2: Probability of capturing eDNA in a sample (A) and probability of detecting eDNA via qPCR (B), as a function of the number of samples and qPCRs, respectively.

These analyses are derived from a limited number of sites (n=6), five of which were occupied by the target species, providing a somewhat limited ability to estimate model parameters. However, these findings do align with those of previous studies in suggesting that around two water samples may be sufficient to achieve a cumulative availability probability = ~0.95 (Lugg *et al.,* 2018; Tingley *et al.,* 2019).

Analysis – qPCR Giant Burrowing Frog

DNA was extracted from filters using a commercially available DNA extraction kit (Qiagen Power Soil Pro Kit). Real-time quantitative Polymerase Chain Reaction (qPCR) assays were used to amplify the target DNA, using a species-specific probe targeting a small region of the mitochondrial DNA from the target species *(Heleioporus australiacus).* Assays were performed in triplicate on each sample. Positive and negative controls were included for all assays.

Results

qPCR Giant Burrowing Frog

A total of 40 samples were analysed from 6 sites using a 1.2 μm manual disc filter. Raw data on per-sample detections and the qPCR results can be found in an accompanying spreadsheet (ED_2312CR1_NRC_GBF Pilot_eDNA Data_Combined.xlsx).

Out of the 6 sites (40 samples) that were analysed for the presence of *Heleioporus australiacus* eDNA, 5 sites returned at least 1 positive replicate sample (2 or 3 positive qPCRs in the sample), indicating the site is positive for the presence of *Heleioporus australiacus*. Positive sites included:

- Sampling occasion 1 – Site 4-2, Site 4-3, Site 4-8, Site 5-8 (Figure 3)
- Sampling occasion 2 – Site 4-12, Site 4-8 (Figure 4)

No *Heleioporus australiacus* DNA (0 positive qPCRs in samples) was detected at one site (Site 4-16) during both sampling occasions (Figure 2 and 3). No tadpoles or frogs were observed at these sites either, with the closest observed tadpoles being 100-400 m upstream.

Two and 3 positive qPCRs (out of 3 assays undertaken for each sample) strongly suggests that a sample is positive for the presence of the target species. Samples with 1 positive qPCR indicates lower levels of target DNA and are classified as a "possible detection". This can signal that the target species is present in low abundance or occupies a site infrequently. Alternatively, low levels of target DNA may arise from sample contamination through sampling or laboratory screening processes (minimised through strict protocols and negative controls), facilitated movement of DNA between waterbodies (e.g., water birds, recreational anglers, water transfers, predator scats), or eDNA dispersal from other sites (e.g., flow, floods). Equivocal results in multiple replicate samples from a site provides greater confidence of the presence of the target species. If greater confidence is required, further sampling is recommended at equivocal sites to confirm the presence of the target species.

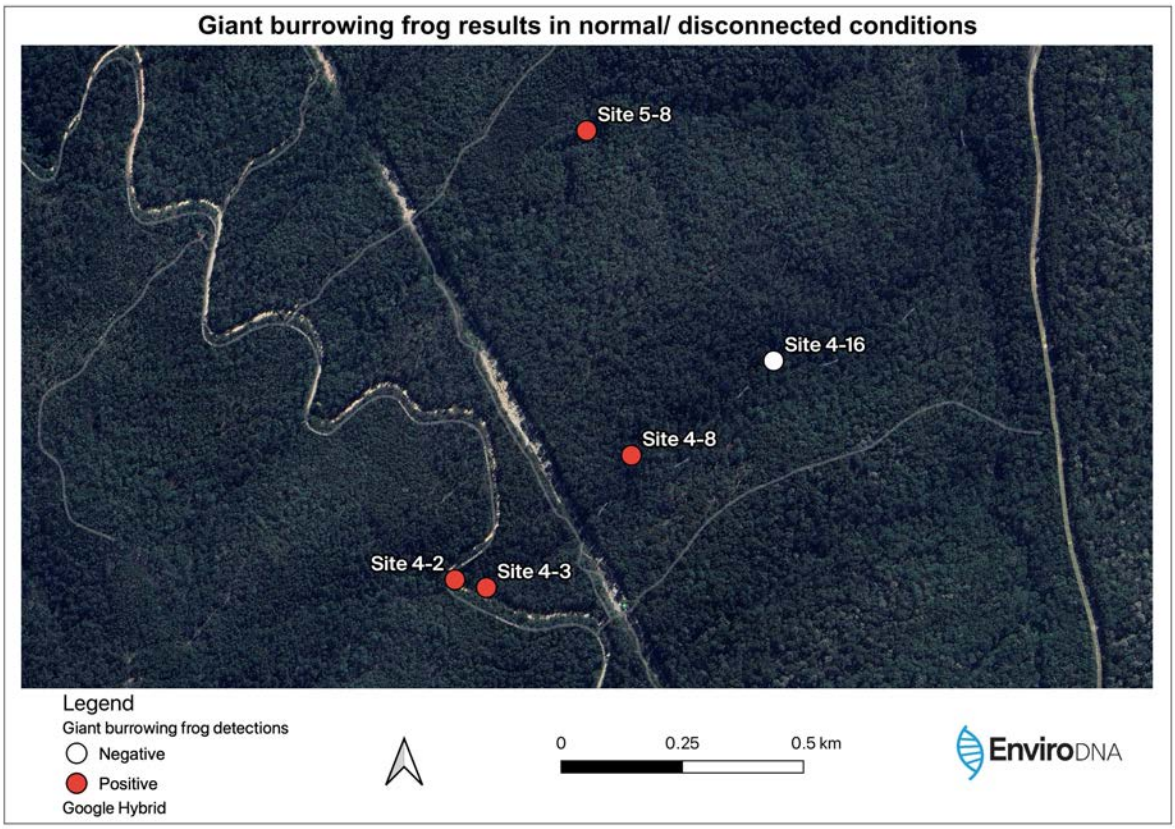

Figure 2: Sampling occasions one (normal/ disconnected) - *Heleioporus australiacus* eDNA detections

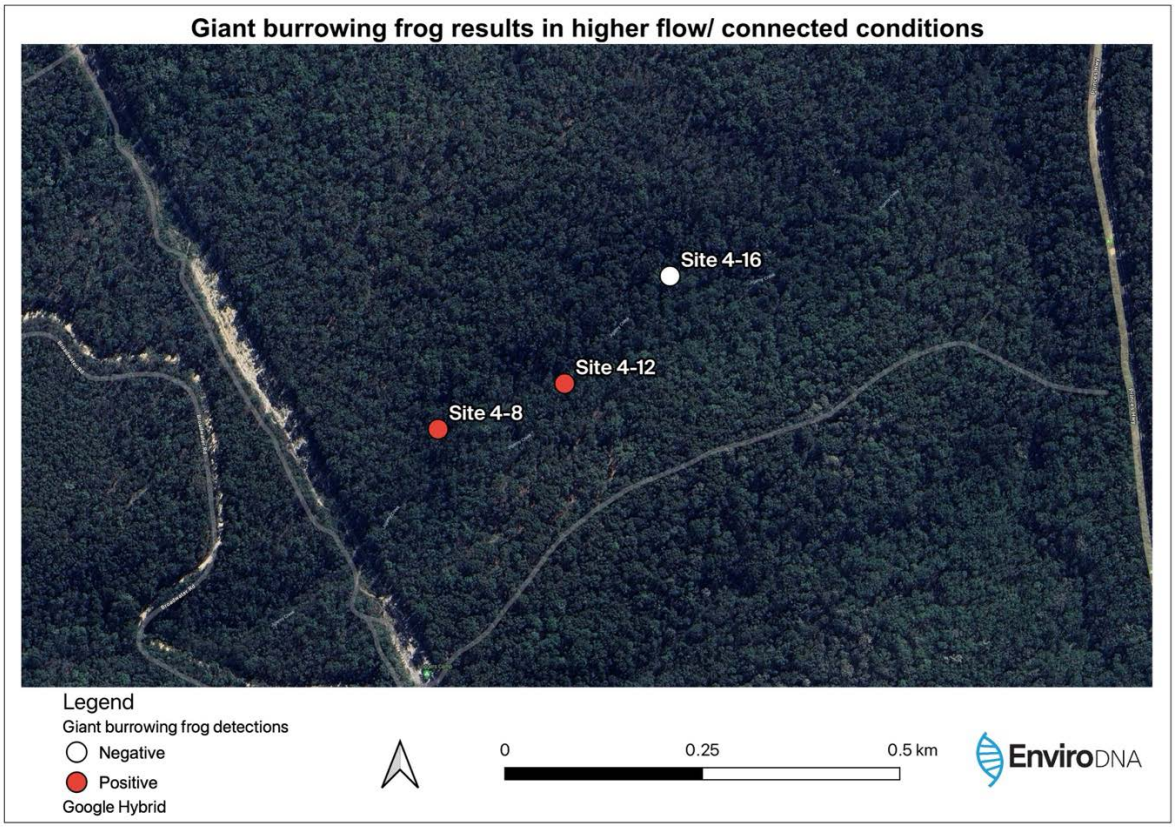

Figure 3: Sampling occasion two (flowing/ connected) - *Heleioporus australiacus* eDNA detections

Quality Assurance

The following provides a summary of the quality assurance undertaken during the qPCR analysis:

- Included in each assay plate were positive control reactions containing a range of *Heleioporus australiacus* DNA concentrations.
- Negative controls were included at all stages (DNA extraction, n = 1 negatives; sample handling, n = 1 negatives; qPCR, n = 1 negatives), so that contamination from laboratory processes could be identified if present.
- No contamination was detected in negative controls.

References

Lugg, W.H., Griffiths, J., van Rooyen, A.R., Weeks, A.R., and Tingley, R. (2018) Optimal survey designs for environmental DNA sampling. Methods in Ecology and Evolution 9(4), 1049-1059.

Tingley, R., Greenless, M., Oertel, S., van Rooyen, A.R. and Weeks, A.R. 2019. Environmental DNA sampling as a surveillance tool for cane toad *Rhinella marina* introductions on offshore islands. Biological Invasions. http://doi.org/10.1007/ s10530-018-1810-4

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EnviroDNA

Table 1. Sample metadata and qPCR assay results by sample - Dry/ Disconnected Pools conditions

									eDNA Results - Giant Burrowing Frog	
Site	**Replicate**	**Latitude**	**Longitude**	**Sample type**	**Notes**	**Collection date**	**Volume**	**Assays**	**Scoring (3 reps)**	**Result**
Site 4-16	1	37.001954	149.902668	Water	No tadpoles observed within 300m upstream - although visibility in some pools was poor	04/27/2023	750	Giant Burrowing Frog	0	Negative
Site 4-16	2	37.001954	149.902668	Water	No tadpoles observed within 300m upstream - although visibility in some pools was poor	04/27/2023	750	Giant Burrowing Frog	0	Negative
Site 4-16	3	37.001954	149.902668	Water	No tadpoles observed within 300m upstream - although visibility in some pools was poor	04/27/2023	750	Giant Burrowing Frog	0	Negative
Site 4-16	4	37.001954	149.902668	Water	No tadpoles observed within 300m upstream - although visibility in some pools was poor	04/27/2023	750	Giant Burrowing Frog	0	Negative
Site 4-16	5	37.001954	149.902668	Water	No tadpoles observed within 300m upstream - although visibility in some pools was poor	04/27/2023	750	Giant Burrowing Frog	0	Negative
Site 4-2	1	37.006	149.895277	Water	~30 tadpoles in pools surveyed	04/26/2023	2000	Giant Burrowing Frog	**3**	**Positive**
Site 4-2	2	37.006	149.895277	Water	~30 tadpoles in pools surveyed	04/26/2023	1000	Giant Burrowing Frog	**3**	**Positive**
Site 4-2	3	37.006	149.895277	Water	~30 tadpoles in pools surveyed	04/26/2023	2000	Giant Burrowing Frog	**3**	**Positive**
Site 4-2	4	37.006	149.895277	Water	~30 tadpoles in pools surveyed	04/26/2023	2000	Giant Burrowing Frog	**2**	**Positive**
Site 4-2	5	37.006	149.895277	Water	~30 tadpoles in pools surveyed	04/26/2023	1350	Giant Burrowing Frog	**2**	**Positive**
Site 4-3	1	37.006145	149.896006	Water	5 or more tadpoles in top Shallow pool (sample 1), 10+ in 3,4 and no visibility for 2 and 5.	04/26/2023	1500	Giant Burrowing Frog	**3**	**Positive**
Site 4-3	2	37.006145	149.896006	Water	5 or more tadpoles in top Shallow pool (sample 1), 10+ in 3,4 and no visibility for 2 and 5.	04/26/2023	1500	Giant Burrowing Frog	**2**	**Positive**
Site 4-3	3	37.006145	149.896006	Water	5 or more tadpoles in top Shallow pool (sample 1), 10+ in 3,4 and no visibility for 2 and 5.	04/26/2023	1500	Giant Burrowing Frog	**3**	**Positive**
Site 4-3	4	37.006145	149.896006	Water	5 or more tadpoles in top Shallow pool (sample 1), 10+ in 3,4 and no visibility for 2 and 5.	04/26/2023	1500	Giant Burrowing Frog	**3**	**Positive**
Site 4-3	5	37.006145	149.896006	Water	5 or more tadpoles in top Shallow pool (sample 1), 10+ in 3,4 and no visibility for 2 and 5.	04/26/2023	1500	Giant Burrowing Frog	**3**	**Positive**
Site 4-8	1	37.003693	149.899369	Water	No tadpoles seen. Creek did dissapear between upstream but was flowing throughout the site	04/27/2023	750	Giant Burrowing Frog	**3**	**Positive**
Site 4-8	2	37.003693	149.899369	Water	No tadpoles seen. Creek did dissapear between upstream but was flowing throughout the site	04/27/2023	750	Giant Burrowing Frog	0	Negative
Site 4-8	3	37.003693	149.899369	Water	No tadpoles seen. Creek did dissapear between upstream but was flowing throughout the site	04/27/2023	750	Giant Burrowing Frog	**2**	**Positive**
Site 4-8	4	37.003693	149.899369	Water	No tadpoles seen. Creek did dissapear between upstream but was flowing throughout the site	04/27/2023	750	Giant Burrowing Frog	0	Negative
Site 4-8	5	37.003693	149.899369	Water	No tadpoles seen. Creek did dissapear between upstream but was flowing throughout the site	04/27/2023	750	Giant Burrowing Frog	0	Negative
Site 5-8	1	36.997688	149.89833	Water	2 tapoles in pool above, 1 tadpole in pool with sample 1, none in subsequent pools sampled	04/27/2023	750	Giant Burrowing Frog	**3**	**Positive**
Site 5-8	2	36.997688	149.89833	Water	2 tapoles in pool above, 1 tadpole in pool with sample 1, none in subsequent pools sampled	04/27/2023	750	Giant Burrowing Frog	**3**	**Positive**
Site 5-8	3	36.997688	149.89833	Water	2 tapoles in pool above, 1 tadpole in pool with sample 1, none in subsequent pools sampled	04/27/2023	750	Giant Burrowing Frog	**1**	**Equivocal**
Site 5-8	4	36.997688	149.89833	Water	2 tapoles in pool above, 1 tadpole in pool with sample 1, none in subsequent pools sampled	04/27/2023	750	Giant Burrowing Frog	**2**	**Positive**
Site 5-8	5	36.997688	149.89833	Water	2 tapoles in pool above, 1 tadpole in pool with sample 1, none in subsequent pools sampled	04/27/2023	750	Giant Burrowing Frog	0	Negative

Table 2. Sample metadata and qPCR assay results by sample - Wet/ Connected/ Flowing conditions

									eDNA Results - Giant Burrowing Frog	
Site	**Replicate**	**Latitude**	**Longitude**	**Sample type**	**Notes**	**Collection date**	**Volume**	**Assays**	**Scoring (3 reps)**	**Result**
Site 4-12	1	-37.00317516	149.9011696	Water	Closest observed tadpoles were ~100m upstream (~10 seen). Creek was flowing lightly.	8/04/2024	750	Giant burrowing frog	0	Negative
Site 4-12	2	-37.00317516	149.9011696	Water	Closest observed tadpoles were ~100m upstream (~10 seen). Creek was flowing lightly.	8/04/2024	750	Giant burrowing frog	0	Negative
Site 4-12	3	-37.00317516	149.9011696	Water	Closest observed tadpoles were ~100m upstream (~10 seen). Creek was flowing lightly.	8/04/2024	750	Giant burrowing frog	**2**	**Positive**
Site 4-12	4	-37.00317516	149.9011696	Water	Closest observed tadpoles were ~100m upstream (~10 seen). Creek was flowing lightly.	8/04/2024	750	Giant burrowing frog	**1**	**Equivocal**
Site 4-12	5	-37.00317516	149.9011696	Water	Closest observed tadpoles were ~100m upstream (~10 seen). Creek was flowing lightly.	8/04/2024	750	Giant burrowing frog	0	Negative
Site 4-16	1	-37.001954	149.902668	Water	Closest tadpoles observed were ~300m upstream	8/04/2024	750	Giant burrowing frog	0	Negative
Site 4-16	2	-37.001954	149.902668	Water	Closest tadpoles observed were ~300m upstream	8/04/2024	750	Giant burrowing frog	0	Negative
Site 4-16	3	-37.001954	149.902668	Water	Closest tadpoles observed were ~300m upstream	8/04/2024	750	Giant burrowing frog	0	Negative
Site 4-16	4	-37.001954	149.902668	Water	Closest tadpoles observed were ~300m upstream	8/04/2024	750	Giant burrowing frog	0	Negative
Site 4-16	5	-37.001954	149.902668	Water	Closest tadpoles observed were ~300m upstream	8/04/2024	750	Giant burrowing frog	0	Negative
Site 4-8	1	-37.003693	149.899369	Water	Numerous tadpoles seen in the pool. Creek was flowing lightly.	8/04/2024	750	Giant burrowing frog	**3**	**Positive**
Site 4-8	2	-37.003693	149.899369	Water	Numerous tadpoles seen in the pool. Creek was flowing lightly.	8/04/2024	750	Giant burrowing frog	**2**	**Positive**
Site 4-8	3	-37.003693	149.899369	Water	Numerous tadpoles seen in the pool. Creek was flowing lightly.	8/04/2024	750	Giant burrowing frog	**3**	**Positive**
Site 4-8	4	-37.003693	149.899369	Water	Numerous tadpoles seen in the pool. Creek was flowing lightly.	8/04/2024	750	Giant burrowing frog	**2**	**Positive**
Site 4-8	5	-37.003693	149.899369	Water	Numerous tadpoles seen in the pool. Creek was flowing lightly.	8/04/2024	750	Giant burrowing frog	**2**	**Positive**