

A performance evaluation of targeted eDNA and eDNA metabarcoding analyses for freshwater fishes

Jonas Bylemans^{1,2,3}  | Dianne M. Gleeson^{1,2}  | Richard P. Duncan¹  |
 Christopher M. Hardy^{2,4}  | Elise M. Furlan^{1,2} 

¹Institute for Applied Ecology, University of Canberra, Canberra, ACT, Australia

²Invasive Animals Cooperative Research Centre, University of Canberra, Canberra, ACT, Australia

³Research and Innovation Centre, Fondazione Edmund Mach, San Michele all'Adige, Italy

⁴CSIRO Land and Water, Canberra, ACT, Australia

Correspondence

Jonas Bylemans, Institute for Applied Ecology, University of Canberra, Canberra, ACT, Australia.
 Email: Jonas.Bylemans@canberra.edu.au

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Abstract

Background: The use of environmental DNA analysis has revolutionized biodiversity monitoring. Initially, eDNA monitoring surveys in aquatic environments utilized a targeted approach, but there has been a steady shift toward whole community assessments (eDNA metabarcoding). Both approaches can increase the detection sensitivity for rare and elusive species, compared to more conventional methods. However, it is important to understand the benefits and limitations of targeted and whole community eDNA monitoring to tailor surveys to research questions and management objectives.

Aims: Here, we aimed to test the relative merits of targeted eDNA analysis versus eDNA metabarcoding in an intermittent river system.

Methods: First, samples collected during different seasons were used to assess the influence of seasonality on the detection probabilities of both methods. Second, detection probabilities from the two monitoring approaches for one focal species were compared to evaluate the sensitivity of both methods. Finally, the data from an eDNA metabarcoding survey conducted across the outer distribution limits of an invasive species were used to evaluate whether species interactions can be inferred by this method.

Results: Analyses showed that sampling intermittent river systems during low flow events increases the performance of the targeted eDNA surveys, while sampling season does not influence the performance of eDNA metabarcoding surveys. Environmental DNA metabarcoding was found to be less sensitive than a targeted monitoring approach, thus making the latter more suitable for generating detailed distribution data. Nevertheless, eDNA metabarcoding survey data can be interpreted in a semiquantitative manner and can provide insights into biological interactions.

KEY WORDS

eDNA metabarcoding, environmental DNA, fishes, real-time PCR

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1 | INTRODUCTION

DNA derived from environmental samples, commonly referred to as eDNA, can be detected and used for biodiversity monitoring (Deiner et al., 2017; Yoccoz, 2012). Environmental DNA monitoring can entail a targeted assay, where the presence of a particular species is assessed using species-specific primers, or a more general approach (eDNA metabarcoding), where entire communities are characterized by amplifying DNA barcode regions for multiple taxa of interest coupled with high-throughput sequencing (HTS) of amplicon libraries (Baird & Hajibabaei, 2012; Cristescu, 2014; Thomsen et al., 2012; Valentini, Pompanon, & Taberlet, 2009). Both approaches have been found to be more sensitive than conventional monitoring methods (Jerde, Mahon, Chadderton, & Lodge, 2011; Lopes et al., 2017; Sigsgaard, Carl, Møller, & Thomsen, 2015; Valentini et al., 2016). However, selecting the most suitable approach to best meet monitoring objectives requires a good understanding of their benefits and limitations (Nichols & Williams, 2006; Yoccoz, Nichols, & Boulinier, 2001).

Biodiversity surveys aim to characterize species present in the environment, and the data are used to understand the biotic and abiotic factors that influence the distribution and abundance of species (Nichols & Williams, 2006; Yoccoz et al., 2001). Surveys can seek to monitor entire communities, although it is a common practice to focus on a limited number of species (indicator species) to evaluate ecosystem health or inform environmental management practices (Siddig, Ellison, Ochs, Villar-Leeman, & Lau, 2017; Simberloff, 1998). Targeted eDNA analysis, using species-specific primers, can be a highly effective method for monitoring rare or elusive species (Ficetola, Miaud, Pompanon, & Taberlet, 2008; Goldberg, Pilliod, Arkle, & Waits, 2011; Jerde et al., 2011) and is highly suitable to accurately map the distribution of the species of interest (Bylemans, Furlan, Pearce, Daly, & Gleeson, 2016; Sigsgaard et al., 2015; Takahara, Minamoto, & Doi, 2013). Additionally, targeted assays can be used to quantify eDNA concentrations within a water body and provide indicators of species abundance (Doi et al., 2017; Lacoursière-Roussel, Côté, Leclerc, & Bernatchez, 2015; Takahara, Minamoto, Yamanaka, Doi, & Kawabata, 2012). Recent studies have demonstrated that multiplexing of species-specific assays can detect multiple species simultaneously but a rigorous evaluation is needed to test the specificity of each assay and the potential negative interactions between primers and probes (Tsuji et al., 2018; Wozney & Wilson, 2017). Consequently, targeted eDNA monitoring of a large number of species can become both time consuming and costly to develop. Furthermore, a targeted approach is by definition restricted and requires prior knowledge and assumptions of species present within a system, and so is unsuitable for the detection of species novel to the survey area.

In contrast, eDNA metabarcoding can be used to characterize entire species assemblages (Cilleros et al., 2019; Deiner et al., 2017; DiBattista et al., 2017; Yamamoto et al., 2017). Environmental DNA metabarcoding can be used to simultaneously determine species distributions, ecologically important patterns of species

diversity, and detect novel species (Blackman et al., 2017; Elbrecht, Vamos, Meissner, Aroviita, & Leese, 2017; Harper et al., 2019). Metabarcoding surveys are able to provide comprehensive presence/absence data, although sampling, laboratory, and analytical protocols can influence biodiversity estimates (Alberdi et al., 2018; Deiner, Walser, Mächler, & Altermatt, 2015). One well-recognized bias in metabarcoding analyses is preferential amplification of a few target sequences, that is, those that are relatively abundant or have few primer-template mismatches (Bylemans, Gleeson, Hardy, & Furlan, 2018; Elbrecht & Leese, 2015). Amplification biases can increase the chance of false-negative detections (failure to detect a species even when it is present) compared to a targeted approach (Harper et al., 2018; Lacoursière-Roussel, Dubois, Normandeau, Bernatchez, & Adamowicz, 2016; Schneider et al., 2016). Concerns also remain as to whether eDNA metabarcoding data can be interpreted in a quantitative manner. Some recent studies have suggested that eDNA metabarcoding can produce semiquantitative data and can be used to monitor species interactions (Häfling, Handley, Read, Hahn, & Li, 2016; Ushio et al., 2018). However, the nature of the community (i.e., richness and evenness) and the PCR primers/polymerases used during the amplification step can have a profound influence on quantitative estimates derived from metabarcoding analyses (Elbrecht & Leese, 2015; Nichols et al., 2018; Piñol, Senar, & Symondson, 2018).

A complicating factor for any eDNA-based monitoring approach is that eDNA concentrations vary seasonally and spatially which in turn influences the detection probabilities (De Souza, Godwin, Renshaw, & Larson, 2016; Furlan, Gleeson, Hardy, & Duncan, 2016). Seasonal fluctuations in eDNA concentrations have been reported for both targeted eDNA and eDNA metabarcoding surveys (Bista et al., 2017; Buxton, Groombridge, Zakaria, & Griffiths, 2017; De Souza et al., 2016; Takahashi et al., 2017), while spatial variations in eDNA concentrations have been reported for both lentic and lotic freshwater systems (Furlan et al., 2016; Handley et al., 2019; Häfling et al., 2016; Takahara et al., 2012). Nonetheless, no information is currently available on the eDNA dynamics in intermittent streams even though large fluctuations in water flow are likely to have strong influence on the abundance and distribution of eDNA in these systems.

Blakney Creek catchment is an intermittent river system in New South Wales (NSW, Australia) and is one of four systems in NSW to hold a self-sustaining population of the endangered southern pygmy perch (*Nannoperca australis*). The presence and continued spread of two invasive fish species, common carp (*Cyprinus carpio*) and redfin perch (*Perca fluviatilis*), is threatening the long-term persistence of this population (Pearce, 2015). A survey conducted in the autumn of 2015, using both traditional and targeted eDNA monitoring, has shown that the upper reaches in this catchment have not yet been invaded by redfin perch, and this information was used to determine the optimal location of a redfin perch exclusion barrier (Bylemans et al., 2016). The previous work conducted in the Blakney Creek system has provided solid background information on the distribution and impact of the present invasive fish species (Bylemans et al., 2016; Pearce, 2015). Consequently, this

system provides a unique opportunity to evaluate and compare the advantages/disadvantages of both eDNA monitoring approaches. More extensive eDNA surveys were conducted in Blakney Creek with the aim of testing three hypotheses. First, if water flow regimes in intermittent streams are the primary factor influencing the abundance and spatial distribution of eDNA, it can be expected that during high flow events eDNA concentrations would be lower (due to a higher dilution effect) and eDNA would be more heterogeneously distributed (due to increased mixing). Second, if the preferential amplification of the DNA from a few species strongly influences eDNA metabarcoding surveys, this monitoring approach is likely to be less sensitive compared to a targeted monitoring approach. Finally, if there truly is a correlation between species abundance and the read counts obtained from eDNA metabarcoding analyses, the read abundance data would be able to reveal the potential negative impacts of the invasive species on the native fish community. Here, these three hypotheses will be tested and the results will be used to assess the suitability of both eDNA monitoring approaches to address question relevant for both ecologists and environmental managers.

2 | MATERIALS AND METHODS

2.1 | eDNA sampling and sample processing

Environmental DNA sampling was conducted at 19 sampling sites within Blakney Creek (BC) and the adjoining Urumwalla Creek (UC) (Figure 1). Sites were selected based on the most up-to-date redfin perch distribution data (Bylemans et al. 2016) to ensure that eDNA samples were collected across the redfin perch invasion front and thus varied in the abundances of this invasive species. Samples were collected over two spring sampling seasons (2015 and 2016) (Figure 1). Eight 2 L water samples were collected for each site and

sampling event. All subsequent analyses were performed using eight eDNA samples per site apart for three instances where only seven samples were available per site (Appendix S1).

Prior to sample collections, all equipment was cleaned using a 20% bleach solution and thoroughly rinsed with UV-treated tap water to remove any contaminant DNA. Each site consisted of a sampling pool, and surface water samples were collected along the entire length of the pool. A blank field control (BFC) was included for each site and consisted of a 2-L sampling bottle filled with UV-treated tap water which was opened on site, closed and submerged in the water. Samples were stored on ice immediately after collection and transported back to the University of Canberra (ACT, Australia). A 1.2- μ m glass fiber filter (Sartorius) was used to capture the eDNA contained in the 2 L samples. Potential contaminant DNA was removed from all filtering equipment as described previously. Negative equipment controls (NEC) were obtained by filtering 500 ml of UV-sterilized water prior to processing the eDNA samples. Filters were stored at -20°C and transferred to a trace DNA laboratory at the University of Canberra where eDNA was extracted using the PowerWater DNA Extraction Kit (MoBio Laboratories). During batch extractions, all BFCs and a subset of the NECs (i.e., one for each sampling site) were processed together with the eDNA samples to monitor for potential cross-contamination arising from the eDNA processing workflow. Environmental DNA extracts (100 μ L) were stored at -20°C until required.

2.2 | Targeted eDNA monitoring

The eDNA samples collected from eight sites (i.e., BC01, BC03, BC06, BC09, BC10, UC01, UC03, and UC05) during autumn 2015 were previously screened for the presence of redfin perch eDNA (Figure 1) (Bylemans et al., 2016). Additional samples were collected from these sites during spring 2015 and analyzed for the

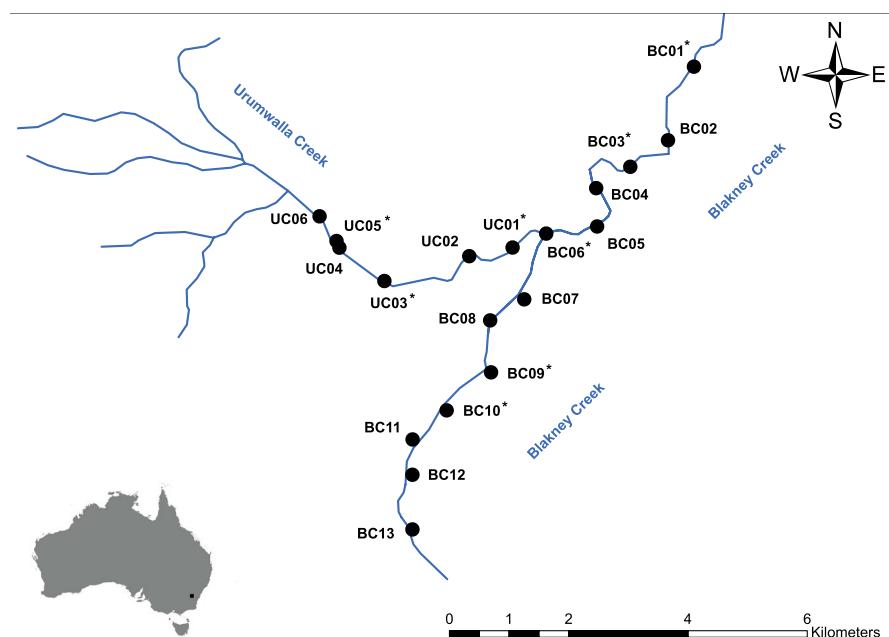


FIGURE 1 Map of sites sampled within Blakney Creek (BC) and Urumwalla Creek (UC) (NSW, Australia) during spring of 2015 and 2016. Sites also sampled during autumn are indicated by an asterisk

presence of redfin perch eDNA following the same procedure (Bylemans et al., 2016). Briefly, three real-time PCR replicates were conducted using 8 µl of template eDNA and a redfin perch-specific TaqMan® real-time PCR assay (Furlan & Gleeson, 2016a). A generic fish assay was included as a positive control, and PCR replicates were only considered valid when amplification was observed in at least one assay (Furlan & Gleeson, 2016b). If all replicates from a sampling site failed to amplify for the redfin perch assay, an additional three PCR replicates were performed for all samples. The total PCR replication for eDNA samples thus ranged from 3 to 6 (see Appendix S1 for full details). For all negative controls (i.e., BFCs and NECs), six PCR replicates were performed to test for potential cross-contamination. Samples were considered positive for the presence of redfin perch DNA if at least one out of all the PCR replicates showed positive amplification for the redfin perch assay.

2.3 | eDNA metabarcoding

All available eDNA samples were used in the eDNA metabarcoding analyses. The MiFish-U fish primers (Miya et al., 2015) were used for the amplification and the construction of the HTS libraries as they were found to perform well in terms of their specificity to fish species and the potential impact of primer biases (Bylemans et al., 2018). Furthermore, the MiFish amplicons are able to distinguish between most fish species occurring in the Murray–Darling Basin (MDB), with the only exception being the *Galaxias* species complex (Bylemans et al., 2018). Sequencing libraries were constructed following the general protocol described in Bylemans et al. (2018) with some minor modifications. Firstly, negative control samples were screened for contaminant fish DNA by performing three PCR replicates per sample. Next, for all eDNA samples and those negative control samples testing positive for fish DNA, HTS libraries were constructed for unidirectional sequencing. A one-step real-time PCR amplification with fusion tagged primers (FTP) was used for library construction. Forward FTP consisted of the P5 sequencing adaptor, a custom forward sequencing primer, a 7 bp Multiplex Identification (MID) tag, and the MiFish-U forward primer. Reverse FTP contained the P7 sequencing adaptor, a 7 bp MID-tag, and the MiFish-U reverse primer. Amplicon libraries were created using three PCR replicates per sample to account for the stochasticity in the PCR amplification.

For all samples used in the targeted monitoring survey, 4 µl of template eDNA was used for each PCR replicate and unique combinations of MID-tags were used for each sample. As template eDNA volumes used during library construction were half the volumes used during the targeted detection, the results obtained from both approaches may not be directly comparable. Consequently, for a subset of the samples (i.e., samples collected from the BC01, BC03, and BC06 sites) HTS libraries were also constructed using 8 µl of template eDNA per PCR replicate and the results were used to determine whether the eDNA template volumes influences the species detection probabilities derived from eDNA metabarcoding analyses. For all remaining samples (i.e., the samples not used in the targeted

survey), HTS libraries were constructed using 4 µl of template eDNA per PCR replicate and unique combinations of MID-tags were used for each site.

The average Ct values for each sample obtained from the real-time PCR analyses were used to quantify and pool equal amounts of amplicon libraries from 8 to 10 samples prior to conducting a library clean-up using the Agencourt AMPure XP Beads (Beckman Coulter) in a 1.2 volume ratio. The presence of a single amplicon with the expected length was confirmed by agarose gel electrophoresis, and band intensities were used to pool approximately equal amounts of each primary pool into a super pool. Two super pools were constructed and sent to the Ramaciotti Centre for Genomics (University of New South Wales) for additional quality checks and unidirectional sequencing with the MiSeq v2 1x300bp sequencing kit. The first sequencing run (MiSeq01) contained the amplicon libraries from the eDNA samples used in the targeted survey, 1 NEC and 3 BFCs. The second sequencing run (MiSeq02) contained the uniquely labeled libraries from all remaining samples together with seven NECs, five BFCs, and five negative PCR controls. Both sequencing runs were conducted as to achieve an approximate sequencing depth of 50,000–60,000 reads per sample or 400,000–480,000 reads per site.

Raw sequencing reads were processed following previously published workflows with some modifications (Bylemans et al., 2018; De Barba et al., 2014). First, technical sequences (sequencing adaptors and primers) were removed using Trimmomatic v.0.36 while simultaneously removing low quality bases (trimming of bases at the end of reads with a quality below 3 and trimming of reads with a sliding window of four bases with an average quality threshold of 15) (Bolger, Lohse, & Usadel, 2014). Two processing pipelines were used for subsequent filtering of the sequencing reads using the OBITOOLS software package (Boyer et al., 2016). Firstly, the data from the MiSeq01 run were processed to obtain fish community data on a per-sample basis. The sequencing reads were binned to their respective samples, and subsequent filtering was done for each sample separately. Short (<150 bp) and low abundance (<100) reads were removed before discarding reads arising from PCR and sequencing errors. Filtering thresholds for low abundance sequences were determined experimentally to ensure that all fish sequences present in the negative controls, likely to arise from spill-over or low levels of contamination, were removed. Taxonomic information was assigned to the sequences using the taxonomic database for vertebrate sequences from the EMBL data repository (release 132) and 12S sequence from all fishes of the MDB (Bylemans et al., 2018). Second, the combined data from the MiSeq01 and MiSeq02 run were used to obtain community data on a per site basis for all sites sampled during the spring surveys. Only those sequences arising from consistent libraries preparations (4 µl of template eDNA per PCR replicate) were binned to their respective sampling sites followed by quality filtering and taxonomic assignments as described previously.

A final clean-up of the eDNA metabarcoding data was done in R version 3.4.1 using the tidyverse package (Appendix S2) (R

Development Core Team, 2010; Wickham, 2016). This additional quality check revealed that some sequences were assigned to commonly consumed fish which are not present within the system (i.e., *Scomberomorus* sp., *Gadus* sp., *Pollachius virens*, *Seriola lalandi*, and *Salmo salar*). These occurrences could be the results of PCR and/or sequencing errors or may have arisen from secondary contamination of the samples and were thus discarded. Additionally, sequence records assigned to the *Hypseleotris* genus (ca. 0.14% of all fish sequences) and *Hypseleotris* sp. *Murray-Darling carp gudgeon* (ca. 0.04% of all fish sequences) were identified at only one sampling site. A closer inspection of the sequences revealed that these are likely to be chimeric sequences and were thus removed (i.e., combination of *Hypseleotris* sp. *Midgley's carp gudgeon* and *Hypseleotris* sp. *Lake's carp gudgeon* sequences). Finally, while only *Nannoperca australis* species occur in the system, some sequences were assigned to the *Nannoperca* genus or to *N. obscura*. As *N. australis* sequence records were highly abundant in the samples giving rise to the ambiguous assignments, the latter are thus likely to result from PCR and/or sequencing errors and were reassigned to *N. australis*.

2.4 | Data analyses

2.4.1 | Seasonal influences

The detections of redfin perch eDNA in the Blakney Creek system were used to evaluate the effect of seasonality on the performance of targeted eDNA monitoring surveys. A Bayesian hierarchical model was used to estimate the concentrations of redfin perch eDNA at each sampling site, evaluate the dispersion of eDNA during the different seasons, and determine the overall sensitivity of the targeted eDNA monitoring survey (Furlan et al., 2016). As the modeling framework relies on between replicate variation in the detection of redfin perch eDNA, sites for which all replicates consistently amplified or failed to amplify redfin perch DNA were excluded. Only the data obtained for four sites (i.e., BC06, BC09, BC10, and UC01) were thus retained for the analyses (Table 1) (see Appendix S1 for further details). Model specifications were as described in Furlan et al. (2016) but with the estimates of the dispersion parameter (r) varying dependent on the sampling season. The R package jagsUI was used to fit the models in JAGS using three chains with 20,000 iterations per chain and discarding the first 10,000 iterations as a burn-in (Kellner, 2015). A detailed description of the analyses can be found in the Appendix S1 and S2.

The eDNA metabarcoding data from all samples used in the targeted survey were used to evaluate the impact of the sampling season on the performance of the eDNA metabarcoding survey (Table 1). For each season and site, the data were used to calculate the detection probabilities for each species (i.e., the proportion of samples indicating the presence of a species' DNA). A paired sample t test was subsequently used to determine if the detection probabilities differed significantly between the two sampling seasons (i.e., autumn vs. spring).

2.4.2 | Detection sensitivity

The redfin perch detection data obtained from the eight sampling sites surveyed during autumn and spring 2015 were used to evaluate whether the overall detection sensitivity differed between the targeted monitoring and eDNA metabarcoding (Table 1). However, as different volumes of template eDNA were used in the targeted and metabarcoding survey the impact of the amount of template eDNA on the eDNA metabarcoding results was evaluated first. Using the eDNA metabarcoding data for the BC01, BC03, and BC06 sites, the detection probabilities for all species were calculated when 4 or 8 μ l of template eDNA was used. Next, a paired sample t test was performed to assess if the volumes of template eDNA influence the detection probabilities for redfin perch.

The relative performance of the targeted eDNA and the eDNA metabarcoding surveys were evaluated by comparing the redfin perch detection probabilities. For all sampling sites ($n = 8$) surveyed during autumn and spring 2015 (Table 1), the redfin perch detection probabilities were calculated as the proportion of samples producing a positive detection for this species. The detection probabilities obtained from the targeted survey were compared to those from the eDNA metabarcoding survey using a paired sample t test.

2.4.3 | Species interactions

The impact of the two invasive fish species present in the system (i.e., common carp and redfin perch) on the native species was evaluated using the proportional read abundances for each site for both the spring 2015 and 2016 sampling season (Table 1). The data obtained for Urumwalla Creek and for *Macquaria ambigua* were excluded from the analyses as no invasive species were detected in the UC system and *M. ambigua* was only detected once (see Appendix S1). The proportional reads for the remaining native fish species (y) were transformed to avoid zero values using Equation 1 with a sample size (N) of 8 samples per site (Smithson & Verkuilen, 2006). A logit-transformation was subsequently used to achieve normality (Equation 2).

$$y' = (y \times (N - 1) + 1/2) / N \quad (1)$$

$$\text{log it } (y') = \log(y' / (1 - y')) \quad (2)$$

As the number of species detected per site will influence the proportional abundance data, the relationship between the logit-transformed data and the total number of species detected was evaluated first. The R package nlme was used to fit linear mixed effect models to the data for each native species (Pinheiro, Bates, DebRoy, Sarkar, & R Core Team, 2019). The logit-transformed proportional abundance data were set as the response variable, the total number of species as the fixed effect and sampling season as a random effect to account for temporal pseudo-replication. The impact of the invasive species was evaluated by fitting a linear mixed effect model for each combination of native and invasive

TABLE 1 Summary of the data used for the different analyses

Site	Seasonal influences		Detection sensitivity		Species interactions	
	Autumn 2015	Spring 2015	Autumn 2015	Spring 2015	Spring 2015	Spring 2016
BC01	HTS	HTS	PCR/HTS	PCR/HTS	HTS	HTS
BC02					HTS	HTS
BC03	HTS	HTS	PCR/HTS	PCR/HTS	HTS	HTS
BC04					HTS	HTS
BC05					HTS	HTS
BC06	PCR/HTS	PCR/HTS	PCR/HTS	PCR/HTS	HTS	HTS
BC07					HTS	HTS
BC08					HTS	HTS
BC09	PCR/HTS	PCR/HTS	PCR/HTS	PCR/HTS	HTS	HTS
BC10	PCR/HTS	PCR/HTS	PCR/HTS	PCR/HTS	HTS	HTS
BC11					HTS	HTS
BC12					HTS	HTS
BC13					HTS	HTS
UC01	PCR/HTS	PCR/HTS	PCR/HTS	PCR/HTS		
UC02						
UC03	HTS	HTS	PCR/HTS	PCR/HTS		
UC04						
UC05	HTS	HTS	PCR/HTS	PCR/HTS		
UC06						

Note: HTS: eDNA metabarcoding using MiFish-U amplicons and high-throughput sequencing; PCR: use of a redfin perch-specific PCR assay.

species. Model specifications were as described previously but with the proportional read abundances of the invasive species set as the fixed effect.

3 | RESULTS

3.1 | Seasonal influences

Based on the results of the targeted survey, redfin perch eDNA concentrations were found to be generally higher during autumn (Figure 2). The estimated eDNA concentration from the BC10 site, however, deviated from this pattern with higher eDNA concentration observed during the spring sampling season (Figure 2). The mean estimates of the dispersion parameter were 17.04 and 18.57 for the autumn and spring sampling season, respectively (with 95% credible intervals of 1.93–35.98 and 3.90–36.03, respectively). The high mean estimates for r and the large credible intervals indicate that during both seasons, redfin perch eDNA is essentially randomly distributed (i.e., mean r values > 10 indicate random dispersion). Finally, the modeling showed a detection likelihood of 95% or greater for our targeted eDNA survey (eight 2 L samples and six PCR replicates per sample), provided redfin perch eDNA concentrations were equal or higher than 2.5 molecules per liter (Appendix S1). The analyses of the detection probabilities derived from the eDNA metabarcoding data showed that even though detection probabilities in the autumn sampling season tend to

be higher, the difference between the sampling seasons was not statistically significant ($t = 0.77$, $df = 46$ and p -value = .4459) (see Appendix S1).

3.2 | Detection sensitivity

The MiSeq01 run generated a total of 12,315,650 sequences with an average sequencing depth of ca. 47,000 reads per sample, an overall Phred Q30 score ≥ 91.17 , and on average 28,918 ($\pm 6,638$) reads per sample were assigned to fish species.

Increasing the volume of template eDNA used during the construction of HTS libraries in the PCR amplification step did not significantly affect the detection probabilities ($t = -1.81$, $df = 38$, p -value = .0779) (Appendix S1). By contrast, redfin perch detection probabilities were significantly higher in the targeted survey than the eDNA metabarcoding results (Figure 3). For example, when the detection probability of the targeted survey ranged from .1 to .9 the eDNA metabarcoding analyses failed to detect redfin perch DNA in six out of eight sites by season combinations (Figure 3).

3.3 | Species interactions

A total of 17,050,364 sequence reads were obtained from the MiSeq02 run with a Phred Q30 score ≥ 79.40 . The lower quality score of the MiSeq02 run is likely due to the inclusion of shorter amplicons

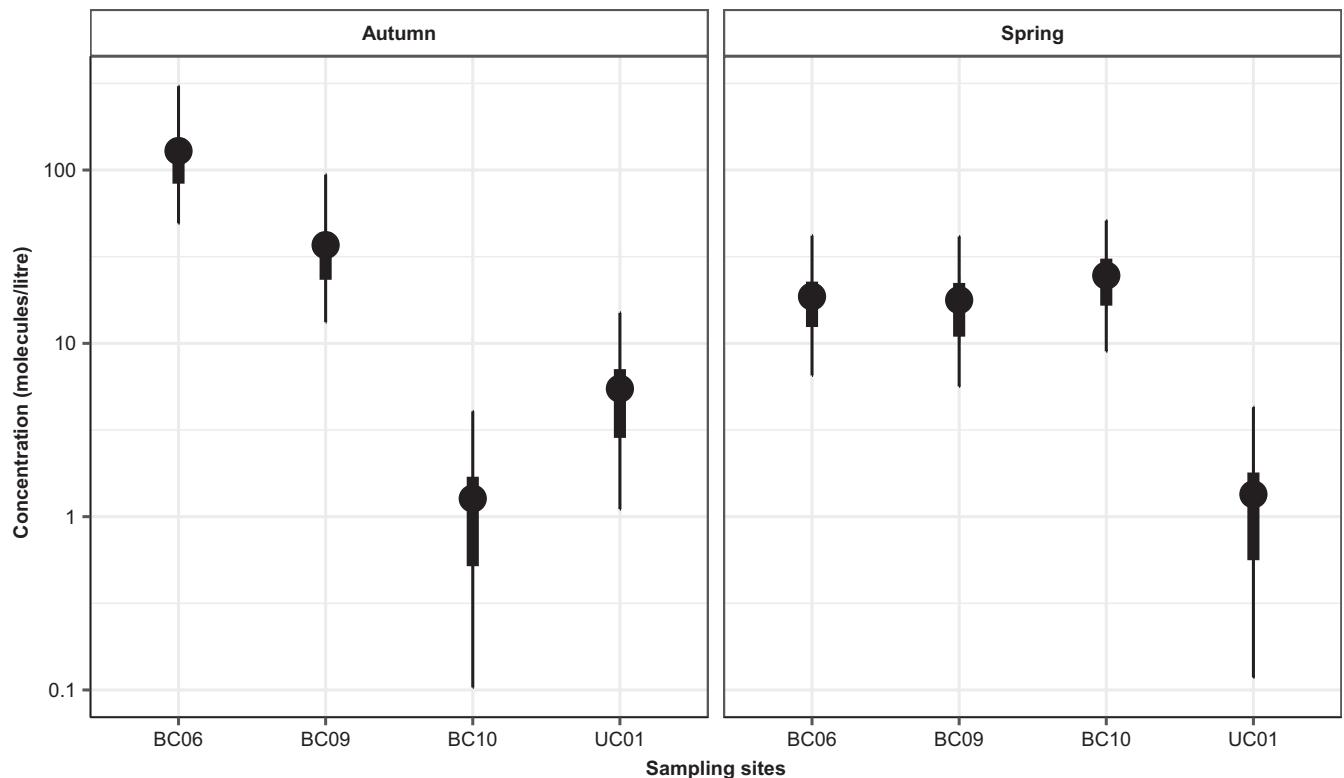


FIGURE 2 Estimated concentrations of redfin perch (*Perca fluviatilis*) eDNA for four sites within the Blakney Creek catchment sampled during spring and autumn of 2015. Estimates are based on the targeted analyses and are shown as the mean eDNA concentrations (solid points) and the 50% and 95% credible intervals (thin and thick lines respectively)

from a different study. On average, 294,826 ($\pm 42,500$) reads per site were assigned to fish species after the bio-informatics filtering of the combined MiSeq01 and MiSeq02 data. A summary figure of the fish community data for each site surveyed during spring 2015 and 2016 can be found in the Appendix S1.

Linear regression analyses based on the proportional read abundance data revealed a negative correlation between the logit-transformed proportion abundances of *N. australis* and the number of species detected per sampling site. For all other native species, no correlation or a slight positive correlation was observed (Figure 4). Regression analyses performed to evaluate the impact of the invasive species showed a similar pattern. Only the proportional read abundance data of *N. australis* showed a negative correlation with the proportional abundance data of both invasive fish species (Figure 4).

4 | DISCUSSION

Seasonal patterns in eDNA abundance were observed for the targeted eDNA survey while no significant effect of sampling season was observed for the eDNA metabarcoding results. The estimated concentrations of redfin perch eDNA were lower during the spring sampling season (Figure 2). The only site that deviated from this (BC10) is located on the edge of the redfin perch distribution, and the increased eDNA concentration may reflect an increased colonization of this site by redfin perch. The observed lower eDNA concentration during

spring may be due to an increased water flow during this time and/or a reduction in the metabolic rates due to lower water temperatures (autumn and spring water temperatures were 21.19°C ($\pm 1.06^\circ\text{C}$) and 12.81°C ($\pm 2.08^\circ\text{C}$), respectively). Previous studies have also found that flow rates influence eDNA concentrations and detection probabilities (Jane et al., 2015; Shogren et al., 2018), while the effects of water temperature are less well understood (Buxton et al., 2017; Jo, Murakami, Yamamoto, Masuda, & Minamoto, 2019; Klymus, Richter, Chapman, & Paukert, 2014). The eDNA metabarcoding results did not reveal a significant effect of sampling season. Overall, eDNA sampling during periods of low flow in intermittent river systems is recommended to maximize the detection probability of eDNA surveys.

Direct comparisons of the detection probabilities for redfin perch, through either a targeted approach or eDNA metabarcoding, confirmed our hypothesis that targeted eDNA analyses have a greater detection sensitivity than eDNA metabarcoding (Figure 3). This is consistent with other studies that have shown that a targeted eDNA monitoring approach is more sensitive compared to eDNA metabarcoding (Harper et al., 2018; Lacoursière-Roussel et al., 2016). Metabarcoding analyses are thus more prone to false-negative detections which are supported by the observation that eDNA metabarcoding failed to detect redfin perch at six sampling sites while targeted eDNA monitoring indicated their presence (Figure 3). Replication levels at various stages of the workflow can influence the detection probabilities of eDNA analyses (Ficetola et al., 2015; Furlan et al., 2016). Here, sampling replication per site

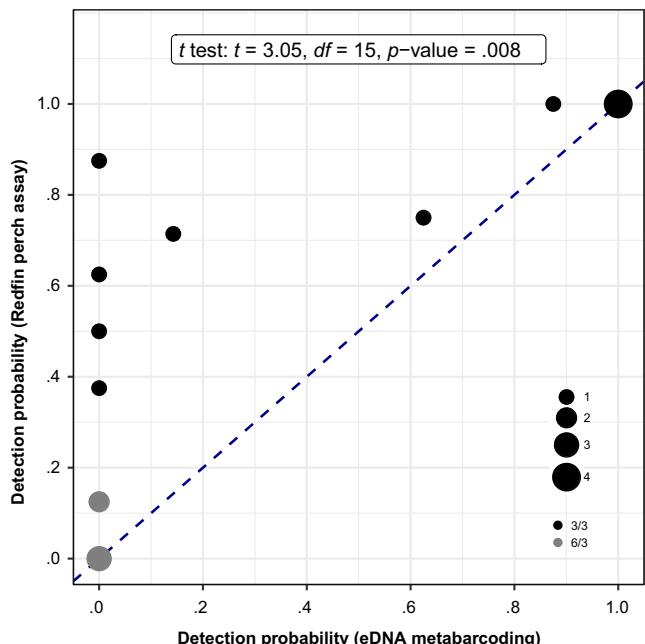


FIGURE 3 Detection probabilities for redfin perch obtained from the targeted eDNA analyses (y-axis) plotted against the detection probabilities obtained from the eDNA metabarcoding analyses (x-axis). Detection probabilities were calculated for each site ($n = 8$) by season ($n = 2$) combination as the proportion of samples testing positive for redfin perch. The size of the solid circles indicate the number of site by season combinations, and the dashed blue line represents the perfect fit if detection probabilities were identical between methods and the shading shows the underlying PCR replication levels of the data (PCR data/eDNA metabarcoding data). The text box shows the results of the paired sample t test

and, for most site by season combinations (i.e., 11 out of 16), the level of PCR replicates used to generate the data were consistent (Figure 3). Furthermore, detection probabilities were calculated as the proportion of samples testing positive for redfin perch eDNA to standardize the data and reduce the impact of varying levels of PCR replication. The differences in the replication levels in the underlying data are thus unlikely to be the main cause of the observed differences. A more plausible reason for the inability to detect redfin perch through the eDNA metabarcoding analyses is preferential amplification of sequences from other fishes present in the eDNA sample (Bylemans et al., 2018; Nichols et al., 2018; Piñol et al., 2018). A reduced amplification efficiency can be due to a relative high number of bp mismatches between the primers and the redfin perch templated DNA (Bylemans et al., 2018; Pinol, Mir, Gomez-Polo, & Agusti, 2015) or the low relative abundance of redfin perch eDNA compared to that of other fish species (Vestheim & Jarman, 2008). When evaluating primer mismatches for all fish species observed in this study, it is clear that the number of bp mismatches between the primers and the redfin perch template DNA is low relative to other species (Appendix S1). The low abundance of redfin perch eDNA relative to all other fish species thus seems the most likely explanation for the reduced detection sensitivity in the eDNA metabarcoding analyses.

Although the detection of rare taxa may be improved by increasing the level of replication (Ficetola et al., 2015), use of unique MID-tag combination for each PCR replicate and increasing the sequencing depth (Grey et al., 2018); all these measurements will increase the costs for eDNA metabarcoding surveys. Overall, the results show that eDNA metabarcoding will be less suitable for the fine-scale monitoring of distribution patterns than targeted eDNA analyses.

The eDNA metabarcoding data and the linear regression analyses showed a negative interaction between the proportional read abundances of *N. australis* and the invasive species common carp and redfin perch. However, several biases need to be taken into consideration to conclude that these invasive species are having a negative impact on *N. australis*. Firstly, species-specific differences in amplification efficiency (i.e., due to primer-template mismatches) will influence proportional read abundances (Bylemans et al., 2018; Elbrecht & Leese, 2015). Nonetheless, multiple studies have indicated that read abundances correlate with species biomass (Evans et al., 2015; Häfling et al., 2016). Second, differences in the number of species detected per sampling unit inherently bias proportional abundance data (i.e., as the total number of species increases, the proportional abundance is expected to decrease). Finally, as invasive species spread, establish, and increase in abundance, they will contribute more toward the total eDNA pool. A relative increase in invasive species DNA may, by itself, reduce the proportional read abundances of the native species even if their absolute abundance remains unchanged. Relatively few primer-template mismatches are present for the species in Blakney Creek for this to impact the eDNA metabarcoding, although patchy detections of one species (*Retropinna semoni*) by eDNA metabarcoding could be attributed to a reduced amplification efficiency from multiple primer-template mismatches (see Appendix S1 for full details) (Bylemans et al., 2018). The absence of any negative correlation for the proportional read abundances of other native species, excluding *N. australis*, suggests that the number of detected species and the relative increase in invasive species eDNA are not inherently reducing the proportional abundance data (Figure 4a,b). Consequently, the negative correlation found between the proportional reads of *N. australis* and the invasive species provides evidence of a real negative impact by common carp and/or redfin perch. Although the relative contribution of the two invasive fish species cannot be assessed from the eDNA data, previous surveys within this catchment have found that predation by redfin perch is the most likely cause (Pearce, 2015). Common carp may also exert additional negative pressure by increasing water turbidity, through their feeding behavior, which in turn reduces the abundance of aquatic macrophytes (i.e., the preferred habitat for *N. australis*) (Price, Stoffels, Weatherman, O'Keefe, & Müller, 2016; Vilizzi, Thwaites, Smith, Nicol, & Madden, 2014).

Finally, a highly unexpected finding of the eDNA metabarcoding results was the exclusive presence of *Hypseleotris* sp. Lake's carp gudgeon in the upstream sites in Urumwala Creek. It was previously believed that this species only occurred as a F1 hemi-clone which requires the presence of a sexually reproducing species to carry on their lineage (Bertozzi, Adams, & Walker, 1997; Schmidt, Bond, Adams, & Hughes, 2011). The data obtained from the current survey

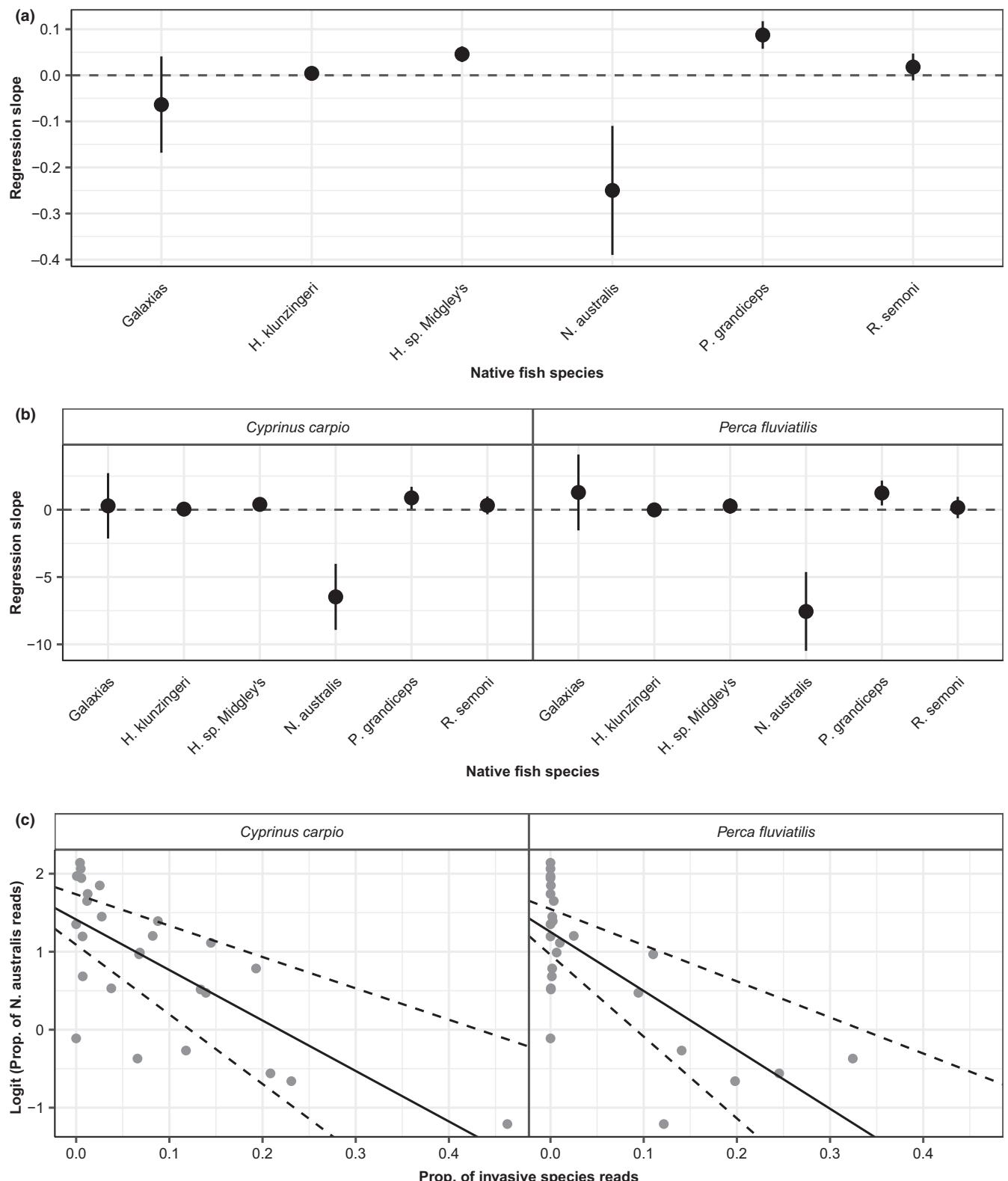


FIGURE 4 Estimated regression slopes showing the relationship between the logit-transformed proportional read abundances for: (a) native species and the number of species detected; (b) proportional read abundances of the two invasive species, common carp (*Cyprinus carpio*) and redfin perch (*Perca fluviatilis*), and (c) relationship between the logit-transformed abundance data for southern pygmy perch (*Nannoperca australis*) and both invasive species. The 95% confidence intervals around the mean estimated regression slopes are shown as a line (a and b) and by dashed lines around the best fitting model (c)

and an extensive sampling survey for the *Hypseleotris* species complex have now provided evidence that sexually reproducing populations of *Hypseleotris* sp. *Lake's carp gudgeon* are still persisting in the MDB although they are restricted in their range (for full details see Unmack et al. 2019). This finding clearly illustrates the ability of eDNA metabarcoding to uncover cryptic diversity. However, it is important to note that the ability to detect cryptic taxa will depend strongly on the taxonomic resolution of barcoding region used.

5 | CONCLUSION

Comparison between eDNA detection methods reveals that a targeted eDNA survey is more sensitive than a whole community metabarcoding approach. This means that management and research questions that focus on indicator species (e.g., invasive and threatened taxa) are best served by adopting a targeted eDNA approach. This would ensure better allocation of time and financial resources to obtain the detailed distribution data required to answer research questions and/or achieve the desired management outcomes (Bylemans et al., 2016; Harper et al., 2018). However, when prior knowledge of the study system is limited, eDNA metabarcoding surveys have the ability to provide baseline information on biodiversity patterns (e.g., cryptic species and novel incursions of invasive species) (Blackman et al., 2017). The obtained information would then allow for the design of more sensitive targeted eDNA surveys designed to address the research and/or management needs. Finally, when surveys are required to study biological interactions, an eDNA metabarcoding survey is most suitable as it has the potential to quantify biotic responses to disturbances (e.g., the impact of invasive species) provided that all biases in the data can be identified.

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AUTHOR CONTRIBUTION

J.B., D.M.G., C.M.H. and E.M.F. contributed to the study and sampling design; J.B. conducted all the field and laboratory work; J.B. performed the data analyses with significant contributions of R.P.D. and E.M.F.; J.B. led the writing of the manuscript with all authors contributing and giving final approval for publication.

DATA AVAILABILITY STATEMENT

Full details of the sampling sites and the number of samples analyses for each aspect of this study are available in the supplementary information together with a detailed description of the data analyses (Appendix S1). The summarized data for both the targeted eDNA and the eDNA metabarcoding surveys, and the Rscripts used to clean-up and analyze the data are also available in Appendix S2.

ORCID

- Jonas Bylemans  <https://orcid.org/0000-0001-6263-0874>
- Dianne M. Gleeson  <https://orcid.org/0000-0002-5093-4405>
- Richard P. Duncan  <https://orcid.org/0000-0003-2295-449X>
- Christopher M. Hardy  <https://orcid.org/0000-0002-7419-566X>
- Elise M. Furlan  <https://orcid.org/0000-0002-1642-9819>

REFERENCES

- Alberdi, A., Aizpurua, O., Gilbert, M. T. P., & Bohmann, K. (2018). Scrutinizing key steps for reliable metabarcoding of environmental samples. *Methods in Ecology and Evolution*, 9(1), 134–147. <https://doi.org/10.1111/2041-210X.12849>
- Baird, D. J., & Hajibabaei, M. (2012). Biomonitoring 2.0: A new paradigm in ecosystem assessment made possible by next-generation DNA sequencing. *Molecular Ecology*, 21(8), 2039–2044.
- Bertozzi, T., Adams, M., & Walker, K. F. (1997). Species boundaries in carp gudgeons (Eleotrididae : Hypseleotris) from the River Murray, South Australia: Evidence for multiple species and extensive hybridization. *Marine and Freshwater Research*, 51(8), 805–815. <https://doi.org/10.1071/MF00039>
- Bista, I., Carvalho, G. R., Walsh, K., Seymour, M., Hajibabaei, M., Lallias, D., ... Creer, S. (2017). Annual time-series analysis of aqueous eDNA reveals ecologically relevant dynamics of lake ecosystem biodiversity. *Nature Communications*, 8, 14087. <https://doi.org/10.1038/ncomms14087>
- Blackman, R. C., Constable, D., Hahn, C., Sheard, A. M., Durkota, J., Häneling, B., & Handley, L. L. (2017). Detection of a new non-native freshwater species by DNA metabarcoding of environmental samples – First record of *Gammarus fossarum* in the UK. *Aquatic Invasions*, 12(2), 177–189. <https://doi.org/10.3391/ai.2017.12.2.06>
- Bolger, A. M., Lohse, M., & Usadel, B. (2014). Trimmomatic: A flexible trimmer for Illumina sequence data. *Bioinformatics*, 30(15), 2114–2120. <https://doi.org/10.1093/bioinformatics/btu170>
- Boyer, F., Mercier, C., Bonin, A., Le Bras, Y., Taberlet, P., & Coissac, E. (2016). obitools: A unix-inspired software package for DNA metabarcoding. *Molecular Ecology Resources*, 16(1), 176–182. <https://doi.org/10.1111/1755-0998.12428>
- Buxton, A. S., Groombridge, J. J., Zakaria, N. B., & Griffiths, R. A. (2017). Seasonal variation in environmental DNA in relation to population size and environmental factors. *Scientific Reports*, 7, 46294. <https://doi.org/10.1038/srep46294>
- Bylemans, J., Furlan, E. M., Pearce, L., Daly, T., & Gleeson, D. M. (2016). Improving the containment of a freshwater invader using environmental DNA (eDNA) based monitoring. *Biological Invasions*, 18(10), 3081–3089. <https://doi.org/10.1007/s10530-016-1203-5>
- Bylemans, J., Gleeson, D. M., Hardy, C. M., & Furlan, E. (2018). Toward an ecoregion scale evaluation of eDNA metabarcoding primers: A

- case study for the freshwater fish biodiversity of the Murray-Darling Basin (Australia). *Ecology and Evolution*, 8(17), 8697–8712. <https://doi.org/10.1002/ece3.4387>
- Cilleros, K., Valentini, A., Allard, L., Dejean, T., Etienne, R., Grenouillet, G., ... Brosse, S. (2019). Unlocking biodiversity and conservation studies in high-diversity environments using environmental DNA (eDNA): A test with Guianese freshwater fishes. *Molecular Ecology Resources*, 19(1), 27–46. <https://doi.org/10.1111/1755-0998.12900>
- Cristescu, M. E. (2014). From barcoding single individuals to metabarcoding biological communities: Towards an integrative approach to the study of global biodiversity. *Trends in Ecology & Evolution*, 29(10), 566–571. <https://doi.org/10.1016/j.tree.2014.08.001>
- De Barba, M., Miquel, C., Boyer, F., Mercier, C., Rioux, D., Coissac, E., & Taberlet, P. (2014). DNA metabarcoding multiplexing and validation of data accuracy for diet assessment: Application to omnivorous diet. *Molecular Ecology Resources*, 14(2), 306–323. <https://doi.org/10.1111/1755-0998.12188>
- De Souza, L. S., Godwin, J. C., Renshaw, M. A., & Larson, E. (2016). Environmental DNA (eDNA) detection probability is influenced by seasonal activity of organisms. *PLoS ONE*, 11(10), e0165273. <https://doi.org/10.1371/journal.pone.0165273>
- Deiner, K., Bik, H. M., Mächler, E., Seymour, M., Lacoursière-Roussel, A., Altermatt, F., ... Bernatchez, L. (2017). Environmental DNA metabarcoding: Transforming how we survey animal and plant communities. *Molecular Ecology*, 26(21), 5872–5895. <https://doi.org/10.1111/mec.14350>
- Deiner, K., Walser, J.-C., Mächler, E., & Altermatt, F. (2015). Choice of capture and extraction methods affect detection of freshwater biodiversity from environmental DNA. *Biological Conservation*, 183, 53–63. <https://doi.org/10.1016/j.biocon.2014.11.018>
- DiBattista, J. D., Coker, D. J., Sinclair-Taylor, T. H., Stat, M., Berumen, M. L., & Bunce, M. (2017). Assessing the utility of eDNA as a tool to survey reef-fish communities in the Red Sea. *Coral Reefs*, 36(4), 1245–1252. <https://doi.org/10.1007/s00338-017-1618-1>
- Doi, H., Inui, R., Akamatsu, Y., Kanno, K., Yamanaka, H., Takahara, T., & Minamoto, T. (2017). Environmental DNA analysis for estimating the abundance and biomass of stream fish. *Freshwater Biology*, 62(1), 30–39. <https://doi.org/10.1111/fwb.12846>
- Elbrecht, V., & Leese, F. (2015). Can DNA-based ecosystem assessments quantify species abundance? Testing primer bias and biomass-sequence relationships with an innovative metabarcoding protocol. *PLoS ONE*, 10(7), 1–16. <https://doi.org/10.1371/journal.pone.0130324>
- Elbrecht, V., Vamos, E. E., Meissner, K., Aroviita, J., & Leese, F. (2017). Assessing strengths and weaknesses of DNA metabarcoding-based macroinvertebrate identification for routine stream monitoring. *Methods in Ecology and Evolution*, 8(10), 1265–1275. <https://doi.org/10.1111/2041-210X.12789>
- Evans, N. T., Olds, B. P., Turner, C. R., Renshaw, M. A., Li, Y., Jerde, C. L., ... Lodge, D. M. (2015). Quantification of mesocosm fish and amphibian species diversity via eDNA metabarcoding. *Molecular Ecology Resources*, 16(1), 29–41. <https://doi.org/10.1111/1755-0998.12433>
- Ficetola, G. F., Miaud, C., Pompanon, F., & Taberlet, P. (2008). Species detection using environmental DNA from water samples. *Biology Letters*, 4(4), 423–425. <https://doi.org/10.1098/rsbl.2008.0118>
- Ficetola, G. F., Pansu, J., Bonin, A., Coissac, E., Giguet-Covex, C., De Barba, M., ... Taberlet, P. (2015). Replication levels, false presences, and the estimation of presence/absence from eDNA metabarcoding data. *Molecular Ecology Resources*, 15(3), 543–556. <https://doi.org/10.1111/1755-0998.12338>
- Furlan, E. M., & Gleeson, D. (2016a). Environmental DNA detection of redfin perch, *Perca fluviatilis*. *Conservation Genetics Resources*, 8(2), 115–118. <https://doi.org/10.1007/s12686-016-0523-1>
- Furlan, E. M., & Gleeson, D. (2016b). Improving reliability in environmental DNA detection surveys through enhanced quality control. *Marine and Freshwater Research*, 68(2), 388–395. <https://doi.org/10.1071/MF1534>
- Furlan, E. M., Gleeson, D., Hardy, C. M., & Duncan, R. P. (2016). A framework for estimating the sensitivity of eDNA surveys. *Molecular Ecology Resources*, 16(3), 641–654. <https://doi.org/10.1111/1755-0998.12483>
- Goldberg, C. S., Pilliod, D. S., Arkle, R. S., & Waits, L. P. (2011). Molecular detection of vertebrates in stream water: A demonstration using Rocky Mountain tailed frogs and Idaho giant salamanders. *PloS ONE*, 6(7), e22746. <https://doi.org/10.1371/journal.pone.0022746>
- Grey, E. K., Bernatchez, L., Cassey, P., Deiner, K., Deveney, M., Howland, K. L., ... Lodge, D. M. (2018). Effects of sampling effort on biodiversity patterns estimated from environmental DNA metabarcoding surveys. *Scientific Reports*, 8(1), 2–11. <https://doi.org/10.1038/s41598-018-27048-2>
- Lawson Handley, L., Read, D. S., Winfield, I. J., Kimbell, H., Johnson, H., Li, J., ... Häneling, B. (2019). Temporal and spatial variation in distribution of fish environmental DNA in England's largest lake. *Environmental DNA*, 1, 26–39. <https://doi.org/10.1002/edn3.5>
- Häneling, B., Lawson Handley, L., Read, D. S., Hahn, C., Li, J., Nichols, P., ... Winfield, I. J. (2016). Environmental DNA metabarcoding of lake fish communities reflects long-term data from established survey methods. *Molecular Ecology*, 25(13), 3101–3119. <https://doi.org/10.1111/mec.13660>
- Harper, L. R., Handley, L. L., Hahn, C., Boonham, N., Rees, H. C., Gough, K. C., ... Häneling, B. (2018). Needle in a haystack? A comparison of eDNA metabarcoding and targeted qPCR for detection of the great crested newt (*Triturus cristatus*). *Ecology and Evolution*, 8, 6330–6341. <https://doi.org/10.1002/ece3.4013>
- Harper, L. R., Handley, L. L., Hahn, C., Boonham, N., Rees, H. C., Lewis, E., ... Häneling, B. (2019). Testing ecological hypotheses at the pondscape with environmental DNA metabarcoding: A case study on a threatened amphibian. *BioRxiv*, 278309. <https://doi.org/10.1101/278309>
- Jane, S. F., Wilcox, T. M., McKelvey, K. S., Young, M. K., Schwartz, M. K., Lowe, W. H., ... Whiteley, A. R. (2015). Distance, flow, and PCR inhibition: eDNA dynamics in two headwater streams. *Molecular Ecology Resources*, 15(1), 216–227. <https://doi.org/10.1111/1755-0998.12285>
- Jerde, C. L., Mahon, A. R., Chadderton, W. L., & Lodge, D. M. (2011). "Sight-unseen" detection of rare aquatic species using environmental DNA. *Conservation Letters*, 4(2), 150–157. <https://doi.org/10.1111/j.1755-263X.2010.00158.x>
- Jo, T., Murakami, H., Yamamoto, S., Masuda, R., & Minamoto, T. (2019). Effect of water temperature and fish biomass on environmental DNA shedding, degradation, and size distribution. *Ecology and Evolution*, 9(3), 1135–1146. <https://doi.org/10.1002/ece3.4802>
- Kellner, K. (2015). *jagsUI: A wrapper around rjags to streamline JAGS analyses*. R Package. Retrieved from <https://github.com/kenkellner/jagsUI>
- Klymus, K. E., Richter, C. A., Chapman, D. C., & Paukert, C. (2014). Quantification of eDNA shedding rates from invasive bighead carp *Hypophthalmichthys nobilis* and silver carp *Hypophthalmichthys molitrix*. *Biological Conservation*, 183, 77–84. <https://doi.org/10.1016/j.biocon.2014.11.020>
- Lacoursière-Roussel, A., Côté, G., Leclerc, V., & Bernatchez, L. (2015). Quantifying relative fish abundance with eDNA: A promising tool for fisheries management. *Journal of Applied Ecology*, 53(4), 1148–1157. <https://doi.org/10.1111/1365-2664.12598>
- Lacoursière-Roussel, A., Dubois, Y., Normandeau, E., Bernatchez, L., & Adamowicz, S. (2016). Improving herpetological surveys in eastern North America using the environmental DNA method. *Genome*, 59(11), 991–1007. <https://doi.org/10.1139/gen-2015-0218>

- Lopes, C. M., Sasso, T., Valentini, A., Dejean, T., Martins, M., Zamudio, K. R., & Haddad, C. F. B. (2017). eDNA metabarcoding: A promising method for anuran surveys in highly diverse tropical forests. *Molecular Ecology Resources*, 17(5), 904–914. <https://doi.org/10.1111/1755-0998.12643>
- Miya, M., Sato, Y., Fukunaga, T., Sado, T., Poulsen, J. Y., Sato, K., ... Iwasaki, W. (2015). MiFish, a set of universal PCR primers for metabarcoding environmental DNA from fishes: Detection of more than 230 subtropical marine species. *Royal Society Open Science*, 2, 150088. <https://doi.org/10.1098/rsos.150088>
- Nichols, J. D., & Williams, B. K. (2006). Monitoring for conservation. *Trends in Ecology and Evolution*, 21(12), 668–673. <https://doi.org/10.1016/j.tree.2006.08.007>
- Nichols, R. V., Vollmers, C., Newsom, L. A., Wang, Y., Heintzman, P. D., Leighton, M. K., ... Shapiro, B. (2018). Minimizing polymerase biases in metabarcoding. *Molecular Ecology Resources*, 18, 927–939. <https://doi.org/10.1111/1755-0998.12895>
- Pearce, L. (2015). Surveys, monitoring and conservation status of Southern Pygmy Perch (*Nannoperca australis*) within Blakney and Pudman Creeks. http://www.dpi.nsw.gov.au/_data/assets/pdf_file/0011/551927/southern_pygmy_perch_web_version.pdf
- Pinheiro, J., Bates, D., DebRoy, S., Sarkar, D., & R Core Team (2019). *nlme: Linear and Nonlinear Mixed Effects Models*. R package version 3.1-141. <https://CRAN.R-project.org/package=nlme>
- Pinol, J., Mir, G., Gomez-Polo, P., & Agusti, N. (2015). Universal and blocking primer mismatches limit the use of high-throughput DNA sequencing for the quantitative metabarcoding of arthropods. *Molecular Ecology Resources*, 15(4), 819–830. <https://doi.org/10.1111/1755-0998.12355>
- Piñol, J., Senar, M. A., & Symondson, W. O. C. (2018). The choice of universal primers and the characteristics of the species mixture determine when DNA metabarcoding can be quantitative. *Molecular Ecology*, 28, 407–419. <https://doi.org/10.1111/mec.14776>
- Price, A., Stoffels, R., Weatherman, K., O'Keefe, R., & Müller, W. (2016). Structural habitat selection by Southern pygmy perch (*Nannoperca australis*). Retrieved from www.mdfrc.org.au/projects/current-projects/images/Southern-Pygmy-Perch-Final-Report.pdf
- R Development Core Team (2010). *R: A language and environment for statistical computing*. Vienna, Austria: R Foundation for Statistical Computing.
- Schmidt, D. J., Bond, N. R., Adams, M., & Hughes, J. M. (2011). Cytonuclear evidence for hybridogenetic reproduction in natural populations of the Australian carp gudgeon (*Hypseleotris: Eleotridae*). *Molecular Ecology*, 20(16), 3367–3380. <https://doi.org/10.1111/j.1365-294X.2011.05206.x>
- Schneider, J., Valentini, A., Dejean, T., Montarsi, F., Taberlet, P., Glaizot, O., & Fumagalli, L. (2016). Detection of invasive mosquito vectors using environmental DNA (eDNA) from water samples. *PLoS ONE*, 11(9), 1–18. <https://doi.org/10.1371/journal.pone.0162493>
- Shogren, A. J., Tank, J. L., Egan, S. P., August, O., Rosi, E. J., Hanrahan, B. R., ... Bolster, D. (2018). Water flow and biofilm cover influence environmental DNA detection in recirculating streams. *Environmental Science and Technology*, 52(15), 8530–8537. <https://doi.org/10.1021/acs.est.8b01822>
- Siddig, A. A. H., Ellison, A. M., Ochs, A., Villar-Leeman, C., & Lau, M. K. (2017). How do ecologists select and use indicator species to monitor ecological change? Insights from 14 years of publication in Ecological Indicators. *Ecological Indicators*, 60, 223–230. <https://doi.org/10.1016/j.ecolind.2015.06.036>
- Sigsgaard, E. E., Carl, H., Møller, P. R., & Thomsen, P. F. (2015). Monitoring the near-extinct European weather loach in Denmark based on environmental DNA from water samples. *Biological Conservation*, 183, 46–52. <https://doi.org/10.1016/j.biocon.2014.11.023>
- Simberloff, D. (1998). Flagships, umbrellas, and keystones: Is single-species management passe in the landscape era? *Biological Conservation*, 83(3), 247–257. [https://doi.org/10.1016/S0006-3207\(97\)00081-5](https://doi.org/10.1016/S0006-3207(97)00081-5)
- Smithson, M., & Verkuilen, J. (2006). A better lemon squeezer? Maximum-likelihood regression with beta-distributed dependent variables. *Psychological Methods*, 11(1), 54–71. <https://doi.org/10.1037/1082-989X.11.1.54>
- Takahara, T., Minamoto, T., & Doi, H. (2013). Using environmental DNA to estimate the distribution of an invasive fish species in ponds. *PLoS ONE*, 8(2), e56584. <https://doi.org/10.1371/journal.pone.0056584>
- Takahara, T., Minamoto, T., Yamanaka, H., Doi, H., & Kawabata, Z. (2012). Estimation of fish biomass using environmental DNA. *PLoS ONE*, 7(4), e35868. <https://doi.org/10.1371/journal.pone.0035868>
- Takahashi, M. K., Meyer, M. J., McPhee, C., Gaston, J. R., Venesky, M. D., & Case, B. F. (2017). Seasonal and diel signature of eastern hellbender environmental DNA. *Journal of Wildlife Management*, 82, 217–225. <https://doi.org/10.1002/jwmg.21349>
- Thomsen, P. F., Kielgast, J., Iversen, L. L., Wiuf, C., Rasmussen, M., Gilbert, M. T. P., ... Willerslev, E. (2012). Monitoring endangered freshwater biodiversity using environmental DNA. *Molecular Ecology*, 21(11), 2565–2573. <https://doi.org/10.1111/j.1365-294X.2011.05418.x>
- Tsuji, S., Iguchi, Y., Shibata, N., Teramura, I., Kitagawa, T., & Yamanaka, H. (2018). Real-time multiplex PCR for simultaneous detection of multiple species from environmental DNA: An application on two Japanese medaka species. *Scientific Reports*, 8(1), 1–5. <https://doi.org/10.1038/s41598-018-27434-w>
- Unmack, P. J., Adams, M., Bytemans, J., Hardy, C. M., Hammer, M. P., & Georges, A. (2019). Perspectives on the clonal persistence of presumed 'ghost' genomes in unisexual or allopolyploid taxa arising via hybridization. *Scientific Reports*, 9(1), 1–10. <https://doi.org/10.1038/s41598-019-40865-3>
- Ushio, M., Murakami, H., Masuda, R., Sado, T., Miya, M., Sakurai, S., ... Kondoh, M. (2018). Quantitative monitoring of multispecies fish environmental DNA using high-throughput sequencing. *Metabarcoding and Metagenomics*, 2, e23297. <https://doi.org/10.1101/113472>
- Valentini, A., Pompanon, F., & Taberlet, P. (2009). DNA barcoding for ecologists. *Trends in Ecology & Evolution*, 24(2), 110–117. <https://doi.org/10.1016/j.tree.2008.09.011>
- Valentini, A., Taberlet, P., Miaud, C., Civade, R., Herder, J., Thomsen, P. F., ... Dejean, T. (2016). Next-generation monitoring of aquatic biodiversity using environmental DNA metabarcoding. *Molecular Ecology*, 25(4), 929–942. <https://doi.org/10.1111/mec.13428>
- Vestheim, H., & Jarman, S. N. (2008). Blocking primers to enhance PCR amplification of rare sequences in mixed samples – A case study on prey DNA in Antarctic krill stomachs. *Frontiers in Zoology*, 5, 12. <https://doi.org/10.1186/1742-9994-5-12>
- Vilizzi, L., Thwaites, L. A., Smith, B. B., Nicol, J. M., & Madden, C. P. (2014). Ecological effects of common carp (*Cyprinus carpio*) in a semi-arid floodplain wetland. *Marine and Freshwater Research*, 65(1), 802–817. <https://doi.org/10.1071/MF13163>
- Wickham, H. (2016). *tidyverse: Easily install and load "Tidyverse" packages*. R Package. Retrieved from <https://github.com/tidyverse/tidyverse>
- Wozney, K. M., & Wilson, C. C. (2017). Quantitative PCR multiplexes for simultaneous multispecies detection of Asian carp eDNA. *Journal of Great Lakes Research*, 43(4), 771–776. <https://doi.org/10.1016/j.jglr.2017.05.001>
- Yamamoto, S., Masuda, R., Sato, Y., Sado, T., Araki, H., Kondoh, M., ... Miya, M. (2017). Environmental DNA metabarcoding reveals local fish communities in a species-rich coastal sea. *Scientific Reports*, 7, 40368. <https://doi.org/10.1038/srep40368>
- Yoccoz, N. G. (2012). The future of environmental DNA in ecology. *Molecular Ecology*, 21(8), 2031–2038. <https://doi.org/10.1111/j.1365-294X.2012.05505.x>

Yoccoz, N. G., Nichols, J. D., & Boulinier, T. (2001). Monitoring of biological diversity in space and time. *Trends in Ecology and Evolution*, 16(8), 446–453. [https://doi.org/10.1016/S0169-5347\(01\)02205-4](https://doi.org/10.1016/S0169-5347(01)02205-4)

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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