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MONITORING NATIVE FISH IN THE LACHLAN CATCHMENT THROUGH ENVIRONMENTAL DNA AND CONVENTIONAL METHODS

SUMMARY OF RESULTS & RECOMMENDATIONS

Rheyda Hinlo, Ben Broadhurst, Jonas Bylemans, Hugh Allan, Dianne Gleeson,
Jason Thiem and Fiona Dyer

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Inquiries regarding this document should be addressed to:

Associate Professor Dianne Gleeson

Ph: 02 6201 2237

e-mail: Dianne.Gleeson@canberra.edu.au

or

Associate Professor Fiona Dyer

Phone: 02 6201 2452

e-mail: Fiona.Dyer@canberra.edu.au

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1. SUMMARY

Environmental DNA (eDNA) metabarcoding was implemented in two reaches of the Lachlan River to demonstrate the application of eDNA as a complementary monitoring technique and compare the technique against conventional fish monitoring undertaken as part of the Long-Term Intervention Monitoring (LTIM) and Short-Term Intervention Monitoring (STIM) programs in the Lachlan River. The project compared species richness and measures of fish abundance obtained through eDNA metabarcoding against those obtained through conventional monitoring and evaluated the potential of eDNA monitoring to answer questions related to the contribution of Commonwealth Environmental Water to the fish community.

Across both reaches, the number of species identified through eDNA metabarcoding was greater than that identified through conventional monitoring (12 vs 11 species). At the reach level, there was a significant difference between methods in terms of presence-absence of species (Global $R = 0.046$, $p = 0.014$). Conventional monitoring was not significantly different to eDNA metabarcoding without the use of Cyprinid blocking primer (CBP) (R statistic = 0.055, $p = 0.065$), but was significantly different to eDNA metabarcoding with CBP (R statistic = 0.082, $p = 0.01$). Two native fish species, the freshwater catfish (*Tandanus tandanus*) and silver perch (*Bidyanus bidyanus*), were only detected in the lower reach by the eDNA metabarcoding technique (without CBP). On the other hand, Australian smelt (*Retropinna semoni*) was only detected in the lower reach by conventional monitoring.

At the reach scale, results of the correlation analysis showed only a significant weak positive relationship between number of eDNA reads (with CBP) and conventional estimates of biomass and abundance in the mid Lachlan. No significant relationships were seen for the lower Lachlan. However, at the species level, a strong relationship was found between abundance and biomass and number of eDNA reads (with and without CBP) for bony herring and Murray cod. Moderate and significant positive relationships were also seen for eastern gambusia and carp gudgeon. These species which had significant positive relationships are either large-bodied and/or abundant in the system.

The Cyprinid blocking primer (CBP) reduced the number of common carp DNA reads by 97% in the lower Lachlan and by 69 % in the mid Lachlan. The CBP effectively blocked all goldfish DNA from amplifying in both reaches. However, the use of CBP did not improve the detection probabilities of native fish in the system. In fact, detection probability for native fish was moderately lower for the mid Lachlan when CBP was used. The freshwater catfish was only detected when CBP was not used.

The integration of eDNA metabarcoding in the 2019 monitoring resulted in more robust species richness data for the lower Lachlan River through the detection of freshwater catfish and silver perch. eDNA metabarcoding has the capacity to improve the accuracy of the evaluations pertaining to native fish populations and survival in the Lachlan system, and therefore can aid in answering the questions related to CEWO's contribution to native fish populations. However, there is important information such as fish recruitment, sizes and age class that can only be gained through conventional monitoring. Hence, eDNA-based monitoring alone has limited capacity to answer CEWO's contribution towards native fish resilience and survival. It is, however, powerful when used in combination with other survey methods as a means to improve detection rates and enhance confidence in the monitoring results.

2. BACKGROUND

The use of environmental DNA (eDNA) techniques to monitor biodiversity is increasing. The eDNA-based monitoring technique is based on identifying the presence of organisms in the environment through the DNA they leave in the environment. All animals leave traces of DNA in their surroundings, whether it be from shed skin, hair, mucus, faeces etc. In water, DNA degrades quite quickly over time, which means that the presence of a species' DNA in the environment signifies a relatively recent presence (Pilliod et al. 2014).

The analysis of eDNA involves a series of steps, including eDNA capture, extraction, amplification, DNA sequencing, and assignment (Fig. 1). In aquatic environments, water samples are taken and filtered to capture the DNA in the water. DNA from the filter paper is then extracted and then amplified through a method called Polymerase Chain Reaction (PCR). PCR makes many copies of the DNA molecules of interest so that it is easier for these DNA to be detected. This is the step wherein species-specific or universal primers are used to target the DNA of interest. The amplified DNA is then sent for sequencing and the specific DNA sequences matched to the target organism/s (Figure 1).

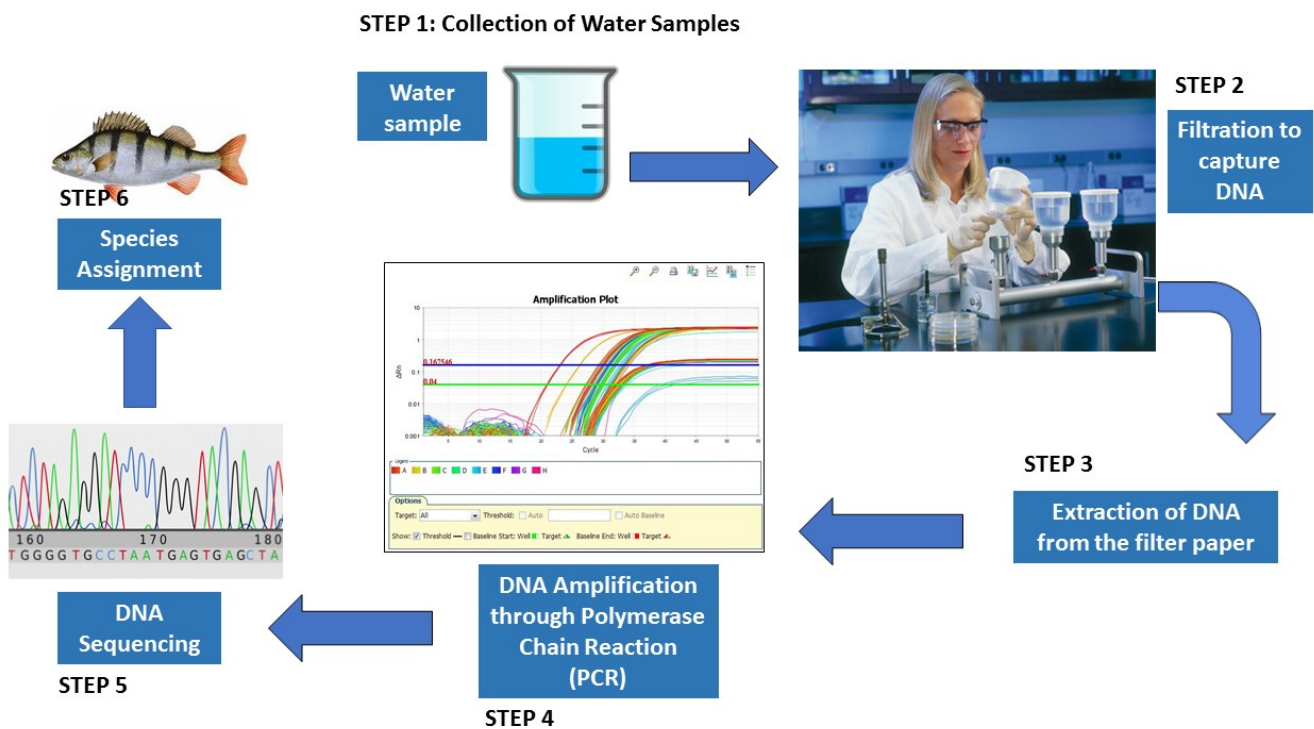


Figure 1. Steps in environmental DNA monitoring.

Environmental DNA-based monitoring can either be a targeted approach (usually called single-species detection) or a community approach where entire communities can be characterized (also called eDNA metabarcoding). The two approaches are similar, except that species-specific primers are used for targeted detection while universal primers and next-generation sequencing are used for eDNA metabarcoding (Thomsen et al. 2012; Valentini et al. 2016).

In this project, eDNA metabarcoding was implemented to demonstrate the application of eDNA as a complementary monitoring technique and compare with conventional fish monitoring techniques undertaken as part of the Long-Term Intervention Monitoring (LTIM) and Short-Term Intervention Monitoring (STIM) programs in the Lachlan River. The main objective of the project was to compare eDNA results with the fish community monitoring in terms of species richness and measures of fish abundance and to evaluate the potential of eDNA monitoring to contribute to answering questions relating to the contribution of Commonwealth environmental water to the fish community. These questions include the following:

- What did Commonwealth environmental water contribute to native fish populations?
- What did Commonwealth environmental water contribute to native fish community resilience?
- What did Commonwealth environmental water contribute to native fish survival?

Recent research have shown that environmental DNA-based monitoring can be an effective tool to complement conventional approaches to monitoring in aquatic ecosystems (Hänfling et al. 2016; Hinlo et al. 2017; Shaw et al. 2016). For example, a metabarcoding study in South Australia found that more species can be detected using both fyke nets and eDNA metabarcoding compared to using one method alone (Shaw et al. 2016). In this study, a universal fish metabarcoding primer specifically developed for fish species occurring in the Murray-Darling Basin was used to target the detection of native species within the study area.

Fourteen species of native fish are historically known to occur in the lower Lachlan River system (Dean Gilligan, NSW DPI, *unpublished data*) (Table 3). Of these, four are believed to be locally extinct or extremely rare (flat-headed galaxias, southern pygmy perch, southern purple spotted gudgeon and the Murray-Darling rainbowfish), whilst three of the 10 extant species occur at very low abundances (olive perchlet, silver perch, freshwater catfish) (NSW DPI, *unpublished data*). Between 2014 and 2019, the LTIM program has regularly detected 6 native fish species, and occasionally 3 other species (Dyer et al. 2019). Given the number of rare species and low catch rates using conventional monitoring, there is considerable potential to use eDNA to improve detection rates for fish species in the lower Lachlan River system.

3. SCOPE OF THIS REPORT

The report presents a summary of the project findings and recommendations on the use of the eDNA method in the future monitoring of the Lachlan catchment. In this report, we compare the species richness and abundance obtained by conventional methods and eDNA metabarcoding during the 2019 monitoring program and present the performance of the Cyprinid blocking primer (CBP) tested in this study. Previous studies within the Murray-Darling Basin (MDB) have shown that within the MDB, the high abundance of common carp DNA can reduce the detection probability of rare native species (Bylemans et al. 2018). To overcome this, CBP was used to selectively block the amplification of carp DNA and increase the chances of detecting rare native species. This blocking primer was tested at two sites within the Murray-Darling Basin previously and was found to increase the detection of priority native species (Bylemans et al., unpublished). A more detailed explanation of the methods and results can be seen in the accompanying manuscript for publication, *Characterising a lowland fish community using conventional sampling and environmental DNA metabarcoding*.

4. METHODS

The study was carried out in two reaches in the Lachlan River; the lower and mid-Lachlan river systems in New South Wales, Australia. Fish community data was collected using conventional and eDNA-based monitoring methods from 18 river channel sites, 9 each from the Lower Lachlan river system from Wallanthery to Hillston, and from the mid-Lachlan River reach from Forbes to Lake Brewster (Fig. 2). Sampling occurred from February to March 2019. Conventional monitoring consisted of a suite of passive and active gears including boat-electrofishing, unbaited bait traps, small fyke nets and large fyke nets. Twelve 1-L water samples per site were taken for eDNA metabarcoding analysis. These samples were stored on ice immediately after collection and eDNA captured within 12 – 24 h by filtering through cellulose nitrate filter papers. DNA extraction and subsequent laboratory processing were done in a dedicated laboratory for trace samples at the University of Canberra, Bruce, ACT. Samples for eDNA metabarcoding were analyzed using two treatments: with and without the Cyprinid Blocking Primer (CBP). Next generation sequencing was performed at the Ramaciotti Centre for Genomics in Sydney, NSW.

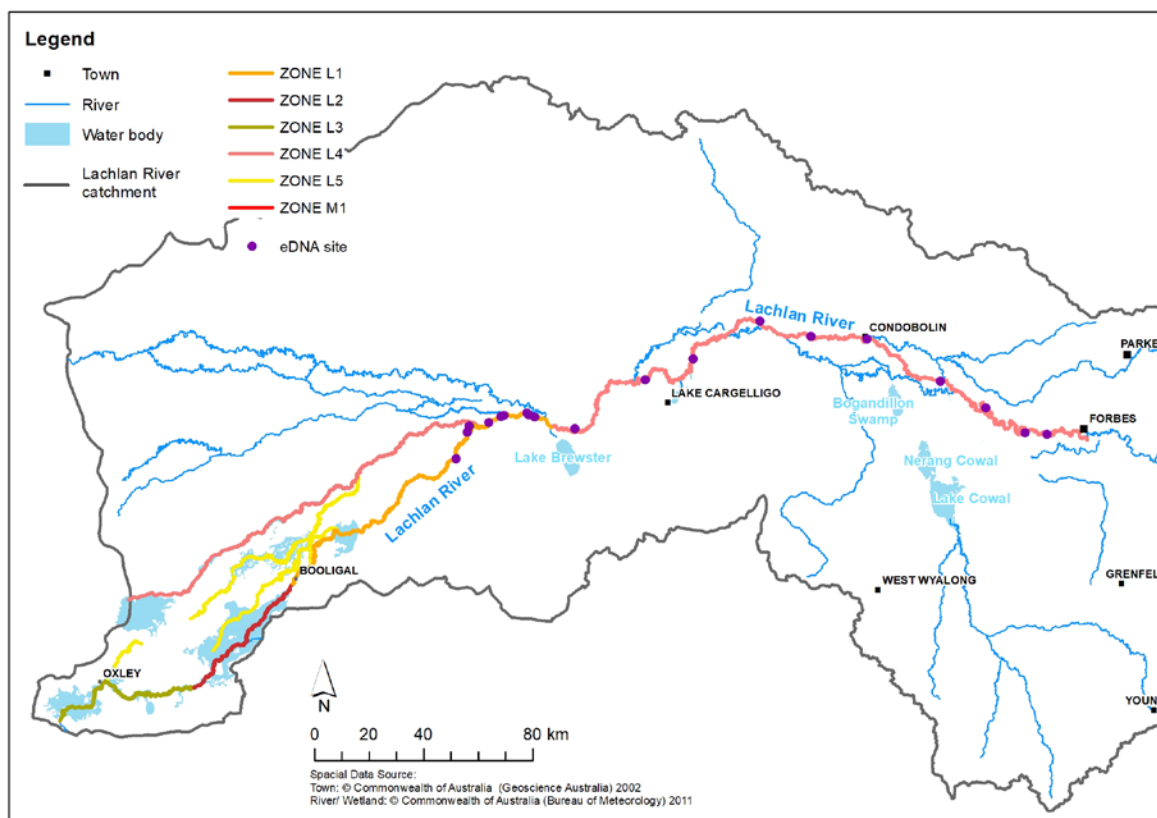


Figure 2. Sampling sites along the lower and mid-Lachlan river systems

5. RESULTS

SPECIES RICHNESS

Across both reaches combined, eDNA metabarcoding detected a total of 12 species compared to 11 species detected by conventional monitoring. At the reach scale, eDNA metabarcoding (without CBP) had the greatest species richness in the lower reach (10 species) compared to conventional monitoring (9 species) and eDNA metabarcoding with CBP (8 species) (Table 1). In the mid Lachlan, the eDNA metabarcoding technique and conventional sampling methods obtained the same species richness (11 species) (Table 1).

Two native fish species, the freshwater catfish and silver perch, were only detected in the lower reach using the eDNA metabarcoding technique (without CBP). Freshwater catfish was last caught in the lower Lachlan in 2015, whereas silver perch has not been caught in the same reach since the LTIM monitoring program commenced in 2015 (Dyer et al. 2019). Conventional monitoring had a generally higher detection rate for Australian smelt and flatheaded gudgeon. Australian smelt was only detected in the lower Lachlan reach using conventional monitoring.

One advantage of eDNA metabarcoding was that it was able to distinguish four species under the genus *Hypseleotris* (carp gudgeons). Morphological identification of carp gudgeon down to the species level is very challenging. Based on eDNA metabarcoding data, four species of carp gudgeons are present in the Lachlan system: Western carp gudgeon (*Hypseleotris klunzingeri*), Midgeley's carp gudgeon (*Hypseleotris* sp. A), Lake's carp gudgeon (*Hypseleotris* sp. B), and Murray-Darling carp gudgeon (*Hypseleotris* sp. C).

Table 1. Species detection in the lower and Mid Lachlan River reaches using eDNA metabarcoding (with and without CBP) and conventional methods. CBP = with cyprinid blocking primer, NO CBP = without cyprinid blocking primer, CONV = conventional monitoring.

	Species	LOWER LACHLAN			MID LACHLAN		
		CBP	NO CBP	CONV	CBP	NO CBP	CONV
NATIVE	Silver perch	Y	Y		Y	Y	Y
	Carp gudgeon complex	Y	Y	Y	Y	Y	Y
	Golden perch	Y	Y	Y	Y	Y	Y
	Murray cod	Y	Y	Y	Y	Y	Y
	Bony herring	Y	Y	Y	Y	Y	Y
	Flatheaded gudgeon	Y	Y	Y	Y	Y	Y
	Australian smelt			Y	Y	Y	Y
	Freshwater catfish		Y				
INVASIVE	Goldfish		Y	Y		Y	Y
	Common carp	Y	Y	Y	Y	Y	Y
	Eastern gambusia	Y	Y	Y	Y	Y	Y
	Redfin perch				Y	Y	Y

At the reach level, there was a significant difference between methods in terms of presence vs absence of species (Global $R = 0.046$, $p = 0.014$). Conventional monitoring was not significantly different to eDNA metabarcoding without the use of CBP (R statistic = 0.055, $p = 0.065$), but was significantly different to eDNA with the CBP (R statistic = 0.082, $p = 0.01$). Species contributing to these differences were flatheaded gudgeon, bony herring and eastern gambusia, which were generally more commonly detected via conventional sampling methods.

The combined use of conventional and eDNA metabarcoding monitoring techniques resulted in the greatest number of native species detections in 2019 (Table 2). The four native species believed to be locally extinct remained undetected for the 4th year of the LTIM project.

Table 2. Pre-European (PERCH = Pre-European Reference Condition for fish) list of the expected native fish species present in the lower Lachlan River system, their associated rarity and detection from 2015-2019. eDNA = eDNA metabarcoding, Conv = conventional technique.

COMMON NAME	SCIENTIFIC NAME	OCCURRENCE ¹	2015 LTIM CENSUS (Conv)	2016 LTIM CENSUS (Conv)	2017 LTIM CENSUS (Conv)	2019 LTIM (eDNA + Conv)
Australian smelt	<i>Retropinna semoni</i>	common	Y	Y	Y	Y
bony herring	<i>Nematalosa erebi</i>	common	Y	Y	Y	Y
carp gudgeon	<i>Hypseleotris spp</i>	common	Y	Y	Y	Y
freshwater catfish	<i>Tandanus tandanus</i>	common	Y			Y*
golden perch	<i>Macquaria ambigua</i>	common	Y	Y	Y	Y
Murray-Darling rainbowfish	<i>Melanotaenia fluviatilis</i>	common				
silver perch	<i>Bidyanus bidyanus</i>	common				Y
Murray cod	<i>Maccullochella peelii</i>	occasional	Y	Y	Y	Y
un-specked hardyhead	<i>Craterocephalus stercusmuscarum fulvus</i>	occasional	Y	Y	Y	
flathead galaxias	<i>Galaxias rostratus</i>	rare				
flat-headed gudgeon	<i>Philypnodon grandiceps</i>	rare		Y		Y
olive perchlet	<i>Ambassis agassizii</i>	rare				
southern purple spotted gudgeon	<i>Mogurnda adspersa</i>	rare				
southern pygmy perch	<i>Nannoperca australis</i>	rare				

¹Descriptions of predominance (occurrence) correspond to reference condition categories for the Murray-Darling Basin Sustainable Rivers Audit program and are used to generate fish condition metrics.

* Detected using eDNA only

SPECIES ABUNDANCE AND BIOMASS

Differences in biomass between conventional monitoring and the eDNA metabarcoding treatments (CBP and no CBP) were based largely around contributions by large bodied species (Murray cod, golden perch and common carp) for both reaches (Table 3). Differences in abundance between conventional monitoring and eDNA metabarcoding were based largely around Murray cod, common carp in the lower reach and flatheaded gudgeon and carp gudgeon in the mid reach. While there are differences in the abundance and biomass estimates using the different monitoring techniques, the general patterns in proportional species composition in the lower and mid Lachlan are somewhat similar (Figure 3).

Table 3. Top 3 fish species with the greatest biomass and abundance (conventional monitoring) and number of DNA sequence reads (eDNA metabarcoding) in the lower and mid Lachlan

REACH	CONVENTIONAL		EDNA METABARCODING	
Reach	Abundance	Biomass	Treatment with Cyprinid blocking primer (CBP)	Treatment without Cyprinid blocking primer (CBP)
Lower Lachlan	1. Bony herring	1. Common carp	1. Bony herring	1. Bony herring
	2. Carp gudgeon	2. Golden perch	2. Murray cod	2. Golden perch
	3. Common carp	3. Murray cod	3. Golden perch	3. Common carp
Mid Lachlan	1. Carp gudgeon	1. Common carp	1. Murray cod	1. Common carp
	2. Flat headed	2. Golden perch	2. Carp gudgeon	2. Murray cod
	3. Common carp	3. Murray cod	3. Bony herring	3. Bony herring

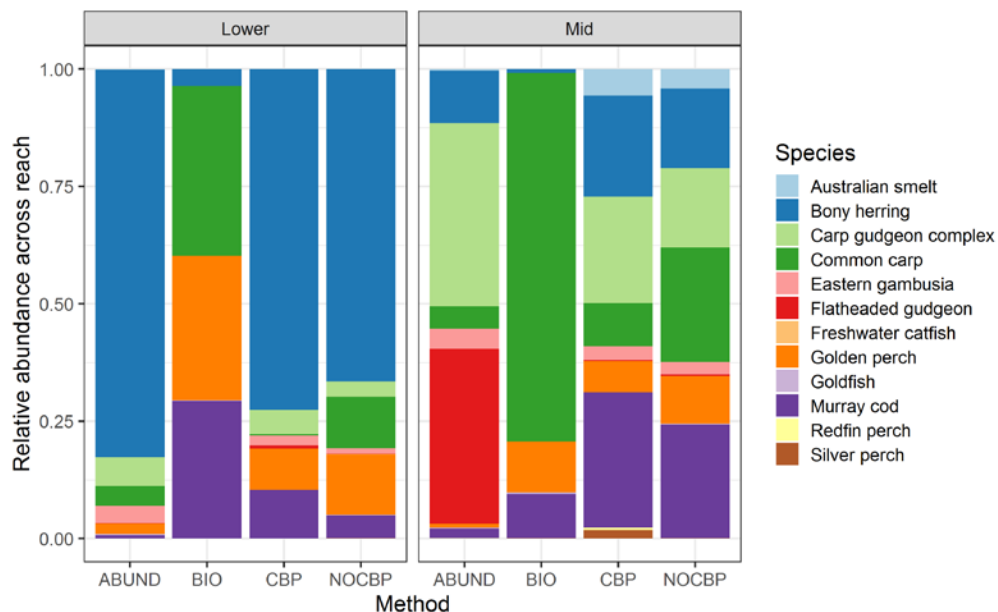


Figure 3. Relative fish community composition of the mid and lower Lachlan River reaches, according to each monitoring method. ABUND = abundance, BIO = total biomass, CBP = eDNA with Cyprinid blocking primer, NOCBP = eDNA without Cyprinid blocking primer.

PERFORMANCE OF THE CYPRINID BLOCKING PRIMER (CBP)

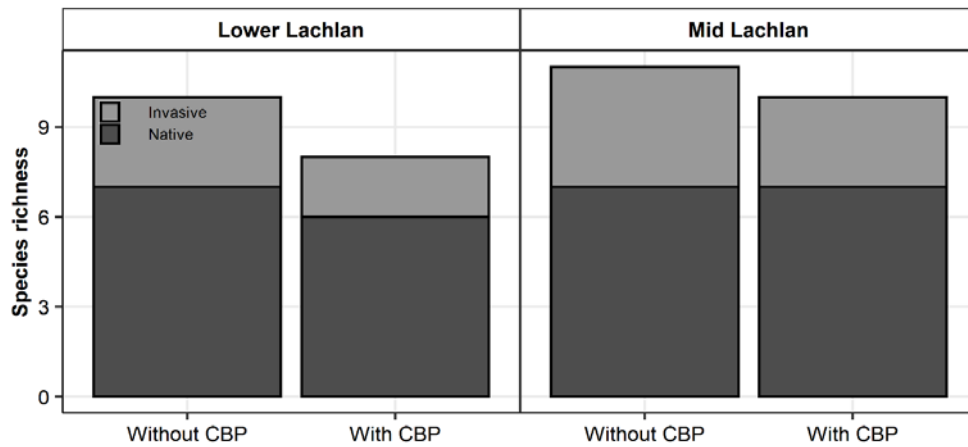


Figure 4. The overall species richness detected at the two reaches with and without the use of the Cyprinid Blocking Primer (CBP).

The use of the CBP decreased the number of invasive species detected in both reaches (Fig. 4). The use of the CBP reduced the number of common carp DNA reads by 97% in the lower Lachlan and by 70 % in the mid Lachlan. The CBP effectively blocked all goldfish DNA from amplifying in both reaches. When evaluating the impact of the CBP on the detection probabilities of native fish species, the paired sample t-test showed a non-significant difference between the samples analysed with and without the CBP for the lower Lachlan ($p = 0.6$, Fig. 4), but a significant difference for the Mid-Lachlan with detection probabilities being moderately lower when the CBP was used ($p = 0.01$, Fig. 5);

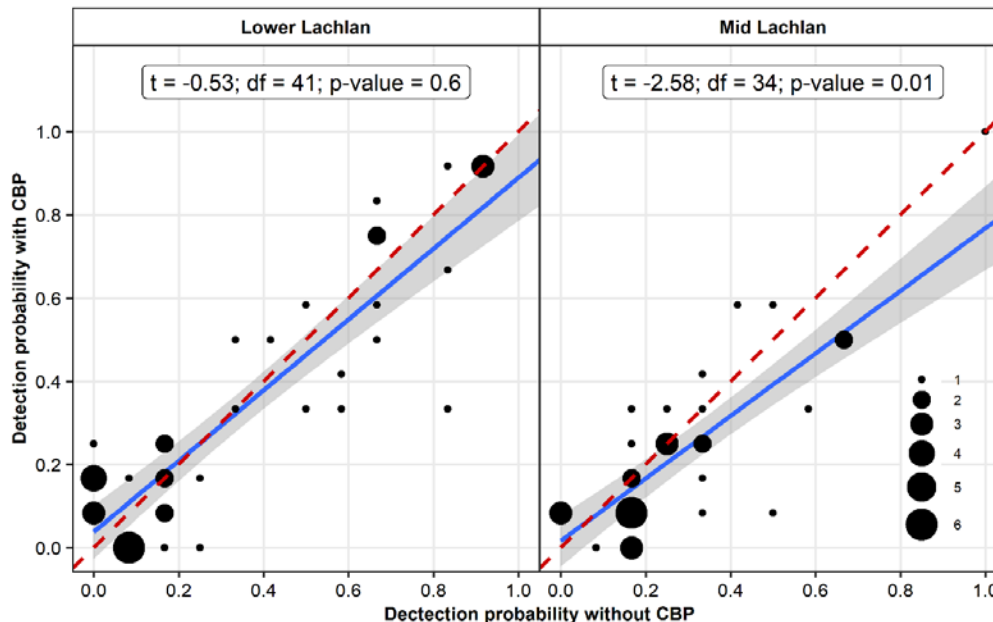


Figure 5. Detection probabilities for native fish species with and without the use of Cyprinid Blocking Primer (CBP), including the results of the paired sample t-test to evaluate the difference in native fish detection with and without the use of CBP. The size of the points indicates the number of occurrences.

RELATIONSHIP BETWEEN EDNA READS AND ABUNDANCE AND BIOMASS

At the reach scale, results of the correlation analysis showed only a significant weak positive relationship between eDNA with CBP and conventional estimates of biomass and abundance in the mid Lachlan reach (Table 2). No significant relationships were seen for the lower Lachlan.

Table 4. Results of the correlation analysis on the relationships between site-based proportions estimated by eDNA and conventional monitoring methods (* denotes significant relationship).

REACH	EDNA METABARCODING	CONVENTIONAL METHOD	P	R	TEST
Lower	with CBP	Abundance	0.296	-0.124	Pearson
Lower	with CBP	Biomass	0.283	-0.127	Pearson
Lower	without CBP	Abundance	0.092	-0.198	Pearson
Lower	without CBP	Biomass	0.058	-0.223	Pearson
Mid	with CBP	Abundance	0.012*	0.300	Pearson
Mid	with CBP	Biomass	0.035*	0.254	Pearson
Mid	without CBP	Abundance	0.567	0.070	Pearson
Mid	without CBP	Biomass	0.221	0.149	Pearson

At the species level, relationships between eDNA methods and conventional methods varied, but there were some strong and moderate significant relationships. The strongest of these relationships existed between conventional monitoring abundance and biomass and eDNA metabarcoding (with and without CBP) for bony herring and between conventional monitoring estimates and eDNA metabarcoding (with and without CBP) for Murray cod. There were also significant moderate positive relationships between conventional monitoring methods and eDNA methods for both eastern gambusia and carp gudgeon complexes. These species which had significant positive relationships are either large-bodied and/or abundant in the system.

6. COLLABORATION AND BROADER IMPACT

The results of this study inform and complement other projects investigating and evaluating the use of environmental DNA as a biomonitoring technique in Australia. To date, this technique has shown to deliver high detection capacity that is non-invasive and relatively cost-effective. However, in order for eDNA to be deployed as a routine tool, standardization is required across all aspects of the workflow from field sampling, lab protocols and bioinformatic analysis. This standardization is necessary to enable comparison between and within studies in order to determine what factors might impact on results obtained. For example, this project tested the same methodology and pipeline used in the New South Wales Department of Primary Industry's (NSW DPI) project on the detection of threatened fish species through eDNA metabarcoding. The NSW DPI project sites focused on rivers and creeks in the northeastern NSW while this project focused on the Lachlan River. While the use of CBP increased the detection probability of priority native species in the NSW DPI project (Bylemans et al. in prep), results in the Lachlan River showed no significant advantages of CBP use. As such, evaluating the eDNA metabarcoding pipeline

across different catchments enables us to determine potential reasons for the variable results seen between landscapes and catchments. This particular study provides a platform for further refinement and eventual standardization of the eDNA metabarcoding technique as a tool for fish biomonitoring within Australia. In line with other international groups such as DNA Aqua-Net in Europe, which is a EU Water Framework Directive, and the US Environmental Protection Agency, Australia has the capability to implement a strategy for the inclusion of molecular based methods into biodiversity assessment and for this method to be routine within the next 10 years. The publication that is expected to come from this project will add to the international literature that seeks to refine methodologies in order advance this approach worldwide and will facilitate collaborations with other research groups to enable coordinated effort both nationally and internationally.

7. CONCLUSIONS AND RECOMMENDATIONS

The results of this research showed that eDNA metabarcoding survey is a robust complementary approach to conventional monitoring at the reach scale. It provided a more robust species richness data for the lower Lachlan River by detecting rare species such as the freshwater catfish and silver perch. In this light, including eDNA monitoring into the monitoring and evaluation of environmental water programs will improve the accuracy of the evaluations of expected outcomes around community composition and species richness (i.e. evaluation questions targeting native fish community resilience and native fish diversity). Furthermore, evidence of the presence of these rare species in the system will improve two of the three Sustainable Rivers Audit (SRA) fish community condition indices (Expectedness and Nativeness), which are used to derive the overall fish community condition index for the reach. The eDNA technique can also distinguish between species that are difficult to differentiate through morphological means alone, paving the way towards greater understanding of species distributions and community dynamics.

We saw areas that can be refined for eDNA metabarcoding. We've seen the failure of eDNA metabarcoding in detecting Australian smelt in the Lower Lachlan, which is a species that is fairly common in the system. This non-detection is likely a result of primer bias (primer-template mismatches) which can cause reduced amplification (Piñol et al. 2015). Primer bias has also been seen in other eDNA metabarcoding studies targeting fish (Shaw et al. 2016). Furthermore, we found evidence that the use of the CBP may have blocked the amplification of the freshwater catfish DNA, limiting the use of CBP in this system. Given that the choice of primers and community characteristics can affect detection and quantitative interpretation of eDNA metabarcoding results, the use of at least two different fish metabarcoding primers in future studies can be explored to maximize the probability of detection.

The results of this study also showed that species richness (reach level) as determined by eDNA metabarcoding and conventional monitoring did not differ significantly. Therefore, targeted eDNA detection of rare species that are difficult to monitor through conventional means could be explored in the future, instead of metabarcoding. Targeted eDNA species detection may offer greater sensitivity and reduced cost compared to the eDNA metabarcoding approach. Species-specific primers for real-time eDNA detection of priority target species in the Lachlan system can be developed to improve monitoring and detection. This study also found some strong positive relationships between proportions of species compositions at sites between the eDNA methods and the conventional monitoring methods. Whilst this doesn't directly transfer to raw eDNA reads to abundance or biomass estimates, it does provide a tool for reasonably accurately estimating the proportional change at a site for bony herring, Murray cod, eastern gambusia and carp gudgeon.

Environmental DNA metabarcoding is a good tool to complement conventional monitoring and can improve the confidence in species detections, particularly rare species. In doing so, it can contribute to answering the specific evaluation questions (as stated in the background section of the report), but it is not, at present, a stand-alone tool for monitoring fish communities. While, it can enhance detection rates, but there is important information such as fish recruitment, sizes and age class that can only be gained through conventional monitoring.

The improved species detection capacity provided by eDNA metabarcoding improves our understanding of native fish populations and could therefore be used in evaluating the contribution of Commonwealth environmental water to native fish populations. If implemented widely throughout a river system, it could be used to complement conventional monitoring of populations, providing providing additional information and confirmation on population extent (in terms of distribution). eDNA metabarcoding could be also used to compliment conventional monitoring for the contribution of Commonwealth environmental water to native fish survival by providing robust reach level species composition information that would be especially useful following interventions around water quality releases to help mitigate blackwater effects.

At this stage eDNA metabarcoding is not suitable to provide robust information on population abundance or demography. This information is critical to determine survival of native fish in response to CEW actions (conventional monitoring provides a more robust estimate of population abundance and demography at this stage). eDNA metabarcoding is still an evolving field and needs refinement and standardization for its adoption as a standard monitoring tool by management agencies.

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