Author/s:
Tingley, R;Coleman, R;Gecse, N;van Rooyen, A;R. Weeks, A

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Accounting for false positive detections in occupancy studies based on environmental DNA: A case study of a threatened freshwater fish (*Galaxiella pusilla*)

Reid Tingley[1] | **Rhys Coleman**[2] | **Nathaniel Gecse**[3] | **Anthony van Rooyen**[4] | **Andrew R. Weeks**[3,4]

[1]School of Biological Sciences, Monash University, Clayton, VIC., Australia

[2]Melbourne Water, Docklands, VIC., Australia

[3]School of BioSciences, University of Melbourne, Parkville, VIC., Australia

[4]EnviroDNA Pty Ltd, Parkville, VIC., Australia

Correspondence
Reid Tingley, School of Biological Sciences, Monash University, Clayton, Victoria 3800, Australia.
Email: reid.tingley@monash.edu

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Abstract

Environmental DNA (eDNA) sampling is a promising method for surveying aquatic fauna. Recent eDNA studies have investigated the likelihood of false negative errors in the laboratory and in the field, but the likelihood of false positives remains poorly studied. We investigated the likelihood of both types of errors in eDNA surveys of an Australian threatened freshwater fish (*Galaxiella pusilla*) using laboratory experiments, field surveys, and recent advances in hierarchical site occupancy-detection modeling. Laboratory experiments revealed high primer/probe specificity; absence of sample contamination in extraction and qPCR blanks; and rapid accumulation and deterioration of eDNA in aquaria. Hierarchical site occupancy-detection models fitted to pilot data collected at 13 wetlands revealed that two water samples, each with two qPCRs, would be required to achieve a cumulative detection probability >.95. A more comprehensive survey, in which we simultaneously used dip netting and eDNA sampling at 29 wetlands, revealed similar mean detection probabilities of the two sampling methods (trapping: 0.74 vs. eDNA: 0.68), and low probabilities of false positive errors at the water sample level (0.0080) and at the qPCR level (0.0039) for eDNA sampling. Collectively, our results illustrate that eDNA sampling can be a sensitive and specific method for monitoring the occurrence of freshwater fauna. Detection probabilities of eDNA sampling were comparable to those of a traditional sampling method, and probabilities of laboratory-induced false positives were low. Future studies employing eDNA sampling should estimate, and properly account for, false positive errors in addition to false negatives.

KEYWORDS

detection, eDNA, environmental DNA, false negative, false positive, occupancy

1 | INTRODUCTION

Understanding spatial and temporal patterns of species occurrence is critical for designing and implementing effective conservation strategies. Many types of occupancy surveys are, however, prone to both false negative and false positive errors. The potential consequences of false negative errors for threatened species monitoring programs are widely appreciated and have been the focus of much research (Bailey, Hines, Nichols, & MacKenzie, 2007; Mackenzie et al., 2002; Mackenzie & Royle, 2005), yet false positive errors have received far less attention. Such errors potentially apply to a wide array of monitoring methods, including volunteer-based surveys (e.g., citizen science), aural surveys, interviews with local experts, and computer algorithms that detect species from recordings. Importantly, false positive errors can induce severe biases in estimates of occupancy, colonization, and extinction, leading to inappropriate conservation actions (Chambert, Miller, & Nichols, 2015; Miller et al., 2015).

Environmental DNA (eDNA) sampling—the detection of nuclear or mitochondrial DNA from environmental samples—is an emerging monitoring method that can be prone to false positive errors (Cristescu & Hebert, 2018; Ficetola et al., 2015; Lahoz-Monfort, Guillera-Arroita, & Tingley, 2016). For example, surveyors or laboratory technicians can contaminate water samples, DNA extractions or PCR wells with a target species' DNA, or lack of marker/primer specificity can lead to species misidentification. Widespread application of eDNA sampling in threatened species monitoring programs therefore requires that we identify the likelihood and potential sources of false positive errors, as well as the implications of such errors for estimates of site occupancy under natural conditions.

Site occupancy-detection models, which are capable of jointly estimating the probability of species occurrence and species detectability (conditional on occurrence), have been richly developed and widely applied to account for false negative errors in threatened species monitoring programs (Bailey et al., 2007; Mackenzie & Royle, 2005). Extensions to this modeling framework that account for false positive detections have also been developed (Chambert et al., 2015; Royle & Link, 2006), but have been infrequently applied in eDNA studies (Chen & Ficetola, 2019; Ficetola et al., 2015; Lahoz-Monfort et al., 2016). Until recently (Guillera-Arroita, Lahoz-Monfort, van Rooyen, Weeks, & Tingley, 2017), a lack of suitable analytical methods to account for false positive detections that also accommodate the hierarchical nature of eDNA samples (in which multiple PCR replicates are nested within multiple environmental samples) has hindered our ability to account for false positives in field-based eDNA surveys.

In this paper, we take an integrative approach to investigate the likelihood of false negative and false positive detections with eDNA sampling, combining recent advances in hierarchical site occupancy-detection modeling (Guillera-Arroita et al., 2017) with field surveys and laboratory experiments. We apply this approach to a south-eastern Australian threatened freshwater fish, the dwarf galaxias (*Galaxiella pusilla*). Specifically, we examine whether false negative and false positive detections could compromise the integrity of eDNA sampling by assessing: (a) primer/probe specificity against closely related species; (b) rates of eDNA accumulation and degradation; and (c) the relative sensitivity of eDNA versus a traditional sampling method (dip netting) under natural field conditions, after accounting for false positive detections.

2 | METHODS

2.1 | Study species

Galaxiella pusilla is a small (adults usually 30–40 mm in length) freshwater fish that typically occurs in shallow habitats (<1 m deep), with still or slow-flowing water and dense cover of submerged and emergent aquatic vegetation such as shallow lakes, wetlands, swamps, billabongs, and small streams (Coleman, Hoffmann, & Raadik, 2015). A common feature of their natural habitats is dynamic wetting and drying cycles, where water levels often substantially contract and expand during dry periods and wet periods, respectively. Frequent fluctuations in the abundances of *G. pusilla* within sites, associated with pronounced wetting and drying regimes, dispersal in floods, short lifespan, and semelparity, indicate an ongoing process of local extinction and recolonization and a strong metapopulation structure (Coleman, Raadik, Pettigrove, & Hoffmann, 2017). *Galaxiella pusilla* is formally recognized as a threatened species at multiple jurisdictional levels across its range, including a conservation status of "endangered" on the IUCN Red List of Threatened Species, with major threats being habitat loss and fragmentation, changes in flow regimes, and invasive species (Coleman, Raadik, & Freeman, R., 2019).

2.2 | Real-time Taqman quantitative PCR assay for G. pusilla

Species-specific primers and a TaqMan® (Life Technologies Australia, Mulgrave, Victoria, Australia) minor groove binding (MGB) probe were developed to target an 82 bp fragment of the mitochondrial cytochrome oxidase subunit 1 (CO1) gene in *G. pusilla* based on sequences from Coleman, Weeks, & Hoffmann, (2013) (Genbank accessions KC282473–KC282620). Forward and reverse primers for *G. pusilla* were 5'– CCCCTCTTTTCTTCTTCTACTTGCA –3' and 5'–TGCTAAAGGCGGGTAAACTGT –3', respectively. The MGB probe (labeled with FAM_{TM}) was 5'– CCCGGCCTCTACGCCAG –3'.

Real-time TaqMan® quantitative PCR (qPCR) assays were conducted using a Roche LightCycler 480 system (Roche Diagnostics Australia, Castle Hill, New South Wales, Australia) in a 384-well format. 10 µl reactions containing 5 µl of 2× Qiagen multiplex PCR Master Mix (Qiagen), 0.5 µl 20 × TaqMan® Gene Expression Assay (final primer and reporter concentration 0.9 µM and 0.25 µM, respectively) (Life Technologies Australia), 2.5 µl RNase-free water (Qiagen), and 2 µl of DNA were prepared in triplicate and included a TaqMan® exogenous internal positive control probe (VIC® labeled) (Life Technologies) to test for the presence of inhibition. Included in each 96-well assay plate

were control reactions containing DNA from 10, 100, 1,000, 10,000, and 100,000 copies of a synthetic oligonucleotide (Intergraded DNA technologies, Baulkham Hills, New South Wales, Australia) and controls with no DNA template. The efficiency of the quantitative PCR was >95% for all the *G. pusilla* assays. The amplification conditions of 15 min at 95°C, followed by 15 s at 95°C and 1 min at 60°C for 50 cycles, were used. The amplification profiles of each PCR were used to determine the crossing point (Cp) value using the Absolute Quantification module of the Roche LightCycler® 480 software package. A standard curve was constructed from the control reactions and the concentration determined for the test samples expressed as the total copies of DNA per water sample.

All extractions and qPCR analyses were undertaken in a room that is dedicated to low-quantity DNA sources, with qPCR setup undertaken in a laminar flow hood. No DNA from *G. pusilla* had been handled in the room previously. Positive controls and standards were added immediately prior to placing in the Roche LightCycler 480 (separate room). Negative controls were included at all stages (DNA extraction, qPCR) so that laboratory contamination could be identified if present.

2.3 | Assay specificity

We first determined that the primers and probe were unique to *G. pusilla* based on NCBI blast searches (Blast.ncbi.nlm.nih.gov, 2016). To further assess the specificity of the assay, we obtained tissue samples (fin clips) and extracted DNA (using the DNeasy Blood & Tissue Kit, Qiagen) from other fish species known to occur within or near our study area east of Melbourne, Victoria, Australia. Samples were diluted to a similar DNA concentration (10 ng/μl) before testing. The following 25 nontarget species were tested with our *G. pusilla* assay; *Galaxiella toourtkoourt*, *Gambusia holbrooki*, *Nannoperca australis*, *Neochanna cleaveri*, *Philypnodon grandiceps*, *Galaxias maculatus*, *Galaxias truttaceus*, *Retropinna semoni*, *Anguilla australis*, *Cyprinus carpio*, *Carassius auratus*, *Tinca tinca*, *Prototroctes maraena*, *Pseudaphritis urvillii*, *Galaxias brevipinnis*, *Galaxias ornatus*, *Gadopsis marmoratus*, *Geotria australis*, *Mordacia mordax*, *Galaxias fuscus*, *Galaxias olidus*, *Oncorhynchus mykiss*, *Salmo trutta*, *Nannoperca obscura*, *Macquaria novemaculeata*, and *Macquaria colonorum*.

2.4 | Rates of eDNA accumulation and degradation

Understanding the rate at which eDNA accumulates and degrades is an important consideration in the context of false negative and false positive detections. If eDNA accumulates slowly in the environment, then it may be an ineffective tool to detect low abundance populations or recent colonizations, leading to false negatives. Additionally, if eDNA degrades slowly in the environment, then it may be detected despite the absence of a viable population, leading to false positives (Darling & Mahon, 2011). Accumulation and degradation studies have been undertaken on many different freshwater fish species (Barnes et al., 2014; Lance et al., 2017; Seymour et al., 2018; Tsuji, Ushio, Sakurai, Minamoto, & Yamanaka, 2017). Thus, we conducted a small laboratory experiment to determine whether rates of *G. pusilla* eDNA accumulation and degradation in aquaria were comparable with those of other species (see Appendix S1).

2.5 | Field studies

Pilot study: In May 2014, we conducted a pilot study using the eDNA assay outlined above at 13 sites in the greater Melbourne (Victoria, Australia) region to determine whether we could detect *G. pusilla* in the wild. Four of those sites were controls, situated in catchments in which *G. pusilla* had never been observed. Based on expert opinion and multiple dip-netting surveys over multiple years, population size estimates for each of the remaining nine study sites were broadly defined as "low," "low–moderate," "moderate," "moderate–high," or "high." Intermediate categories (i.e., "low–moderate" and "moderate–high") were those in which population sizes fluctuated between categories in different years. Importantly, these estimates were made by one of the authors (RC) prior to sampling.

We collected three water samples and filtered each separately using Sterivex-GP 0.22μm filter units with a Polyethersulfone membrane (Merck Millipore, Bayswater Victoria, Australia) attached to a 60 ml luer lock syringe (Livingstone International, Sydney, New South Wales, Australia) during a single visit to each site. Sterile disposable equipment was used for each sample. This included latex gloves, and separate filter units and syringes. We collected 300–500 ml samples by passing consecutive 50 ml samples through the filter. Excess water was removed by repeatedly passing 50 ml of air through the filter unit. We then placed the filter unit back into its packaging, which was then taped over and sealed in a plastic zip-lock bag and placed at 4°C within a dark closed portable cooler until processing (<48 hr).

DNA was extracted from the Sterivex® filters using the Qiagen DNeasy Blood & Tissue Kit. 540 μl ATL buffer (Qiagen) and 40 μl of proteinase *K* (Qiagen) were added to each filter unit. The units were then sealed and incubated at 56°C for 3 hr with constant agitation. Following incubation, 500 μl of the lysis solution was transferred into 2 ml eppendorf tubes. Thereafter, the same procedure was followed as above using the Qiagen DNeasy Blood & Tissue Kit (manufacturers protocol) with the following minor volume adjustments: 500 μl AL buffer, 500 μl ethanol, and final elution in 50 μl AE buffer. Three replicate qPCR assays were undertaken on each water sample. All other details regarding eDNA sample processing (e.g., Taqman PCR assay and primer) were identical to those outlined above for the laboratory experiments.

2.5.1 | Field experiment

After obtaining promising results from our pilot study, we decided to undertake a more comprehensive survey encompassing a greater

number of sites; more eDNA samples at each site; and a direct comparison with dip netting. Between 29 March and 21 April 2016, we attempted to survey 33 sites east of Melbourne with dip nets and eDNA (Figure 1); however, four sites did not contain any water at the time of sampling. One of us (RC) selected equal numbers of sites at which the species was presumed to be present and absent based on knowledge of the presence or absence of *G. pusilla* from multiple long-term surveys. Seven of the 29 sites that contained water were the same as those sampled in the 2014 pilot study. Sites were representative of the semi-urbanized study area and consisted of floodplains, rivers, streams, creeks, wetlands, and roadside drains. The field surveyor (NG) was kept blind to the occurrence of *G. pusilla* at each site, until after field sampling and eDNA analyses were completed. The field surveyor was also kept naive to expectations of where *G. pusilla* were likely to occur (based on habitat and distribution). Each site was visited twice, with approximately two weeks between site visits. Rainfall occurred over the survey period; however, site connectivity did not change between site visits.

A double-blind assessment was undertaken for water samples; water samples were coded in the field, and the person who collected the samples (NG) was different from the person who processed these samples in the laboratory (AvR). Protocols for water sample collection were identical to those used in the 2014 pilot study.

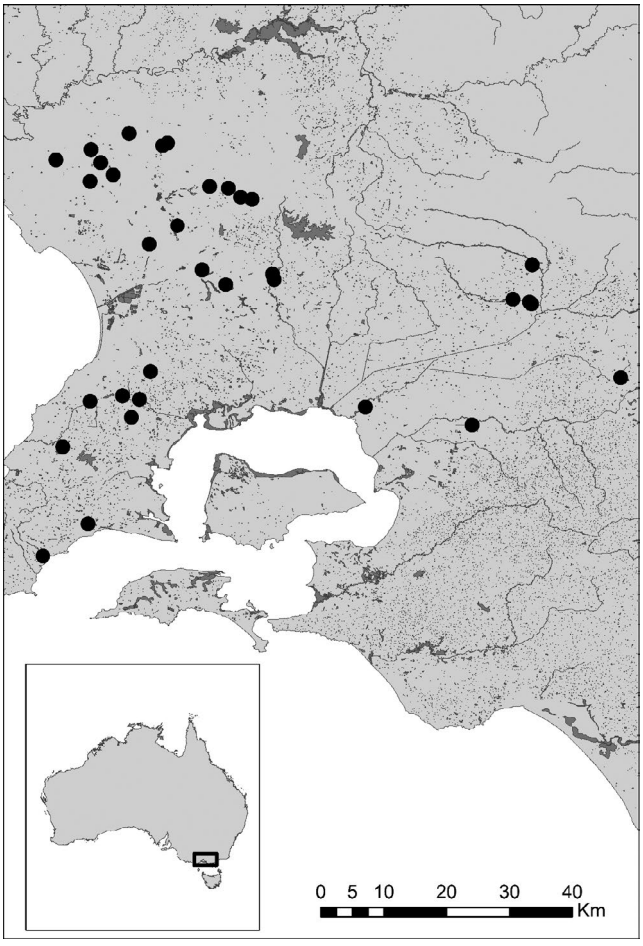

FIGURE 1 Study sites in Victoria, Australia, surveyed in 2016

Four qPCR assays were conducted on each water sample. Following eDNA sampling, *G. pusilla* were sampled for 30 min at each site using a dip net. All equipment that came in contact with the water body was sprayed with a 10% bleach solution, rinsed with sterile water, and dried thoroughly between sites.

2.6 | Statistical modeling

We used a recently developed hierarchical site occupancy-detection modeling framework (Guillera-Arroita et al., 2017) to estimate the probability of species occupancy, while simultaneously estimating the probability of false negative and false positive detections. We assume that the probability that a target species occupies a site is defined by ψ; the probability of its absence is therefore $1 - \psi$. If present at a given site, the probability of a species' eDNA being captured in a water sample during a survey occasion is θ_{11}. However, we also need to consider the reverse possibility; that sample contamination could result in eDNA being present in a water sample when it is in fact absent, with probability θ_{10}. Furthermore, we need to define the probabilities of true and false positive detection at the qPCR level. When a species' eDNA is present within a water sample, the probability of a qPCR run detecting that eDNA is p_{11}. Conversely, false positive detections may occur when a species' eDNA is not available for detection with probability p_{10}. A hierarchical representation of these processes is as follows (Guillera-Arroita et al., 2017):

for i in 1:S {
  $z_1[i] \sim \text{Bernoulli}(\psi)$
  for j in 1: $V[i]$ {
    $z_2[i, j] \sim \text{Bernoulli}(z_1[i] * \theta_{11} + (1 - z_1[i]) * \theta_{10})$
    $d[i, j] \sim \text{Binomial}(L[i, j], z_2[i, j] * p_{11} + (1 - z_2[i, j]) * p_{10})$
  }
}

where S represents the number of sites surveyed; V_i is the number of water samples collected at site i; L_{ij} is the number of qPCRs run on water sample j from site i; and d_{ij} is the number of qPCR detections per site and water sample. Here, z_1 indicates a latent (unobserved) variable that defines whether the species occupies a site S, and z_2 indicates the latent occurrence of eDNA within a sample. If the species is present at the site ($z_1 = 1$), z_2 takes value 1 with probability θ_{11}. If, however, the species is absent ($z_1 = 0$), then z_2 takes value 1 with probability θ_{10}. Similarly, detections d are generated by one of two mutually exclusive paths, depending on the state of z_2.

Guillera-Arroita et al. (2017) showed that, without additional information that informs some of these parameters, this model is unidentifiable (multiple solutions exist). One way to help overcome this lack of identifiability is to use an additional survey method that yields unambiguous detections (i.e., results in no false positive detections). This informs the most appropriate value of the parameter ψ, but does not resolve identifiability with respect to θ_{11} versus θ_{10}, nor p_{11} versus p_{10}. One solution to further resolve the identifiability issue is to conduct calibration experiments, for example, running

blank water samples through the same filtration, extraction, and qPCR process as field samples.

Including unambiguous detections and calibration experiments in the model described above results in the following hierarchical model (Gurutzeta Guillera-Arroita et al., 2017):

for i in 1: S{
z_1[i]~Bernoulli(ψ)
for j in 1: $V[i]$ {
$z_2[i, j]$~Bernoulli($z_1[i] * \theta_{11} + (1 - z_1[i]) * \theta_{10}$)
$d[i, j]$~Binomial($L[i, j], z_2[i, j] * p_{11} + (1 - z_2[i, j]) * p_{10}$)
}
$\delta[i]$~Binomial($K[i], z_1[i] * r_{11}$)
}
for j in 1: $V^{[c1]}$ {
$z_2^{[c1]}[j]$~Bernoulli(θ_{10})
$d^{[c1]}[j]$~Binomial($L^{[c1]}[j], z_2^{[c1]}[j] * p_{11} + (1 - z_2^{[c1]}[j]) * p_{10}$)
}
$d^{[c2]}$~Binomial($L^{[c2]}, p_{10}$)

where, in addition to the parameters defined above, K_i and δ_i are the number of dip-netting replicates and positive detections at site i, respectively; r_{11} is the probability of detecting the species using dip netting on a single survey occasion; $V^{[c1]}$ is the number of blank water samples processed in the calibration experiment at the water sample level; $L^{[c1]}$ and $d^{[c1]}$ are, respectively, the number of qPCRs and positive detections in blank water sample j; and $L^{[c2]}$ and $d^{[c2]}$ are, respectively, the number of qPCR blanks and detections in the calibration experiment at the qPCR level. Note that the model of Nichols et al. (2008), which was first applied to eDNA data by Schmidt, Kéry, Ursenbacher, Hyman, and Collins (2013) and has since been applied widely (Hunter et al., 2015; Lugg, Griffiths, van Rooyen, Weeks, & Tingley, 2017; Strickland & Roberts, 2019), considers false negative detections at both the water sample level and the qPCR level, but assumes no false positive detections are present.

At the time of our 2014 pilot study, we were primarily interested in the sensitivity of our eDNA assay and therefore conducted limited calibration experiments. Specifically, we filtered and extracted a single distilled water sample, following the same procedure as field samples. Three qPCR assays were then conducted on that sample. In addition, we pipetted distilled water into two wells of a 96-well plate to assess potential qPCR contamination. Neither the extraction blank nor the two qPCR blanks amplified the species' DNA. Because of the limited number of calibration trials conducted, and because we did not have unambiguous survey data from the time of eDNA sampling, we fixed parameter values of θ_{10}, p_{10}, and r_{11} equal to 0 in our analysis of the pilot data, making our model equivalent to that of Nichols et al. (2008).

In our more comprehensive 2016 case study, we conducted a greater number of calibration experiments focusing on DNA filtration/extraction and qPCR. For filtration/extraction blanks, eight distilled water samples were filtered and extracted, following the same procedure as field samples. Four qPCRs were then run on each blank sample; none of these amplified the species' DNA. This rate was used as calibration data in the model, indirectly informing θ_{10}, p_{11}, and p_{10}. Twelve qPCR blanks were run to control for potential qPCR contamination, with none testing positive for the species' DNA. This rate was used as calibration data for p_{10} in the model. Dip netting was treated as an unambiguous survey method.

We used profile log-likelihoods for parameter estimation (see Griffin, Matechou, Buxton, Bormpoudakis, & Griffiths, 2020 for a Bayesian implementation). Profiles were constructed in steps of 0.001, and at each step, we ran the optimization procedure 25 times with different starting values (normally distributed around 0.5 with a standard deviation = 2, on the logit scale), ensuring the true maximum in the function was identified (Guillera-Arroita et al., 2017). We estimated conditional cumulative availability (θ^*_{11}) and qPCR detection probabilities (p^*_{11}) for eDNA sampling, and cumulative detection probabilities for dip netting (r^*_{11}), using mean estimates of dip-netting detection probability (r_{11}), eDNA availability probability (θ), and qPCR detection probability (p), respectively:

$$\theta^*_{11} = 1 - (1 - \theta_{11})^V$$

$$p^*_{11} = 1 - (1 - p_{11})^L$$

$$r^*_{11} = 1 - (1 - r_{11})^K$$

where V is the number of water samples collected; L is the number of qPCRs run on each water sample; and K is the number of dip-netting replicates. We use mean estimates of model parameters to estimate the number of water samples or dip-netting replicates required to achieve availability/detection probabilities >.95; depending on the goals of the study and the level of certainty required, studies may wish to ensure the lower limit of the confidence interval of availability and/or detection probability is >.95.

For our 2016 survey data, we used mean values of the occupancy parameter estimate and of the four eDNA detection parameters (ignoring the dip-netting data by setting K_i and $\delta_i = 0$) to compute the probability of *G. pusilla* presence at each of the 29 surveyed sites, conditional on the observed eDNA data:

$$\frac{\psi_i \left\{ \prod_{j=1}^{V_i} \left(\theta_{11} p_{11}^{d_{ij}} (1-p_{11})^{L_{ij}-d_{ij}} + (1-\theta_{11}) p_{10}^{d_{ij}} (1-p_{10})^{L_{ij}-d_{ij}} \right) \right\}}{\psi_i \left\{ \prod_{j=1}^{V_i} \left(\theta_{11} p_{11}^{d_{ij}} (1-p_{11})^{L_{ij}-d_{ij}} + (1-\theta_{11}) p_{10}^{d_{ij}} (1-p_{10})^{L_{ij}-d_{ij}} \right) \right\} + (1-\psi_i) \left\{ \prod_{j=1}^{V_i} \left(\theta_{10} p_{11}^{d_{ij}} (1-p_{11})^{L_{ij}-d_{ij}} + (1-\theta_{10}) p_{10}^{d_{ij}} (1-p_{10})^{L_{ij}-d_{ij}} \right) \right\}}$$

All analyses were conducted in R v3.6.2 (R Core Team, 2019). Code for fitting different types of site occupancy-detection models that account for false positive detections can be found in the supplement of Guillera-Arroita et al. (2017). We present code (and

data) to fit a subset of those models (those used in our case study) in Appendices S2 and S3.

3 | RESULTS

3.1 | Assay specificity

Template DNA from each of the 26 nontarget species that could potentially occur within the sampled region (Figure 1) did not amplify in the qPCR assay for *G. pusilla*. Tissue from *G. pusilla*, which was included in the assay as a positive control, came up at a Crossing Point (Cp) value of 22.96. Therefore, the results confirm the high specificity of the *G. pusilla* assay with no likely nontargets amplified.

3.2 | Field surveys

We detected *G. pusilla* eDNA at 9/13 sites included in our 2014 pilot survey. Dip-netting surveys around the time of eDNA sampling detected *G. pusilla* at all nine of those sites; the species was not detected at the remaining four sites with either method. The estimated mean eDNA availability probability at the water sample level (θ_{11}) was similar to the mean probability of detection at the qPCR level (p_{11}) (Table 1a). Two eDNA samples, each with two qPCRs, would be required to achieve a cumulative availability probability >.95. Abundance was positively correlated with mean copy number (Spearman's $\rho = 0.96$) and number of qPCR detections per site (Spearman's $\rho = 0.94$) (Figure 2). Similar (albeit weaker) results were apparent when the four control sites with no positive detections were excluded from the analysis (mean copy number, Spearman's $\rho = 0.88$; number of qPCR detections, Spearman's $\rho = 0.80$).

Of the 29 sites that were surveyed in 2016, we detected *G. pusilla* with dip nets at 8 sites. *Galaxiella pusilla* was detected at 7 of those sites on both site visits. In contrast, we detected the species' DNA at 11 sites. At 9 of those 11 sites, at least one of the three water sample replicates tested positive for the species' DNA on both survey occasions. On one survey occasion at a single site, *G. pusilla* eDNA was not detected despite the species being caught with a dip net. Long-term survey records indicated that *G. pusilla* had been recorded at 15 of the 29 study sites and are consistent with positive DNA detections at the 11 sites during the 2016 surveys. *Galaxiella pusilla* populations at the four sites at which the species had been historically recorded, but eDNA was not detected in our 2016 survey, typically occur at lower abundance. Of the seven sites that were surveyed in both 2014 and 2016 (all of which were known *G. pusilla* populations), *G. pusilla* eDNA was detected in both years. Dip netting in 2016 detected the species at six of the seven sites that were surveyed in both 2014 and 2016.

TABLE 1 Means and 95% confidence intervals of parameters from the model fitted to the 2014 pilot data, and the 2016 data. Estimates were obtained using the profile likelihood method

	Mean	Lower 95% CI	Upper 95% CI
(a) 2014 pilot data			
ψ	0.70	0.43	0.91
θ_1	0.78	0.58	0.92
p_{11}	0.77	0.65	0.86
(b) 2016 data			
ψ	0.35	0.19	0.53
θ_{11}	0.68	0.54	0.80
θ_{10}	0.0080	0.0001	0.0371
p_{11}	0.81	0.74	0.89
p_{10}	0.0039	0.0001	0.0153
r_{11}	0.75	0.53	0.90

The mean eDNA availability probability at the water sample level (θ_{11}) based on our 2016 data was lower compared to the estimate from our model fitted to the 2014 data (0.68 vs. 0.78). However, this comparison should be treated with caution, as the 2014 model did not account for false positive detections, and the 95% confidence intervals of the 2014 and 2016 estimates overlapped considerably (Table 1). The mean probability of detection using qPCR (p_{11}) estimated from the 2016 data was slightly higher than the estimate based on 2014 data, although the same caveats apply as above (Table 1).

The mean estimate of the probability of detection with dip netting (r_{11}) was slightly higher than the mean eDNA availability probability, although 95% confidence intervals overlapped considerably (Table 1b). Three eDNA samples (Figure 3a), each with two qPCRs (Figure 3b), would be required to achieve a cumulative availability probability > 0.95 at a single location. Three netting surveys (30 min each, 1 operator) would be required to achieve a similar cumulative detection probability (Figure 3c). False positive probabilities (θ_{10} & p_{10}) were extremely low (Table 1b).

Probability of *G. pusilla* occurrence, conditional on the observed eDNA data, was 0 for 18 sites and 1 for 9 sites. Intermediate probabilities of occurrence of 0.71 and 0.38 were predicted for the remaining two sites. eDNA was detected in 3/6 water samples from the former site (3/4, 1/4, 1/4 qPCRs) and in 2/6 samples from the latter (4/4 and 1/4 qPCRs).

4 | DISCUSSION

False positive detections are an important, yet frequently neglected issue in eDNA studies. This neglect has, until recently, been partially due to a lack of appropriate statistical methods. The present study demonstrates that new advances in site occupancy-detection models (Guillera-Arroita et al., 2017) are an effective approach to account for false positives in field-based eDNA studies.

Multiple lines of evidence suggest that the probability of false positive detections was low in our case study. First, our results illustrate that primer specificity was not an issue. The *G. pusilla* assay did not amplify the DNA of 26 nontarget species in the study area and was

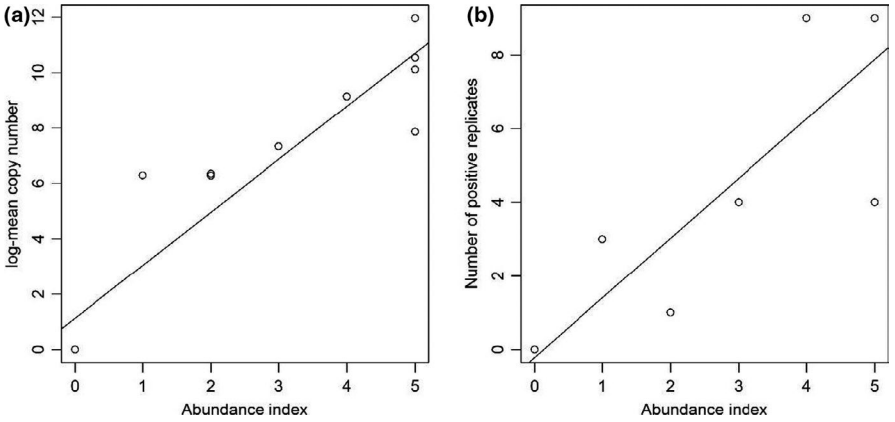

FIGURE 2 Relationship between log-mean copy number (a) and number of qPCR detections (b) and abundance

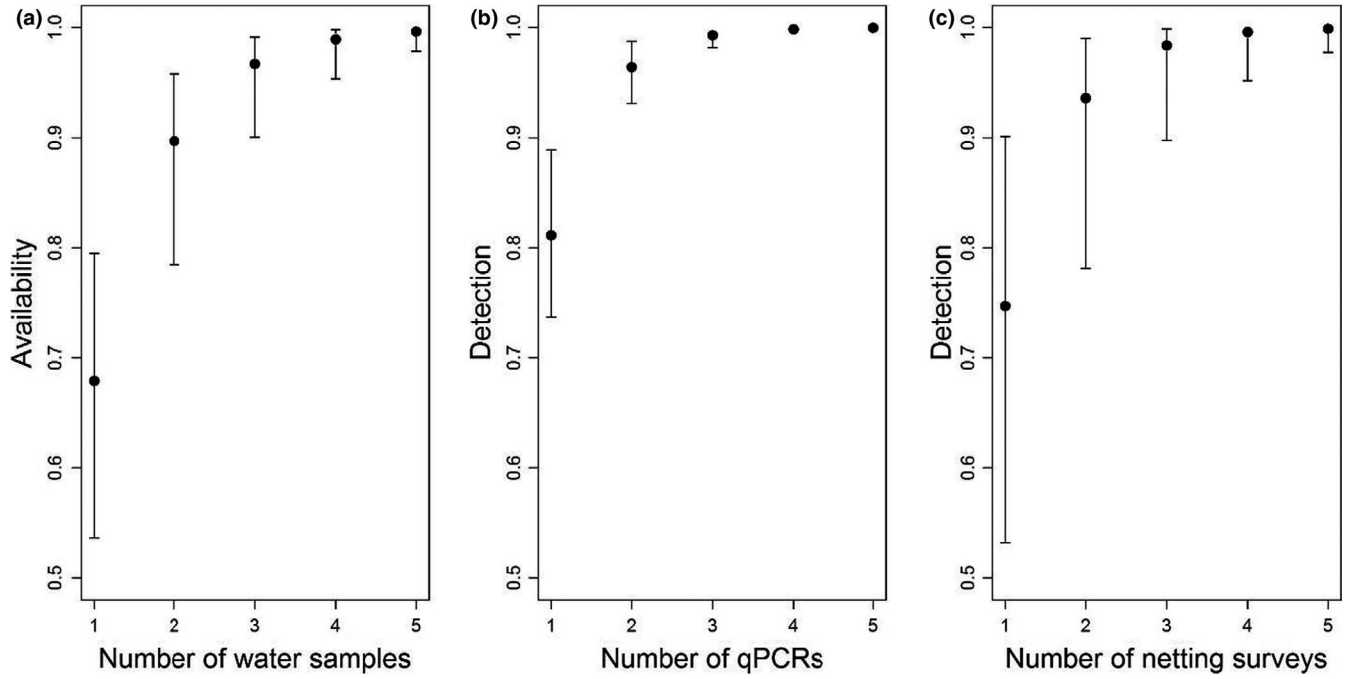

FIGURE 3 Estimated relationships between (a) the number of water samples taken at a site and the mean (95% CI) cumulative availability probability θ_{11}; (b) the number of qPCR assays conducted on a water sample and the mean (95% CI) cumulative detection probability p_{11}; and (c) and the number of repeat visits to dip net a site and the mean (95% CI) cumulative detection probability r_{11}.

unique to *G. pusilla* based on NCBI blast searches. Second, eDNA accumulated and degraded rapidly (within 120 hr at 21°C) in laboratory trials (Appendix S1), as in other studies on freshwater fish (e.g., Seymour et al., 2018; Tsuji et al., 2017), suggesting that our eDNA assay provides a close to real-time detection method that is unlikely to detect extirpated populations (although persistence times can be much longer in sediment: Turner, Uy, & Everhart, 2015). Third, eDNA was only detected at sites where *G. pusilla* have been captured (either during our 2014 and 2016 dip-netting surveys, or during long-term surveys). If false positive detection rates were high, we would not expect such high concordance between eDNA surveys and an independent survey method. Fourth, *G. pusilla* eDNA was not detected in any of our calibration experiments (water sample and qPCR blanks), and thus, laboratory contamination of field samples is highly unlikely. Finally, site occupancy-detection models estimated very low probabilities of false positive detections at both the water sample and qPCR level. Collectively, these results suggest that eDNA sampling can be a specific monitoring tool in freshwater environments.

Interestingly, Guillera-Arroita et al. (2017) applied the same modeling approach to four frog species, and generally found higher false positive probabilities than observed here for *G. pusilla*. This was partially due to the fact that rates of water sample contamination were higher in the calibration experiments conducted in the frog case study of Guillera-Arroita et al. (2017) than those estimated here. While false positive probabilities may be expected to vary between laboratories and technicians, all samples used in this study were processed by the same individual (AvR). One potential reason for the disparity between the results of the two studies could be the recent improvements made to sample filtering and DNA extraction protocols. Guillera-Arroita et al. (2017) passed water samples collected in the field through a cellulose nitrate filter using a filter funnel and peristaltic pump, whereas here,

we used an encased Sterivex filter unit attached to a disposable luer lock syringe. The latter procedure is less prone to contamination and is expected to significantly reduce the probability of false positive detections (Spens et al., 2017). Sample collection and extraction method may therefore be important for limiting false positives in eDNA surveys.

Our results add to a growing body of literature illustrating that, despite the fact that eDNA detection probabilities are almost always less than one, eDNA sampling can achieve similar (or greater) detection probabilities than traditional sampling methods (Lugg et al., 2017; Smart, Tingley, Weeks, Van Rooyen, & McCarthy, 2015; Wineland et al., 2019; Wittwer et al., 2018). The mean probability of detecting *G. pusilla* eDNA in a single water sample was only slightly lower than the probability of detecting one or more *G. pusilla* individuals in 30 min of dip netting. These results were likely influenced by the fact that *G. pusilla* often occur in small, shallow waterbodies at high densities, when present; such conditions are particularly conducive for detection with dip netting. We might expect that eDNA sampling would have even greater sensitivity than dip netting in larger waterbodies, and/or for species that occur at lower densities.

Our field studies suggested that achieving a cumulative availability probability >.95 with eDNA sampling required taking two (2014 pilot data) or three (2016 data) water samples on a single visit. Achieving a cumulative detection probability > 0.95 with dip netting required visiting a site for 30 min on three separate occasions (although detection probabilities might be improved by using multiple surveyors on each sampling occasion). Travel and personnel costs are therefore much greater with dip netting than with eDNA sampling, important considerations when designing monitoring strategies to maximize the probability of detection at a set of sites (Smart et al., 2016). Future studies wishing to employ eDNA sampling to understand patterns of occurrence need not employ an unambiguous survey method, such as dip netting, as calibration experiments at the qPCR level and water sample level are sufficient to identify the core modeling parameters (G. Guillera-Arroita et al., 2017). Alternatively, one can use informative priors in a Bayesian framework (Griffin et al., 2020).

Environmental DNA sampling not only achieved similar detection probabilities to dip netting, but also detected eDNA at three sites where we did not capture *G. pusilla* individuals. All three of these additional sites are unlikely the result of false positive detections, given the low probabilities of false positives in our model and historical records of *G. pusilla* occurrence on multiple occasions using traditional survey methods. Probabilities of *G. pusilla* occurrence at each of the 29 surveyed sites, conditional on the observed data, largely support this assertion. In addition, the results of our 2014 pilot survey support the general finding that qPCR can provide information on organismal abundance (Chambert, Pilliod, Goldberg, Doi, & Takahara, 2018; Lacoursière-Roussel, Côté, Leclerc, & Bernatchez, 2016; Pilliod, Goldberg, Arkle, Waits, & Richardson, 2013; Tillotson et al., 2018). Nonetheless, direct capture methods, such as dip netting, provide additional information on population health (e.g., age structure, recruitment, sex ratios), and are typically less subject to ambiguous detections, assuming sufficient taxonomic expertise.

The results of this study illustrate that eDNA sampling can be a sensitive and specific method for monitoring the occurrence of threatened freshwater fauna. Our results also highlight the power of site occupancy-detection models when applied to eDNA data. Although probabilities of laboratory-induced false positives were low in the present study, future eDNA studies should still estimate the likelihood of, and properly account for, false positive detections using modeling approaches such as the one used here.

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DATA AVAILABILITY STATEMENT

All field results, laboratory results, and R code are available in Appendices S2 and S3.

ORCID

Reid Tingley https://orcid.org/0000-0002-7630-7434
Rhys Coleman https://orcid.org/0000-0001-5072-3504
Andrew R. Weeks https://orcid.org/0000-0003-3081-135X

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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