Estimating the biomass of fish stocks using novel genetic techniques

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Executive summary

Concept

This project represents the first detailed study exploring the relationship between eDNA concentrations and the biomass and/or abundance of some economically and ecologically important (primarily freshwater) fish species in Australia. The work was conducted over four-and-a-half-years as part of a collaboration between the New South Wales Department of Primary Industries and Regional Development (NSW DPIRD), the University of Canberra (UC) and the Australian Museum. Broadly, the project concept was to address a critical need for accurate and timely non-lethal population assessments involving environmental DNA (eDNA) to compliment and/or eventually replace fishery-dependent monitoring methods. Justification for this concept was based on positive outcomes from recent and ongoing international efforts.

Background

Estimating the biomass and/or abundance (defined as the total weight and number of a species in defined space and time, respectively) of fish stocks is a crucial prerequisite for effective management. Various fishery-dependent and -independent monitoring approaches are used to census populations; each with various advantages and limitations. In the last decade, there has been a focus on the potential to use traces of DNA left in the water to estimate not only the presence of a target species, but also their relative biomass and/or abundance. This method is appealing because it does not impact aquatic-fauna welfare, and large numbers of sites can be quickly sampled—often more cost effectively than conventional methods. Nevertheless, like for all assessment methods, there are numerous abiotic and biotic factors affecting the strengths of relationships between eDNA concentrations and biomass and/or abundance.

While there have been very few Australian studies with a primary or secondary objective to quantify relationships between eDNA concentrations and the biomass and/or abundance of fish, most have identified positive associations. However, no studies have involved aquaria trials to first validate and then define the extent of relationships between eDNA concentrations and biomass and/or abundance under controlled conditions. Doing so is important because it can help to identify abiotic and biotic factors that may affect relationships under natural conditions.

The deficit in available information justified the present study; which sought to investigate the utility (and any influencing factors) of eDNA methods for assessing the relative biomass and/or abundance of four fish species of conservation and economic importance in Australian freshwater and estuarine environments – Mulloway (*Argyrosomus japonicus*), Murray Cod (*Maccullochella peelii*), Golden Perch (*Macquaria ambigua*) and Bony Bream (*Nematalosa erebi*). Ultimately, the project contributes towards the growing Australian and international literature suggesting eDNA technology has great promise for non-invasive stock assessments of numerous fish species.

Aims/objectives

1. Assess the species-specific precision and importance of key influencing abiotic and biotic factors for determining abundances of Mulloway and Murry Cod using eDNA under controlled conditions.

2. Compare the utility of eDNA against directed fishery-dependant methods for assessing relative abundances of Mulloway, Murray Cod and Golden Perch across appropriate spatio-temporal scales in the wild.

3. Based on results of 2 above, determine what additional factors affect the concentrations of eDNA such as depth of eDNA water sampling.

4. Assess if eDNA of other selected species collected from samples obtained during 2 above, correspond to routinely collected catch-and-effort data in New South Wales.

Methodology

The project was carried out in two phases, starting with a comprehensive literature review (phase 1) to summarise the research to date on the utility of eDNA concentrations for estimating fish biomass and/or abundance and the key influencing factors. The review identified various biotic and abiotic factors that could affect the interpretations of relationships, which guided formulating hypotheses and research objectives for aquaria (phase 2a) and field trials (phase 2b) on focus species.

Based on the conclusions and recommendations from the literature review, four experiments were conducted for Mulloway and five experiments for Murray Cod to investigate relationships between their eDNA concentrations and their known biomasses and, for field trials, also their abundances. Species-specific PCR primers were developed and validated to ensure the assays met required standards for eDNA quantification. All laboratory work was conducted in a dedicated eDNA facility to minimise contamination risks. The Murray Cod experiments showed substantial variation in DNA shedding rates among individuals—reasons for which were unclear, but may have included variable behavioural profiles and DNA shedding rates. This variable shedding rate hindered establishing a useful relationship between eDNA concentration and biomass. Therefore, Golden Perch was included in the project due to its larger population sizes and thus perceived lower intra-specific variability among shedding rates. Aquaria and pond trials with Golden Perch, although constrained by budget limitations, aimed to explore the relationships between eDNA concentrations and absolute biomass for adults (in aquaria tanks) and juveniles (in ponds).

Results/key findings

The literature review identified 63 relevant papers investigating relationships between eDNA and biomass and/or abundance, with biases towards salmonids and cyprinids, and mostly from the United States and Japan. These studies predominantly utilised species-specific detection methods and reported positive correlations for various fish species in the field (60% of publications), controlled environments (25%) or a combination of both (14%). However, the review also highlighted uncertainties regarding absolute biomass or abundance estimates due to environmental factors affecting DNA dynamics in freshwater and marine ecosystems—emphasising the need for controlled pilot studies to address these limitations. Consequently, the design of phase-2a aquaria experiments assessed the importance of key factors such as water temperature on eDNA concentrations.

During the phase-2a aquaria work, species-specific assays were developed for Murray Cod, Mulloway, and Golden Perch. The results indicated there was a significant quadratic relationship between biomass and eDNA concentration for Murray Cod, whereby eDNA increased in tanks containing up to 180 g of

Murray Cod, but then decreased as Murray Cod weights exceeded 200 g. This outcome was attributed to excessive variability in DNA shedding rates among individuals and possibly exacerbated by a species-specific response to confinement. In contrast, promising findings were observed for Golden Perch and Mulloway, with significant positive relationships between eDNA concentrations and biomass, despite some variability in shedding rates among the latter species.

Phase-2b field trials further validated the potential of eDNA as an indicator of fish biomass, and in some cases, abundance. Notably, experiments with Golden Perch revealed a useful significant positive quadratic relationship between eDNA concentration and biomass, while the Mulloway trials showed a significant positive relationship between eDNA concentration and biomass and an almost significant positive relationship with abundance, but (for both) only during autumn. Bony Bream also had useful positive linear relationships between their eDNA concentration and biomass and abundance and a significant negative relationship between eDNA and flow. Similarly, and despite few attributable impacts detected in aquaria, in their natural environment Murray Cod biomass (but not abundance) was characterised by a significant (but weaker) positive linear relationship with eDNA concentration, as well as a significant but weak negative relationship with flow.

Implications for relevant stakeholders

The mostly consistent results for the studied species during the field-based trials imply the potential of eDNA-based methods for enabling the non-lethal, fishery-independent monitoring of their stocks into the future. This is particularly relevant for the spatially complex estuarine and inland waterways of NSW, where a monitoring method that is both non-lethal and applicable across diverse environments would be highly advantageous, potentially enhancing cost-effectiveness and overall efficiencies.

Nevertheless, the existing methods are not sufficiently refined to begin routine population estimates and further calibration should be carried out alongside capture-based surveys. Further, for Mulloway assessed in one estuary, the results cannot be extrapolated to marine environments owing to variable factors that might affect eDNA concentrations (such as greater depths and hydrological processes).

Despite these challenges, it is clear from the exponential increase in global initiatives to assess eDNA in the three years since our review (in phase 1) that the required technology is constantly being refined and updated. Because of these global efforts, we propose that eDNA will become an important tool for non-invasive population monitoring in NSW and nationally. Such an implication will benefit all stakeholders, including recreational and commercial fishers, managers and other groups directly or indirectly concerned with resource sustainability.

Recommendations

1. **Owing to consistent intra- and interspecific variability in relationships between eDNA and abundance, prioritise investigating relationships between eDNA and biomass.**

- Substantial variability in the sizes of large-bodied fish in some systems might preclude the meaningful utility of investigating relationships between eDNA and the abundance of some species. So, like for traditional fish-dependant methods, efforts might focus on assessing relationships with relative weights (biomass).

2. **Collect eDNA samples when electrofishing in freshwater rivers across all seasons.**

- To increase the available data and thus enable refinements to relationships between eDNA concentrations and biomasses to be made, additional data are required.

- This work could be done relatively easily alongside existing large-scale electrofishing projects, which already collect basic water-quality data.

3. **Collect eDNA samples during commercial fishing operations for Mulloway in estuaries across all seasons.**

- As for freshwater species, additional data are required to refine the relationship between eDNA concentrations and Mulloway biomasses.
- This work could be done as part of routine observer programs for Mulloway and potentially other priority species where data are recorded.

4. **Develop a protocol to facilitate collecting eDNA samples from freshwater and marine environments.**

- The protocol should specify the number of samples required and from which locations, including the preferred timing of collection (season, flow conditions, tide etc.). In addition, this protocol should include training requirements to minimise the risk of sample contamination.

5. **Validate hydroacoustics for estimating Mulloway biomass in estuaries.**

- The hydroacoustic method for estimating Mulloway biomass in this study has yet to be validated.
- This work could be done using tank and/or cage experiments (and involve other co-occurring species). There is a need to determine if hydroacoustic techniques can be refined to detect Mulloway <40 cm TL. Not only would this provide increased confidence around comparisons with eDNA concentrations, but hydroacoustics may be a viable method for not only evaluating Mulloway biomass and abundance in estuaries, but also various other priority species.

Keywords

Environmental DNA, fishery-independent biomass/abundance estimates, biotic and abiotic factors, Murray Cod, Golden Perch, Mulloway, Bony Bream.

1. Introduction

Australia's commercial and recreational fisheries are annually valued at 3.42 billion (Cao et al., 2023), and recreational fishers contribute ~11 billion a year and generate >100,000 jobs to the Australian economy (Moore et al., 2023). Ensuring the sustainability of our fisheries resources requires regular stock assessments underpinned by robust data. Classical fishery-independent survey techniques, to obtain these data, tend to be costly and labour-intensive, and may also involve invasive sampling techniques. Consequently, obtaining regular and reliable data to support stock assessments can be challenging. The development of low-cost, non-invasive sampling methods that could supply basic demographic and morphometric data to inform stock assessments can help address these challenges. This project sought to facilitate a pathway forward for rapidly providing relative biomass and/or abundance estimates (defined as the weight or number of a particular species in defined space and time) using a non-invasive method, focusing on two priority freshwater (Murray Cod, *Maccullochella peelii*, and Golden Perch, *Macquaria ambigua*) and one estuarine species (Mulloway, *Argyrosomus japonicus*), as case studies.

All three species are large-bodied, high-order predators with key ecosystem roles, have iconic statuses, are of considerable economic value and in NSW their stocks are classified by the 'Status of Australian Fish Stocks' (SAFS) reports as either 'recovering' (Mulloway) or 'depleted' (Murray Cod and Golden Perch). More specifically, the NSW populations of Mulloway have substantially declined over recent decades and are considered depleted to ~13% of the unfished biomass (Earl et al., 2024). Similarly, a combination of fishing pressure and environmental drivers have resulted in fluctuating abundances of Murray Cod and Golden Perch throughout NSW (Crook et al., 2023). Commercial fishing for Murray Cod and Golden Perch in NSW ceased in 2000 and 2021, respectively. However, there is still a commercial fishery for Golden Perch in South Australia, and both species remain important for recreational fishing. Each have complex life histories that predispose their stocks to over exploitation (Koehn & Nicol, 2016; Reynolds, 1983).

Population declines for all three species warrant rigorous and representative monitoring and assessment. Yet owing to their conservation statuses, all three species are challenging to monitor. Mulloway are caught by both recreational and commercial fishing sectors, although the primary source of monitoring data has been derived from the latter. However, as part of recovery efforts, there have been incentives to reduce commercial effort via refined gear selectivity, commercial fishing exclusions within key estuaries, and removing bycatch allowances preventing harvesting individuals smaller than the minimum legal length (MLL). These changes have evoked variations in catch-per-unit-of-effort (CPUE) and therefore discontinuity in the consistency of time-series data.

The absence of widespread and consistent freshwater commercial fishing effort means that population assessments of Murray Cod and Golden Perch are now limited to fishery-independent electrofishing and sporadic recreational creel surveys (Crook & Schilling, 2023; Forbes et al., 2015). But both species are costly to representatively sample. There are clear benefits to developing alternative or ancillary population-monitoring approaches for all three species (and indeed a plethora of others, subject to validation) that simultaneously encompass sufficient spatial coverage, are cost-effective, and provide indices of either relative or absolute biomass and/or abundance.

The need for accurate population estimates extends more broadly across all economically or ecologically important species as an implicit prerequisite for their effective management. Nevertheless, even after more than a century of effort, estimating the weight of number of fish in a population remains one of the greatest challenges facing fisheries scientists. Traditional methods for estimating population sizes include parametric and empirical approaches that often rely on extensive time-series of size-at-age data (e.g. from otoliths). Acquiring these data can be expensive and

therefore restricted to species that are most economically important or of conservation concern. Due to the cost of independent sampling, indicators of abundance usually only involve the types of fishery-dependent data listed above, which have numerous biases that can affect accurately quantifying population trends.

One applicable, novel, method that might facilitate population assessments involves modern genetics to estimate the absolute number of individuals in a population (e.g. so-called 'close-kin'; FRDC projects 2007-34; 2014-024; 2016-044; 2021-015; 2023-068 and 2021-111). Such work is in its infancy, but with lowered costs as technology improves there is a need for new approaches to be explored that increase the accuracy and speed of assessments over traditional approaches. More specifically, there is potential for estimating absolute or relative biomasses and/or abundances from the concentration of environmental DNA (eDNA) collected from water samples (Carraro et al., 2018; Takahara et al., 2012). Put simply, this method does not require labour-intensive sampling—instead relying on capturing DNA shed by the target species into the water and then using these data to estimate their weight or abundance as an index for that space and time (and so facilitate monitoring relative changes).

The clear need for accurate and rapid stock assessments is increasingly apparent in the current management paradigm of rationalising competing commercial, recreational and conservation interests. Specifically, marine protected areas have been implemented across all NSW coastal bioregions. These areas are a source of political and public contention owing to perceived inequalities in access to (and allocation of) fisheries resources, particularly among species shared by commercial and recreational fishers. Also, recent fish kills in NSW due to hypoxic water have created debate over the cause of poor water quality and longer-term effects on freshwater habitats. Rapidly quantifying existing and/or changing stock abundances of key species will be essential to inform recovery actions and advise on the sustainable exploitation of commercial and recreational species. Sampling eDNA could meet these needs by facilitating cost-effective relative biomass and/or abundance estimates for population assessments in NSW where fishery-dependent data are scarce or unavailable.

2. Objectives

The original contracted objectives were to:

1. Assess the species-specific precision and importance of key influencing abiotic and biotic factors for determining abundances of Mulloway and Murry Cod using eDNA under controlled conditions.

2. Compare the utility of eDNA against directed fishery-dependant methods for assessing relative abundances of Mulloway and Murray Cod across appropriate spatio-temporal scales in the wild.

3. Based on results of 2 above, determine what additional factors affect concentration of eDNA such as depth of eDNA water sampling.

4. Assess if eDNA of other selected species collected from samples obtained during 2 above, correspond to routinely collected catch-and-effort data in NSW.

However, owing to substantial variation in the amount of DNA shed by individual Murray Cod during initial aquaria trials, within a ratified project variation, we also included Golden Perch as a focus species (objective 2). Further, owing to availability, Bony Bream (*Nematalosa erebi*) was assessed within objective 4 in addition to Murray Cod.

3. Methods

This was a four-and-a-half-year project that was conducted using a phased 'stop/go' approach to assess the potential for eDNA to cost-effectively underpin stock assessments for the focus species, and any extension to other species (Table 1). Recognising the novelty of the approach, the first four months of the project (phase 1) were used to complete a comprehensive literature review to define all possibilities, and outline challenges and a proposed framework moving forward (Appendix 1). The review supported refined experimental designs for phase-2a aquaria trials (Appendices 2–4) and Phase 2b field trials (Appendices 5 and 6) to address the four project objectives.

As stated in the objectives, the original project plan focussed on Murray Cod and Mulloway, but because the aquaria trials with the Murray Cod failed to detect a useful relationship between eDNA concentration and biomass (Appendix 3), we included Golden Perch (Appendix 5). Nevertheless, we still maintained ancillary assessment of Murray Cod within objective 4. Summaries of the methods relevant to each objective are provided below and in Table 1.

3.1. Phase 1: literature review

A search of the peer-reviewed literature was conducted on the Web of Science with pre-determined keywords to identify publications mentioning eDNA and any quantification of biomass and/or abundance between January 2000 and October 2020. The search produced 631 publications, which were filtered to those mentioning 'fish'. The papers were read and summarised before the key methods, outcomes and/or limitations of each were tabulated, and the most common themes were used to partition topics for the review (Appendix 1). Ultimately, the review was used to structure the research in phases 2a and 2b.

3.2. Phase 2a: aquaria trials with priority species

The literature review facilitated developing hypotheses to be tested under controlled conditions to determine whether progression to phase 2a was warranted (which it was). The experimental designs formulated to address these hypotheses are described in Table 1. The initial experiments investigated for any relationships between Murray Cod and Mulloway eDNA concentrations and biomasses using either captive-bred young-of-year fish or adult broodfish.

Species-specific PCR primers were first developed and validated to ensure the assays met the appropriate standard for use in eDNA surveys to measure eDNA concentrations (Figure 1, Table 1 and Appendix 2). All laboratory work was carried out in a purpose-built eDNA facility at the Narrandera Fisheries Centre (NFC) (Figure 2a and b). This facility contains three UV-sterilised rooms for each process (DNA extraction, polymerase chain reaction (PCR) preparation and DNA template loading) to minimise the risk of contamination. Murray Cod aquaria experiments were undertaken at the NFC, while the Mulloway experiments were done at the Port Stephens Fisheries Institute (PSFI).

Murray Cod presented substantial variation in the DNA shedding rate of individuals in aquaria that was not explained by their weights, which resulted in no useful relationship between eDNA concentration and their known biomass; (see Results and Appendix 3). Subsequently, following consultation with the FRDC, we added Golden Perch to the study (also using the aquaria and ponds at the NFC) based on the logic that this species tends to be present in larger numbers than Murray Cod in the wild and therefore eDNA concentrations in the water may not be as heavily impacted by individual variation in shedding rates.

Regardless of the species, the aquaria experiments involved stocking juvenile or adult fish into tanks or ponds (also called 'mesocosms') at various biomasses to assess the influence of biotic and abiotic factors on eDNA concentrations via five experiments for Murray Cod and four experiments for Mulloway (Figures 3–4, Table 1). Because Golden Perch were not originally included in the project, budgetary limitations precluded the same level of replication as for the Murray Cod and Mulloway aquaria experiments and we did not assess the impacts of eDNA sampling day or temperature or DNA shedding rates for individual fish. Rather, we conducted simple aquaria and pond experiments with Golden Perch to determine if there was any evidence of a useful relationship between eDNA concentrations and biomass for adults and larvae prior to commencing field experiments (Table 1). This work consisted of three flow-through tanks containing various adult Golden Perch biomasses and three ponds containing different larval Golden Perch biomasses (Table 2 and Figure 5).

Table 1. Summary of the project objectives, experimental locations (all in NSW) questions asked, treatments and relevant appendix containing the published or draft manuscript. Some of the questions were relevant to more than one project objective and are indicted as such. NA, not applicable.

Project objective	*Locations*	*Questions asked*	*Treatments tested*	*Appendix*
Review international literature	NA	Does the published literature support eDNA concentrations being correlated with fish biomass and/or abundance in controlled and/or natural environments and, if so, what are key influencing abiotic and biotic factors?	NA – literature review	1
1–3	NA	Can effective species-specific eDNA assays be developed for Murray Cod, Golden Perch and Mulloway?	Species-specific assay development.	2, 5
1–3	Narrandera Fisheries Centre	Are Murray Cod eDNA concentrations correlated with known biomasses in aquaria or ponds, and are there abiotic or biotic influences?	Four aquaria experiments and a mesocosm experiment were conducted to investigate variability in the relationship between Murray Cod eDNA concentration and biomass due to biotic and abiotic factors. 1. Temporal variation: two densities: low (n = 4 individuals tank^{-1}); and high (n = 8 individuals tank^{-1}); each replicated across five experimental tanks. One 100 mL water sample was collected from each tank at one, two, three, six, nine and 12 days post-stocking (Fig. 1). 2. Effects of water temperature: six Murray Cod were held in each of four tanks at low (14 ± 1°C) and high (24 ± 1°C) temperatures for nine days. Three replicate 100 mL water samples were collected tank^{-1} (Fig. 1). 3. Stocking density: Murray Cod were held at nine stocking densities: two to 18 fish, for nine days. Three replicate 100 mL water samples were collected tank^{-1} on day nine (Fig. 1). 4. Intra-specific variation: a single Murray Cod was held in each of 10 tanks. Five replicate 100-mL water samples were collected from each tank^{-1} after nine days (Fig. 1). 5. Variable population size: seven mesocosms containing a range of biomasses of Murray Cod broodfish. Four replicate 2-l water samples were collected from each mesocosm.	3
1	Narrandera Fisheries Centre	Are Golden Perch eDNA concentrations correlated with known biomasses in tanks or ponds?	Tank and mesocosm experiments were conducted to investigate the relationship between Golden Perch eDNA concentrations and biomasses for adult and larval fish. 1. Relationship between adult Golden Perch eDNA concentration and biomass: three tanks containing a range of biomasses of broodfish. Four replicate 2-l water samples were collected tank^{-1} (Fig. 5a). 2. Relationship between larval Golden Perch eDNA concentration and biomass: three mesocosms containing a range of biomasses of larval fish. Four to six replicate 2-l water samples were collected from each mesocosm (Fig. 5b).	Main text

1 and 3	Port Stephens Fisheries Centre	Are Mulloway eDNA concentrations correlated with known biomasses in tanks, and are there abiotic or biotic influences?	Four aquaria experiments were conducted to investigate variability in the relationship between Mulloway eDNA concentration and biomass due to biotic and abiotic factors. 1. Temporal variation: two densities: low (n = 20 individuals $tank^{-1}$); and high (n = 40 individuals $tank^{-1}$); each replicated across four experimental tanks. One 1-l water sample was collected from each tank at one, two, three, six, nine and 12 days post-stocking (Fig. 2). 2. Effect of water temperature: twenty Mulloway were held in each of seven tanks at low (16 ± 1°C) and high (24 ± 1°C) temperatures for eight days. Four replicate 1-l water samples were collected $tank^{-1}$ on day eight (Fig. 2) 3. Mulloway were held at 18 stocking densities: two to 60 fish, for six days. Three replicate water samples were collected $tank^{-1}$ on day six (Fig. 2). 4. A single Mulloway was held in each of 10 tanks. Four replicate 1-l water samples were collected from each $tank^{-1}$ after nine days (Fig. 2).	4
2	Lachlan, Murrumbidgee, and Edward-Wakool rivers	Are Golden Perch eDNA concentrations correlated to fish biomasses or abundances estimated by electrofishing across three randomly selected rivers?	Five sites in each of three rivers were water sampled (8 × 2-l replicates $site^{-1}$) prior to electrofishing to compare estimates of relative biomass and abundance during autumn 2021 and 2022.	5
2	Hawkesbury River	Are Mulloway eDNA concentrations correlated to fish biomasses or abundances estimated by hydroacoustic surveys in one estuary during two seasons?	Twelve sites in the Hawkesbury River with ten replicate water samples $site^{-1}$ (2-l target volume), were water sampled immediately prior to hydroacoustic surveys during autumn and spring 2021 and 2022.	6
4	Lachlan, Murrumbidgee, and Edward-Wakool rivers	Are Murray Cod and Bony Bream eDNA concentrations correlated to biomasses or abundances estimated by electrofishing across three randomly selected rivers?	Five sites in each of three rivers were water sampled (8 × 2-l replicates $site^{-1}$) prior to electrofishing to compare estimates of relative biomass and abundance during autumn 2021 (Edward-Wakool and Murrumbidgee rivers) and 2022 (all three rivers).	Main text

(a) Using an eDNA backpack water filter (b) The 5-µm filter used to collect eDNA

Figure 1. Photographs of (a) filtering water using the eDNA backpack in the upper Murrumbidgee River for the field negative site for testing the Murray Cod and Golden Perch assays, and (b) the 5-µm self-preserving eDNA filter from Smith-Root.

(a) The PCR preparation room (b) The QuantStudio3 qPCR machine

Figure 2. Photographs of the (a) workstation in the PCR preparation room, and (b) QuantStudio3 qPCR machine at the Narrandera Fisheries Centre.

(a) An experimental tank

(b) Experimental-tank configurations

(c) Example of the juvenile Murray Cod used in the aquaria experiments

Figure 3. Photographs showing (a and b) the Murray Cod closed-system aquaria configuration for experiments 1–4, and (c) an example of a juvenile Murray Cod used in all aquaria experiments. Each experimental tank was positioned inside an 81-l 'outer' tank filled with 45 l of flowing temperature-regulated water (via an external heater-chiller unit) that maintained experimental tank water temperatures at 18.5–19.5°C during most experiments.

(a) Four experimental tanks

(b) Individual shedding-rate experiment

(c) An example of a juvenile Mulloway used in the aquaria experiments

Figure 4. Photographs showing (a) an example of the Mulloway experimental tank set-up for experiments 1–3, (b) the experimental setup for experiment 4 (individual shedding rate), and (c) a juvenile Mulloway used in the experiments.

Table 2. Volume, number of fish, mean and total weights and quantity of DNA for Golden Perch in three tanks containing adults and three ponds containing larvae which were sampled to determine if there was any relationship between eDNA concentrations and biomasses.

Variable	*Tank 1*	*Tank 2*	*Tank 3*	*Pond 1*	*Pond 2*	*Pond 3*
Volume	3500 l	1500 l	1500 l	1.02 ML	1.14 ML	5.6 ML
Number of fish	11	5	13	21,000	37,000	203,200
Mean individual weight (g)	1095.45	1900.00	1269.23	0.64	0.49	0.44
Total weight (kg)	12.05	9.50	16.50	13.48	18.13	89.40
Quantity of DNA (ng l^{-1})	1.11×10^{-4}	1.08×10^{-3}	4.78×10^{-4}	1.66×10^{-6}	3.41×10^{-6}	2.61×10^{-6}

(a) Golden Perch broodfish tanks

(b) Golden Perch larval pond

Figure 5. Photographs of (a) Golden Perch broodfish tanks and (b) filtering water from a Golden Perch larval pond at the Narrandera Fisheries Centre.

3.3. Phase 2b: field trials with all species

The typical method for estimating the biomasses and abundances of freshwater fish in NSW is via electrofishing (Figure 6a and b). A large project funded by the Commonwealth Environmental Water Office generates extensive electrofishing datasets (2015–2024) for these species in three river systems in the southern MDB (Murray–Darling Basin; Murrumbidgee, Lachlan and Edward-Wakool rivers). This initiative provided an opportunity to conduct simultaneous eDNA sampling for the priority species (Murray Cod and Golden Perch) and another selected species (Bony Bream) within relevant experiments, and so address objectives 2 and 4.

Unlike for freshwater species, indices of Mulloway biomass are typically estimated using commercial fishing landings (mostly from gillnets), which is becoming increasingly limited due to management changes, including closures within key estuaries, and more recently, reductions in effort. We instead explored the potential for non-lethal fisheries-independent hydroacoustic surveys to first estimate the abundance and then biomass (based on lengths) of Mulloway across discrete space and time.

Along with Mulloway, spatio-temporally stratified designs were used to examine the utility of eDNA as an indicator of relative biomasses and abundances at the system scale for Golden Perch, Murray Cod, and Bony Bream (Appendices 5 and 6). Water samples were collected at five replicate sites within three river systems in the MDB (to target Golden Perch, Murray Cod and Bony Bream; Appendix 5) and 12 sites in the Hawkesbury River estuary (to target Mulloway, Appendix 6). The freshwater species were targeted during March to May 2021 and February to May 2022 to avoid the peak spawning season of Golden Perch. Mulloway eDNA samples were collected in November and December 2022 prior to the spawning season and again in May 2022 once spawning was complete. Immediately following eDNA collection, the relative biomass and abundance data were collected using standardised electrofishing (for Golden Perch, Murray cod and Bony Bream) and hydroacoustic surveys (for Mulloway) (Table 1, Appendices 5 and 6).

(a) An electrofishing boat

(b) Adult Golden Perch

Figure 6. Photograph of (a) an electrofishing boat used to stun (b) Golden Perch during field trials.

3.4. Data collected

3.4.1. Environmental DNA

Prior to all eDNA sampling, two replicate water-quality parameter measurements were collected for temperature, salinity (as conductivity) and dissolved oxygen. For aquaria experiments, water samples were collected nine (Murray Cod) and six days (Mulloway) after juveniles were introduced into experimental tanks based on the results of temporal experiments (Table 1, Appendices 3 and 4). Fish were held in captivity for appropriate periods prior to eDNA sampling and weighed within days of experiment terminations. Field eDNA samples were collected immediately prior to electrofishing (Murray Cod, Golden Perch and Bony Bream) or simultaneously with hydroacoustic surveys (Mulloway). Various volumes of water and replicate samples were collected for each experiment using a Smith Root eDNA sampler^{TM} and 5-µm polyethersulfone (PES) filters (Table 1). The DNA was extracted from filters using standard procedures at the NFC and the amount of DNA l^{-1} of water filtered was determined using a standard curve constructed with synthetic DNA of known concentration (Appendices 3–7).

3.4.2. Electrofishing

For the Golden Perch, Murray Cod and Bony Bream field trials, electrofishing was conducted across a 200-m reach immediately following water-sample collection (Appendix 5). The reach was broken into eight operations, each carried out in 50-m long 'cells' with electrofishing beginning at the downstream end of the site (Appendix 5). In each cell, 90 electrofishing seconds 'on time' was conducted, and any inert fish displaced to the surface were removed (defined as 'caught') and held in a live well for up to 10 min. If large catches of a species occurred, all individuals were measured in the operation where the 50th fish was reached and then the first 20 fish of that species were measured and weighed in each subsequent operations and remaining individuals were only counted. Fish were measured (to the nearest 1 mm TL or fork length; FL) and weighed (to the nearest 1 g). After every two operations, fish were released downstream of the survey reach to minimise the risk of them being recaptured in subsequent operations. Fish that were displaced to the surface but were not inert and could not be retrieved were recorded as 'observed'. The total caught and observed weights from cells 1–8 were used as the biomass estimates for each site. The abundances included the observed numbers of fish.

3.4.3. Hydroacoustic surveys

We conducted a pilot study in November 2021 using InfoFish Pty Ltd to collect bathymetric and hydroacoustic data at high and low tides at 12 sites in the Hawkesbury River and to analyse the data to determine whether this method was suitable for enumerating and measuring Mulloway (Figure 7a and b, Appendix 6). Length-weight relationships from von Bertalanffy growth curves were then used to estimate the weight of fish detected. The results of this pilot work indicated that hydroacoustic surveys were an acceptable method to identify Mulloway ≥40 cm total length (TL). Fish <40 cm TL could not be confidently identified, but no other nonlethal method effectively samples this size class. Nevertheless, the goal of this component of the work was not to compare the eDNA concentration to the absolute amount of Mulloway, but rather to provide a comparative analysis of the two approaches to provide relative biomasses and abundances of larger fish, assuming some seasonal homogeneity of smaller fish.

To minimise the effect of water flow on eDNA concentrations and maximise the probability of hydroacoustic detections at each site, the surveys were timed to occur as close to no tide as possible (prioritised from downstream to upstream sites). Hydroacoustic surveys to detect Mulloway abundance proceeded along ten transects covering a 1-km reach and with concurrent bathymetry surveying to check for any changes in site characteristics. Data detections of individual Mulloway were collected using the BioSonics DT-Z split beam echo sounder and managed with the Visual Acquisition software package (BioSonics), which also concurrently collected bathymetry data (Appendix 6). Total lengths were then converted to weights using the relationship provided by Silberschneider et al. (2009) to produce biomass. The Mulloway biomass volume^{-1} (kg m^{-3}) and abundance (no. m^{-3}) were then estimated for each transect and pooled to provide abundance and biomass estimates site^{-1} (Appendix 6).

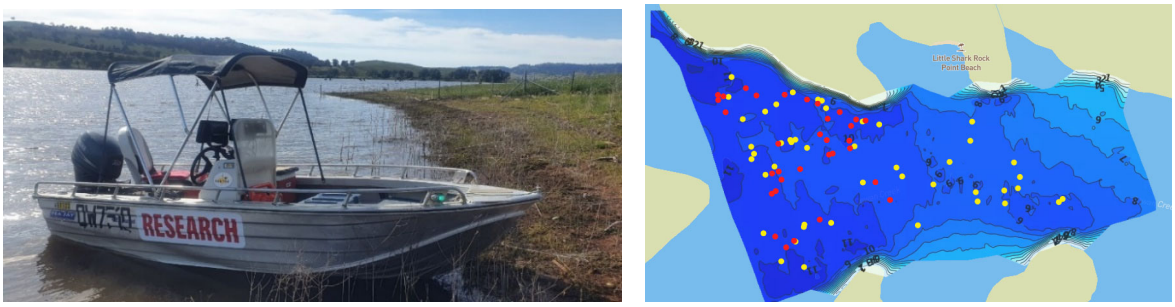


Figure 7. (a) A photograph of the InfoFish research vessel and (b) the detections of Mulloway at high tide (yellow dots) and low tide (red dots) during the pilot survey at one of 12 sites (Shark Rock) in the Hawkesbury River during November 2021.

3.5. Data analyses

For most analyses, eDNA concentrations, and biomass and/or abundance data were log-transformed and analysed using appropriate linear (LM) or linear mixed models (LMM). Fixed effects included those of interest (Table 1), while random blocking effects incorporated space (e.g. 'rivers' or 'tanks') and in some cases time (e.g. 'years'). The significance of fixed effects of interest were assessed using Wald-*F* or likelihood ratio tests. Models were fitted within glmmTMB (v. 1.1.7) or AsReml in R using penalised quasi-likelihood and with model-checking (i.e. over-dispersion) and diagnostics (residual plots) (Brooks et al. 2017; R core team, 2022). For some models, marginal and conditional r2 was estimated using the function r.squaredGLMM in the MuMIn package (v. 1.47.5, Barton, 2023). For Murray Cod and Bony Bream, eDNA concentration was multiplied by 1×10^5 to assist analysis of extremely small values, however figures were reverted to the nominal scale.

4. Results

4.1. Phase 1: literature review

Initially, 631 eDNA related papers were identified using our search terms. Refinement of these papers to those mentioning “fish” resulted in 63 papers, which formed the basis of the review. The first published study assessing for a correlation between fish eDNA and biomass was in 2012 (Takahara et al., 2012). Most subsequent studies were completed in the United States of America (USA; 53%) and Japan (25%) and were biased towards experiments done in the field (rivers, lakes, estuaries and oceans; 60%) followed by those in controlled environments (aquaria or ponds; 25%). The remaining studies (14%) were conducted across combination of field and controlled environments, but all within economically developed nations.

The commonly studied families were Salmonidae (includes salmon, trouts, chars, freshwater whitefishes and graylings) and Cyprinidae (includes carps, the true minnows and their relatives) (Appendix 1). Most studies used species-specific detection methods (51 or 81% across 46 species) rather than metabarcoding methods (12 or 19% across >200 species; Appendix 1) to evaluate eDNA concentrations, with the latter restricted to estimating only relative biomass or abundance.

Regardless of the experimental location, only six (10%) of the 63 studies postulated no relationships between eDNA and the biomass or abundance of focal species. There was consistent support for the utility of the approach with 46 (90%) and 11 (92%) studies incorporating species-specific or metabarcoding methods, respectively and reporting positive correlations between eDNA concentrations or read counts and biomasses or abundances (Appendix 1).

The review highlighted uncertainty around absolute (versus relative) biomass and/or abundance estimates due to various biotic and abiotic processes affecting the production, degradation and transport of DNA in the environment (Appendix 1). Some of this variability was encapsulated in random effects, but there were also clear fixed impacts, that included various parameters such as water temperature, salinity, and flow, and species-specific stocking densities and natural histories. Further, methodological considerations such as choice of filter can impact assessments. Such variability highlighted a clear need to conduct pilot studies in controlled conditions as a precursor to identifying the potential for detecting coherent relationships in the wild. The outcomes and recommendations were incorporated into all subsequent field experiments.

4.2. Phase 2a: aquaria trials with priority species

Valid species-specific assays were developed for Murray Cod, Mulloway and Golden Perch (Appendices 2 and 5). We carried out a total of 11 aquaria/pond experiments assessing for relationships with between eDNA and biomass–comprising five for Murray Cod four for Mulloway and two for Golden Perch. The outcomes of these experiments played pivotal roles in determining progression to field trials (Appendices 3 and 4).

The results of the Murray Cod experiments revealed there was a significant (although non-useful) quadratic relationship between biomass and eDNA concentration with the latter increasing in tanks containing up to 180 g of fish and then decreasing in tanks containing >200 fish ($p < 0.05$; Table 3, Appendix 3). There was a significant difference in the amount of DNA detected in tanks containing single Murray Cod of similar sizes (weights), suggesting variable DNA shedding rates may be responsible for the failure to detect a linear relationship between eDNA concentration and biomass

($p < 0.05$; Table 3, Appendix 3). However, when data from all experiments was pooled, a significant quadratic relationship was detected ($p < 0.05$). Initially, it was not clear from the results whether the perceived shedding-rate differences were inherent to the species biology or influenced by their behaviour during confinement. Subsequent field trials implied the latter (see below).

Owing to budget limitations, the Golden Perch experiments lacked the same level of replication as those for Murray Cod. Therefore, these did not assess the impacts of eDNA sampling day or temperature, precluding formal analyses. Nevertheless, a positive relationship was observed between eDNA concentrations and the biomass of adult Golden Perch in tanks, although the clarity of this trend was less apparent in the pond data involving larval Golden Perch (Figures 8 and 9). Despite these limitations, the results were deemed sufficiently promising to advance to field trials for this species. Similarly, the results from the Mulloway aquaria experiments were also promising, revealing a significant positive relationship between eDNA concentration and biomass despite some variability in shedding rates among similar-sized fish ($p < 0.05$; Table 3, Appendix 4).

4.3. Phase 2b: field trials with all species

The results from the Golden Perch field trial indicated no relationship with abundance, but confirming the trend in the aquaria work, there was a significant quadratic relationship with biomass ($p < 0.05$). Specifically, there was a positive relationship between ~1.1 and 5.5 kg, after which there were consistent eDNA concentrations to 10.3 kg (Table 4, Appendix 5). The Mulloway field trials indicated a significant positive relationship between eDNA concentration and biomass and an almost significant positive relationship with abundance, but only during autumn ($p < 0.05$; Table 4, Appendix 6). The Murray Cod field trial revealed a significant positive linear relationship between eDNA concentration and biomass ($p < 0.001$) (Table 4, Figure 10a) contrasting with the aquaria trials (Appendix 3). However, there was no significant relationship with abundance ($p > 0.05$). There was a general negative relationship between eDNA concentration and flow (Table 4, Figure 10b), suggesting Murray Cod eDNA was diluted as flow increased. A very similar relationship was detected for Bony Bream for biomass ($p < 0.001$), abundance ($p < 0.001$) and flow ($p < 0.01$), though these relationships were slightly steeper for Bony Bream (Table 4, Figure 11a and 11b).

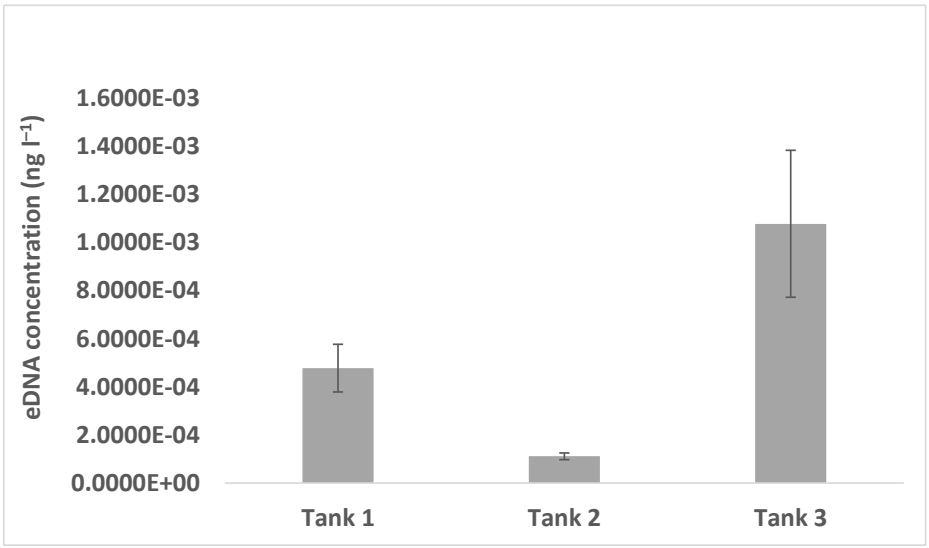
Table 3. Summary of the results of the phase 2a: aquaria trials for Murray Cod, Golden Perch and Mulloway. NA, not applicable.

		Effect of treatment(s) on eDNA concentrations and/or relationships with biomass				
Objectives	***Treatments***	***Murray Cod***	***Golden Perch***	***Mulloway***	***Bony Bream***	***Comme***
1	Temporal variation	There was a significant effect of time on eDNA concentration (p < 0.05).	NA	There was a significant effect of time on eDNA concentration (p < 0.05).	NA	The eDN stabilise nine for day six f
1	Effects of water temperature	There was no significant effect of temperature on eDNA concentration (p > 0.05).	NA	There was a significant effect of temperature on eDNA concentrations (p < 0.05).	NA	Effects r ~1.5 × g concent warmer
1	Effects of stocking density	There was a significant but not useful significant relationship between eDNA concentration and weight (p > 0.05).	There was a general trend of a positive relationship between eDNA concentration and the biomass of adult fish in tanks (Fig. 8a and b) and between eDNA concentration and biomass of larvae in ponds (Fig. 9a and b).	There was a significant positive linear relationship between log eDNA concentration and the log weight of fish (p < 0.05).	NA	For Mur all exper was a sig quadrat between concent weight. Perch, th sufficien relations trials.
1	Individual shedding rate	There were significant differences in eDNA concentrations in tanks with individual fish not explained by weight p < 0.05).	NA	There were significant differences in eDNA concentrations in tanks with individual fish not explained by weight p < 0.05).	NA	Individu Mullowa Cod she differen DNA.

Table 4. Summary of the results of the phase 2b: field trials for Murray Cod, Golden Perch, Mulloway and Bream Bony Bream. NA, not applicable.

Objectives	***Treatment***	***Murray Cod***	***Golden Perch***	***Mulloway***	***Bony Bream***	***Comment***	***Appendix***
2–4	eDNA vs fishery-independent methods (electrofishing or hydroacoustics) and estimates of biomass and abundance.	There was a significant but weak positive relationship between eDNA concentration and electrofished-estimated biomass $p < 0.05$). There was no significant relationship with abundance ($p > 0.05$).	There was a significant quadratic relationship between eDNA concentration and electrofished-estimated biomass ($p < 0.05$). There was no significant relationship with abundance ($p > 0.05$).	There were significant positive and negative relationships between eDNA concentration and hydroacoustically estimated biomass in autumn and spring, respectively ($p < 0.05$). There was no significant relationship with abundance ($p > 0.05$).	There were significant positive relationships between eDNA concentration and electrofished-estimated biomass and abundance ($p < 0.001$) (Fig. 11 and b).	Golden Perch: There was a positive relationship between ~1.1 and 5.5 kg l^{-1}; after which eDNA concentrations were consistent to 10.3 kg.	5, 6, and main text
2–4	Effects of flow on eDNA concentration	There was a significant negative relationship between eDNA concentration and flow ($p < 0.05$).	NA	NA	There was a significant negative relationship between eDNA concentration and flow ($p < 0.05$).	Presumably faster flow meant DNA was evacuated more quickly.	NA (main text)

(a)



(b)

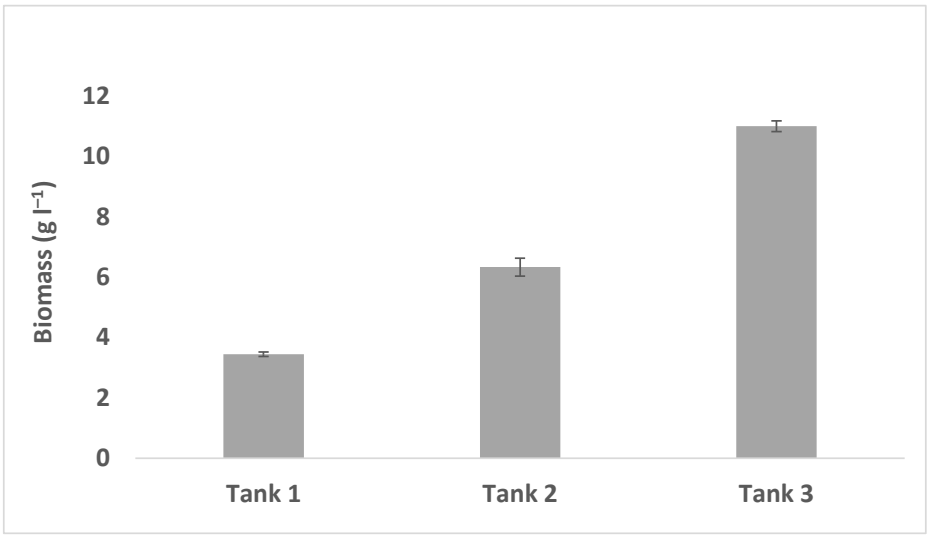
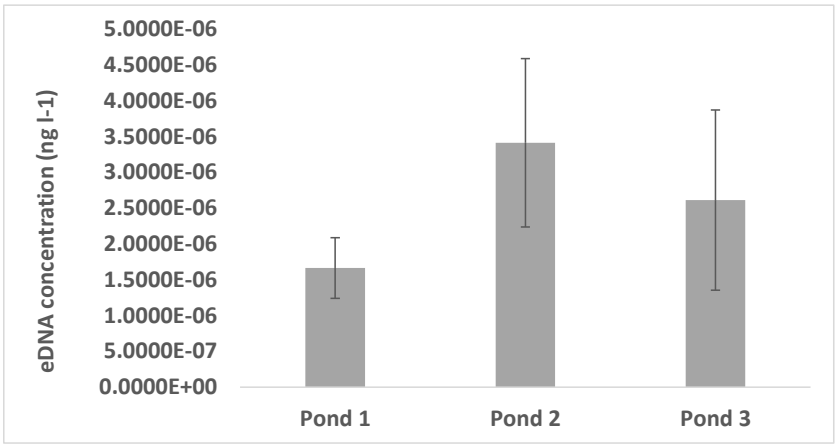


Figure 8. (a) Mean (± SE – where applicable) concentrations of eDNA, and the absolute (b) biomasses of adult Golden Perch in three flow-through tanks at the Narrandera Fisheries Centre.

(a)



(b)

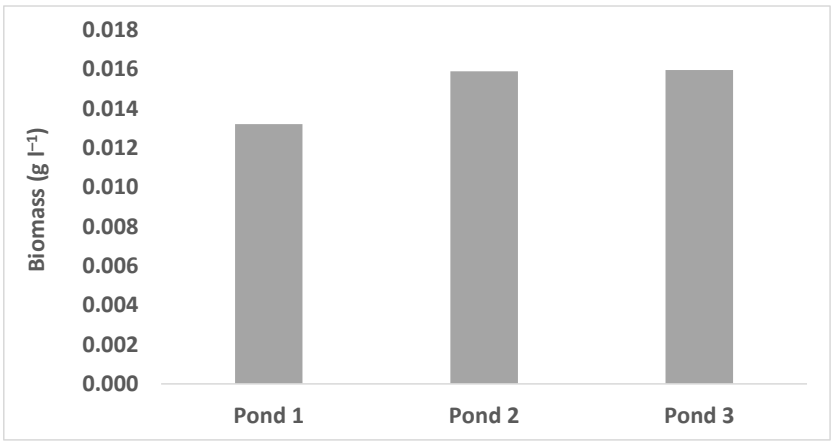


Figure 9. (a) Mean (± SE – where applicable) concentrations of eDNA and the corresponding absolute (b), biomasses of Golden Perch larvae in three mesocosms at the Narrandera Fisheries Centre.

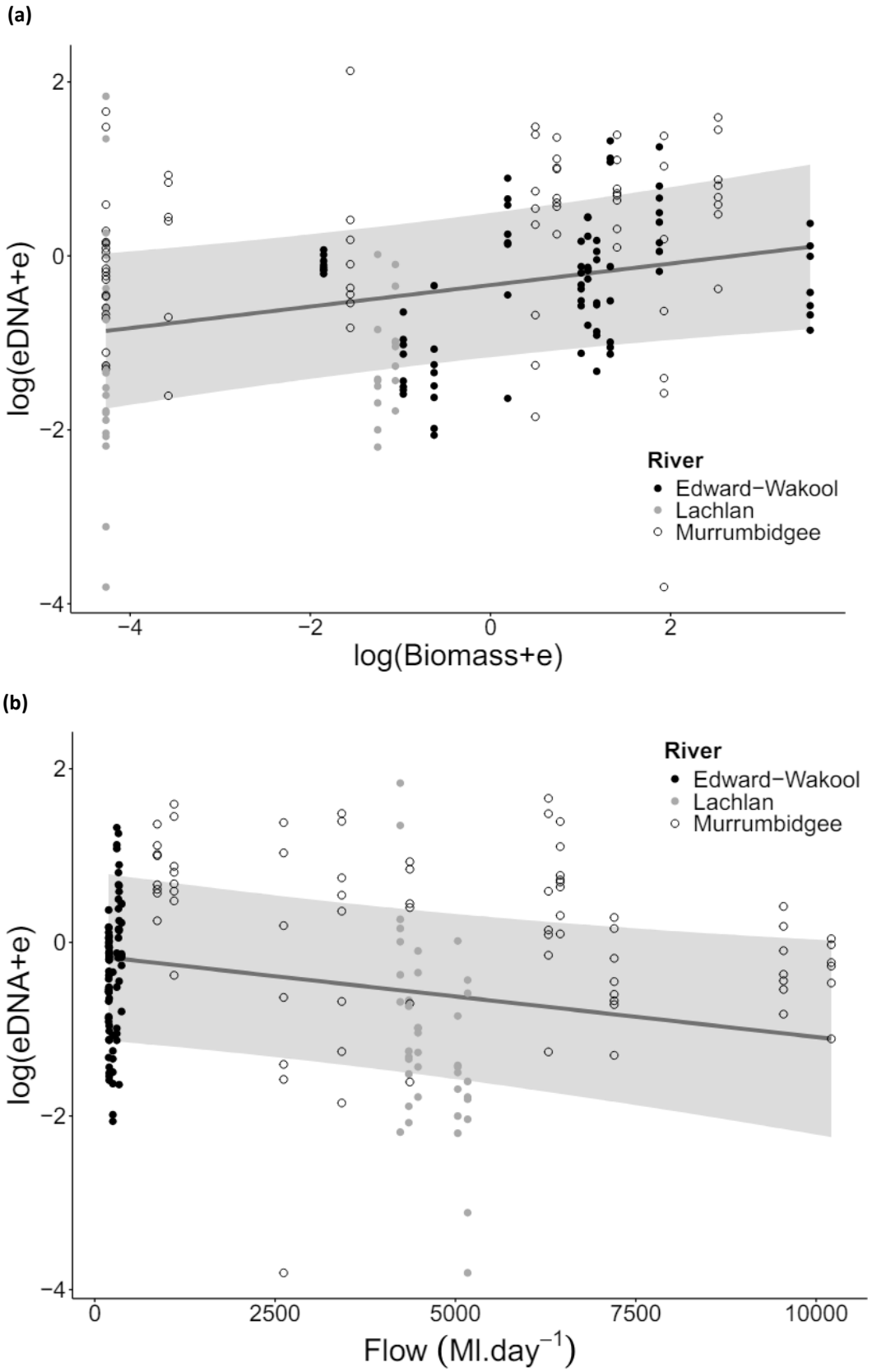


Figure 10. Significant linear relationships between log eDNA concentrations and (a) Murray Cod biomass, and (b) water flow in three rivers ($p < 0.05$).

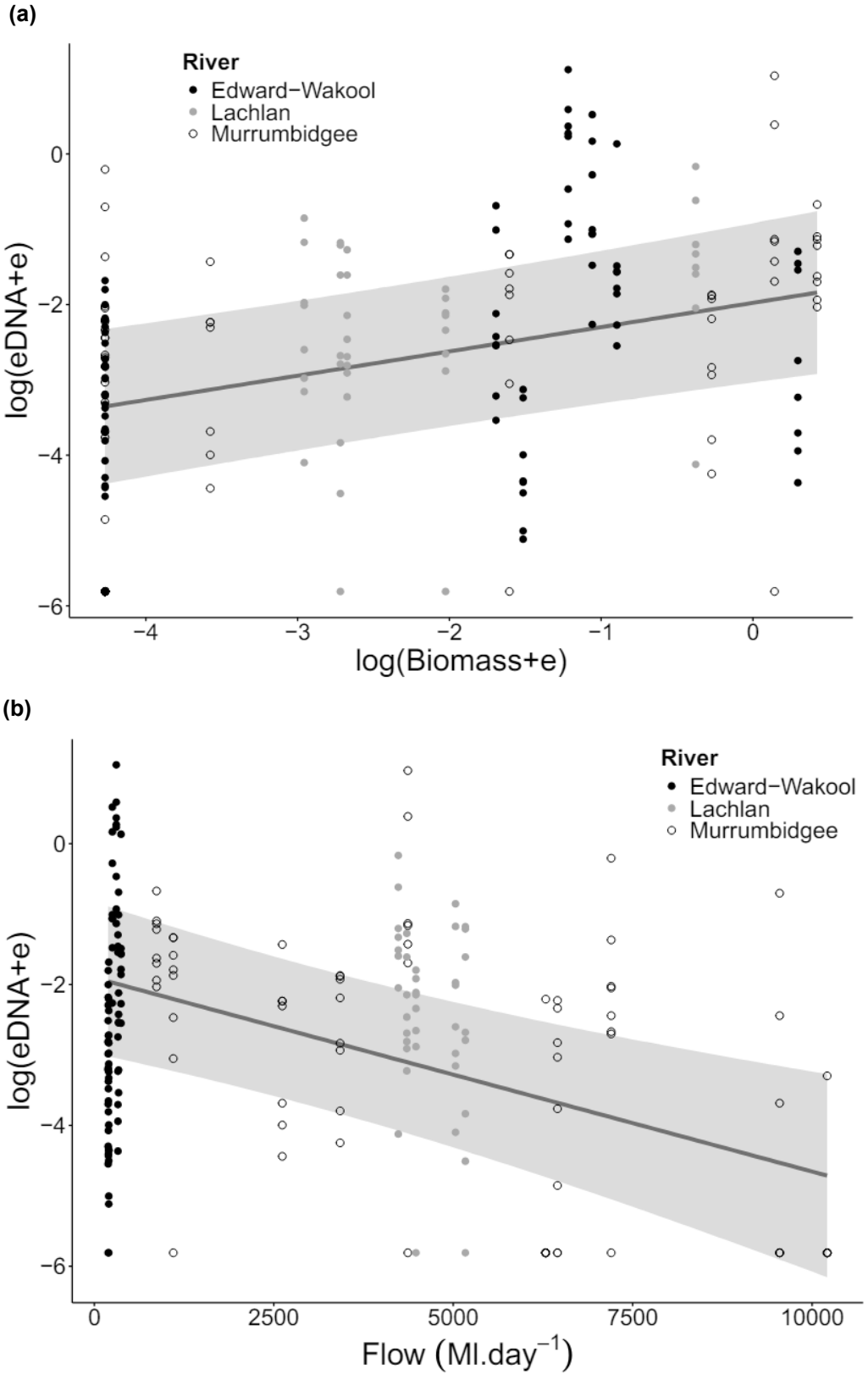


Figure 11. Significant linear relationships between log eDNA concentration and (a) Bony Bream biomass, and (b) water flow in three rivers ($p < 0.05$).

5. Discussion

Estimating the population size of fish stocks is fundamental to developing policies for managing their exploitation. Various fishery-dependent and independent monitoring approaches are used to census populations; each with various advantages and limitations. In this study, we conducted definitive and rigorous aquaria trials and field trials for four fish species of conservation and economic importance in Australia–Mulloway, Murray Cod, Golden Perch and Bony Bream—to determine the potential for estimating their relative biomasses and abundances using eDNA. The summarised body of work supports the general observations in the international literature of a positive relationship between eDNA concentration and either biomass or abundance (Appendix 1), but the strength of relationships varied among species and there were key affecting factors, notably flow, temperature and season (Appendices 3–6). Despite these caveats, there is clear evidence to support the assertion that monitoring eDNA concentrations is a valid method for estimating the relative biomass of teleosts. Ongoing refinement of protocols will facilitate future use in Australia's estuarine and freshwater environments.

5.1. Phase 1: literature review

The impact of this project has been substantial, as evidenced by the overwhelming response to our definitive literature review which, in the 36 months since publication, has accumulated ~250 citations. This exponential increase in research activity in the field underscores the growing interest in the utility of eDNA for estimating biomass and/or abundance. While the exact nature of citing papers varies, ranging from manipulative experiments to targeted reviews, most were the former. Such a trajectory suggests that thousands more papers could emerge on this topic in the next decade, affirming our position and this research project as being at the forefront of the field. Despite initial doubts about the project necessitating a stop/go approach, the success, as demonstrated by the review (i.e. 90% of reviewed papers identified useful relationships) and subsequent results, unequivocally validates the approach taken and justifies future regional research efforts.

Notwithstanding the clear trend identified, most studies noted at least some uncertainty around absolute (versus relative) biomass and/or abundance estimates. Such uncertainty was due to the processes affecting the production, degradation and transport of DNA in the environment (Hansen et al., 2018; Klymus et al., 2015; Wood et al., 2020), as well as the choice of eDNA capture, extraction methods and primer amplification biases (Eichmiller et al., 2016; Kelly et al., 2014). In some cases, this uncertainty might be considered acceptable (e.g. generating a relative index for temporal comparisons), but in other cases could produce unacceptable biases (e.g. attempting to understand the population size of an endangered, threatened or protected species).

The review identified relatively few Australian studies aimed to quantify relationships between eDNA concentrations and population sizes (a shortfall subsequently addressed). Nevertheless, of Australian studies published prior to 2021, most detected positive relationships between eDNA concentrations and estimates of relative biomass and/or abundance (Hinlo et al., 2017; Piggott, 2016; Rojahn et al., 2021) but see Hinlo et al., (2018). A further two studies published since our review have also showed positive relationships between eDNA concentration and biomass (Green et al., 2024; Uthicke et al., 2022). However, none of these studies involved aquaria trials to first validate the relationship between eDNA concentration and biomass under controlled conditions or to determine key affecting abiotic and biotic factors. To address this shortfall, we subsequently designed aquaria experiments to focus on several factors that could influence eDNA concentrations, including stocking density, temperature and individual shedding rates for two of the priority species (Murray Cod and Mulloway). Based on the outcome of these experiments, we then designed relevant field experiments for these species as well as the non-priority species.

5.2. Phase 2a: aquaria experiments

Contrary to the general consensus of our literature review, for our first studied species, Murray Cod, we did not detect a useful relationship between their eDNA concentrations and biomass. Specifically, while we identified a significant quadratic relationship between eDNA concentration and fish weight, this had no applied utility simply because eDNA concentrations $tank^{-1}$ increased with fish weight to ~180 g, but then subsequently declined as weights increased to a maximum of 311 g. Thus, our aquaria data implied Murray Cod was not a suitable candidate for using eDNA to quantify their biomass and so, following the logic in previous studies (e.g. Danziger et al., 2022), we determined (incorrectly in hindsight) that field studies were not warranted, or at least should not be prioritised.

There are at least three possible factors that contributed towards our interpretation of poor correlations between eDNA and biomass in aquaria for this species. First, the closed system which might have stressed the fish and created an imbalance between eDNA accumulation and decay. However, similar studies on other teleosts in closed systems have detected positive relationships between eDNA concentration and biomass (Doi et al., 2015; Karlsson et al., 2022; Takahara et al., 2012). Second, it is also possible that our method of collecting eDNA samples or PCR inhibitors could have influenced results, though we argued this was unlikely. Third and most likely, we speculated that the lack of a relationship could be due to the aggressive and territorial behaviour of Murray Cod which evokes variable activity among individuals (and subsequent variation in individual DNA shedding rates) in aquaria.

Regardless of the contributing factors, the observed outcomes from the aquaria trials for Murray Cod remain fairly unique among similar studies of teleosts in controlled environments (Doi et al., 2015; Horiuchi et al., 2019; Klymus et al., 2015; Mizumoto et al., 2018; Takahara et al., 2012). Rather, the few previous studies reporting the lack of coherent positive relationships were conducted under much more variable field conditions in lakes (Capo et al., 2019; Perez et al., 2017), rivers (Deutschmann et al., 2019; Hinlo et al., 2018) and oceans (Fraija-Fernandez et al., 2020; Knudsen et al., 2019). Clearly, while positive results in aquaria trials tend to imply the same in the field, the converse is not necessarily true. Perhaps the benefit of doing both is to gauge the extent to which variability is likely to be quantifiable in the field. More specifically, because Murray Cod can display measurable intra-specific variability in DNA shedding (potentially related to behavioural differences among individuals), this should be considered when interpreting field-based data. Indeed, the field-based trials still showed that this species had the least decisive relationship between eDNA and biomass, and certainly abundance (i.e. no relationship).

Based on the poor outcome for Murray Cod (in aquaria), we redirected our focus to Golden Perch under the assumption that any variation in individual shedding rates would be tempered by its generally greater densities than Murray Cod in the wild. We did not have the budget for comprehensive aquaria trials, but the general pattern of eDNA concentration in ponds containing juvenile Golden Perch and tanks containing adult Golden Perch were favourable and suggestive of a positive relationship between eDNA concentrations and biomass.

Like for Golden Perch and in contrast to Murray Cod, we detected a strong positive relationship between juvenile Mulloway eDNA concentrations and their biomass under controlled conditions, despite significant variation among individual shedding rates and influences of temperature. Similar to Murray Cod, there was substantial variability among tanks containing individual fish that was not explained by their weight. Despite this variation in shedding, the overall positive relationship between eDNA concentration and biomass remained strong for Mulloway. The results of the controlled experiments indicated directed field experiments were clearly warranted for Mulloway and Golden Perch. While Murray Cod results were not as encouraging, we postulated that field experiments may have a different outcome if behaviour did indeed affect outcomes and thus, we

included analyses of this species along with Bony Bream during the field experiments with Golden Perch.

5.3. Phase 2b: field experiments

It is important to note that for the field work (and unlike in the aquaria studies where absolute fish numbers and weights were known), neither eDNA, electrofishing nor hydroacoustic methods provided absolute estimates of biomass or abundance. Rather, our focus in the field was comparative analyses of two approaches for providing indices of relative quantities. This approach was similar in theme to studies comparing traditional active (trawls and seines) or passive (hooks, traps and gillnets) fishing gears (e.g. Gray et al. 2005, Bies et al. 2016). Ultimately, such comparisons can help to ascertain whether alternative methods offer statistically equivalent biomass or abundance indices and might be preferred because of not only lower operational costs, but also logistical benefits. Implicit within the latter is adequate understanding of the limitations of each method. For example, we clearly showed eDNA concentration estimates can be affected by numerous biotic and abiotic factors including water temperature, presence of PCR inhibitors, flow and choice of filter (Appendices 1, 3–6). Similarly, electrofishing and hydroacoustic methods are also affected by biotic and abiotic factors including flow, temperature and difficulty in classifying small individuals to species level (hydroacoustics) (Fernandez et al., 2023; Lyon et al., 2014; Martignac et al., 2015; Perivolioti et al., 2020; Rourke et al., 2022), which is discussed further below.

Nevertheless, all four assessed species showed relationships between their eDNA concentrations and biomasses in the field trials, though the nature and strength of the relationships varied between species, and only Bony Bream showed a relationship with abundance. The consistent relationship between eDNA and biomass across all species probably reflects the direct correlation with the number of cells produced (rather than the number of fish). Specifically, the eDNA shed by fish originates from all DNA-producing material in their bodies, which is directly determined by their weight. But without homogenous sizes (like for Bony Bream), the relationship between the number of fish and eDNA would intuitively be less clear; whereby a large number of very small fish will weigh the same as one larger fish (and so confound any relationship with eDNA). For this reason, biomass might be the most appropriate response variable (at present) for population monitoring via eDNA samples.

The Mulloway field experiments provided some support for the positive correlations between eDNA concentration and biomass previously detected in the controlled aquaria experiments (Appendices 4 and 6). By including seasonal effects in our analyses, we also identified considerable negative influences of extraneous factors. Specifically, while a significant positive relationship between eDNA concentration and biomass was noted in autumn, the opposite occurred in spring (Appendix 6). The latter anomaly was primarily driven by high eDNA concentrations when there were very few (or no) hydroacoustic detections of Mulloway. At least two possible explanations for this observation include: (1) relatively larger abundances of undetected Mulloway <40 cm TL; and/or (2) variability in eDNA persistence at a site. Considering the first hypothesis, following spawning in December, and based on penaeid-trawl bycatch, the greatest abundances of juvenile Mulloway (~5–15 cm TL) in the Hawkesbury River typically occur in autumn (when fish are up to ~6 months old), rather than summer (Kennelly, 1993). But, notwithstanding high natural mortality, many of these 0+ fish would be >20 cm TL by spring (and still undetectable by hydroacoustic scans) when they have lower trawl catchability (Broadhurst & Kennelly, 1994). If present, these larger juvenile fish might be expected to shed relatively more DNA than adult fish (Rourke et al., 2022).

The second possible explanation relates to the impact of environmental parameters on DNA persistence at a site. Assuming the hydroacoustic scans remained similarly efficient between seasons, any discrepancy between eDNA concentrations and hydroacoustic detections of Mulloway at a site in

spring could simply reflect greater rainfall. Lower salinity across most sites indicates greater freshwater flows during spring. These faster flows can dilute and/or transport eDNA from further upstream to the sampling location. Such effects may explain the low eDNA concentrations detected at some sites despite many Mulloway. Additionally, biotic and abiotic factors can influence eDNA production and persistence and are known to fluctuate between seasons (Barnes et al., 2014; Joseph et al., 2022). Various unmeasured variables (including UV light, chemicals, microorganisms and biofilms) may have also affected DNA persistence differently between seasons, leading to variation in detected DNA concentrations.

The Golden Perch field experiments also detected a significant relationship between eDNA concentration and electrofished biomass. However, the relationship was not a simple linear relationship, but rather asymptotic. This asymptotic relationship deviates from patterns observed in many similar studies comparing eDNA concentration to biomass, where linear relationships have been reported, although competing model fits are often not described. Nevertheless, the quadratic relationship reported here is similar to that reported for silver carp (*Hypophthalmichthys molitrix)* in a large river, assessed using eDNA and an acoustic survey (Coulter et al., 2019), and may be influenced by various factors, including but not limited to water temperature or flow, and eDNA production or degradation (Rourke et al., 2022).

Despite the stated limitations, there was a clear relationship between eDNA concentration and biomass for all species included in the field trials. The key factors that influenced the strength of the relationship are considered below.

5.4. Considerations for all experimental approaches

The comprehensive evaluation encompassing both controlled aquaria experiments and field trials inform relationships between fish eDNA concentration and biomass, highlighting the influence of various factors. Notwithstanding the possibility for some variability (e.g. the anomaly observed for Murray Cod), typically most relationships between eDNA concentration and biomass might be expected to be weaker in the natural environment than aquaria. This is simply because there are many uncontrolled factors, including water temperature and flow and different sizes/age-classes of fish that contribute towards the production and degradation of DNA (Yates et al., 2019). Nevertheless, it is more likely than not that for most species, there will be a relationship between eDNA concentration and biomass, although understanding key affecting factors such as those influencing the results in this study (temperature, seasonality, flow and individual shedding rates) remains cryptic. For example, season was a key factor in the positive relationship detected for Mulloway in autumn, but sampling across seasons was not carried out for the other three species. It is possible there may be seasonal effects that we have not detected.

Consequently, the challenges to applying eDNA methods to estimate biomass persist, particularly in the complexity of natural environments (including both freshwater and marine systems) where multiple factors affect eDNA dynamics. The contrasting relationship between eDNA concentration and biomass across seasons, underscores the need for ongoing refinement and validation of eDNA methodologies to ensure their reliability and accuracy in informing fisheries management strategies. By addressing these challenges and continuously refining our understanding of the eDNA-biomass relationship, we can maximise the full potential of eDNA as a powerful tool for sustainable fisheries management. Nevertheless, definitive conclusions regarding the superiority or otherwise of eDNA methods over fishery-dependent methods cannot be drawn without absolute biomass estimates in the field.

Despite the above limitations, eDNA methods for estimating relative biomass could see routine implementation in the coming years, provided additional data are collected to refine our understanding of relationships. More specifically, the method might find initial application for

estimating the relative biomass of Golden Perch populations where biomasses do not exceed 5.5 kg at a site, as well as Bony Bream (no biomass limit based on current data).

It is important to reiterate that virtually all current fishery-dependant or -independent methods do not estimate absolute abundance (Lyon et al., 2014; Rose & Kulka, 1999). Compared to many other methods, eDNA estimates may offer potential advantages for estimating relative or even absolute biomass in some systems, given the typically strong correlation in aquaria studies with known weights of fish (e.g. for Mulloway and other species mentioned in our literature review). But definitive conclusions regarding the superiority of eDNA methods over fishery-dependent methods cannot be drawn without adequate validation across field conditions (Yates et al., 2021). Implicitly, such work requires ongoing wide-scale spatiotemporal assessments to encompass the full range of influencing factors.

6. Conclusions

This project evaluated the potential for eDNA methods to estimate the relative biomass and/or abundance of key fish species in freshwater and estuarine environments. It is important to consider that the conclusions reached here are only applicable to the studied species and should not be extrapolated to other species in freshwater or estuarine/marine environments.

Our first objective, to assess the precision of correlations between eDNA concentrations and quantities of fish and to identify the key abiotic and biotic factors affecting eDNA concentration in controlled conditions, has been satisfied through an extensive literature review and the aquaria experiments that were peer-reviewed and published. The aquaria results were varied, with Mulloway and Golden Perch results supporting progression to field trials, while Murray Cod results were poor, and we initially reasoned (incorrectly) that field trials were not warranted.

The second objective of the project was to conduct field trials for priority species to compare eDNA concentrations to biomass and abundance estimates from fishery-dependent methods, and has also been satisfied. We conclude both Mulloway and Golden Perch are likely to be good candidate species for relative biomass estimates using eDNA, but with certain caveats. Specifically, for Golden Perch, the positive relationship with biomass tapered off at biomasses >~5.5 kg site^{-1}. In addition, we were unable to discern whether the inability to hydroacoustically detect juvenile Mulloway <40 cm TL could have affected our ability to detect a stronger relationship between eDNA concentration and biomass.

The third objective of the project to identify some of the factors affecting relationships between eDNA concentration and biomass and/or abundance indicated various biotic and abiotic factors were statistically influential. Similarly, the fourth objective of the project to assess if eDNA was correlated with biomass and/or abundance for other species has also been achieved. Despite the poor results for Murray Cod in aquaria, there was a positive correlation between their eDNA concentration and electrofished biomass; although the relationship was the weakest among the four species tested. Nevertheless, the field is advancing rapidly as evidenced by an upsurge in the published literature since our review was published in 2022. Methods are being continually refined and it is anticipated that future work will enable eDNA-based methods of estimating relative biomass to be a feasible option for Murray Cod.

Unlike for Murray Cod, the results here suggest eDNA-based methods for estimating the relative biomass of Golden Perch and Bony Bream could be adopted immediately; or at least introduced concurrent to electrofishing to generate greater comparative data sets. The positive results for Mulloway in autumn imply additional research (involving other fishery-dependant or -independent methods) could assist in unravelling those factors affecting the relationship and identify the most suitable period for eDNA sampling to estimate relative biomass.

7. Implications

This work has substantial implications for the future of non-lethal, fishery-independent monitoring of fish stocks. The results underscore the potential of eDNA-based methods for estimating the relative biomasses of Australian freshwater and estuarine fish stocks. However, if the method was to be applied to marine or open ocean environments, this would require substantial field testing for each candidate species to test its suitability given the contrast to riverine environments tested here and possible species-specific difference in eDNA shedding. Such work should be encouraged because developing any expensive, non-lethal stock survey method will benefit all stakeholders, including recreational and commercial fishers, managers and other groups directly or indirectly concerned with resource sustainability.

For Mulloway, adopting non-lethal monitoring methods presents some clear advantages over current lethal methods (in estuarine environments), allowing for more rigorous population monitoring and a deeper understanding of stock status. This, in turn, would enable fisheries managers to refine planning strategies, promoting both profitability and sustainability within the Mulloway fishery. Nevertheless, additional data should be collected to help refine relationships (see Recommendations). It is also important to consider that current eDNA technology does not yet provide information on size structures (which can be collected with lethal methods). Ancillary size-at-age data would still be required to compliment eDNA methods.

For the freshwater species examined in this study, while traditional methods such as electrofishing have proven effective, exploring alternative, non-capture-based options holds considerable appeal. This is particularly the case in the spatially complex coastal and inland waterways of NSW. A monitoring method that is both non-lethal and applicable across diverse environments would be highly advantageous, potentially enhancing cost-effectiveness and overall monitoring efficiency.

8. Recommendations

Based on the collected data, the recommendations for the project are as follows:

1. **Owing to consistent intra- and interspecific variability in relationships between eDNA and abundance, prioritise investigating relationships between eDNA and biomass.**

- Substantial variability in the sizes of large-bodied fish in some systems might preclude the meaningful utility of investigating relationships between eDNA and the abundance of some species. So, like for traditional fish-dependant methods, efforts might focus on assessing relationships with relative weights (biomass).

2. **Collect eDNA samples when electrofishing in freshwater rivers across seasons.**

- To increase the amount of data available and thus enable refinements to relationships between eDNA concentrations and biomasses to be made, additional data are required.
- This work could be done relatively easily alongside existing large-scale electrofishing projects, which already collect basic water-quality data.

3. **Collect eDNA samples during commercial fishing operations for Mulloway in estuaries across seasons.**

- As for freshwater species, additional data are required to refine the relationship between eDNA concentrations and Mulloway biomasses.
- This could be done as part of routine observer work for Mulloway and potentially other priority species where data are recorded.

4. **Develop a protocol to facilitate collecting eDNA samples from freshwater and marine environments.**

- The protocol should specify the number of samples required and from which locations, including the preferred timing of collection (season, flow conditions, tide etc.). In addition, this protocol should include training requirements to minimise the risk of sample contamination.

5. **Validate hydroacoustics for estimating Mulloway biomass in estuaries.**

- The hydroacoustic method for estimating Mulloway biomass in this study has yet to be validated.
- This could be done using tank and cage experiments (and involve other co-occurring species). There is a particular need to determine if hydroacoustic techniques can be refined to detect Mulloway <40 cm TL. Not only would this provide increased confidence around comparisons with eDNA concentrations, but hydroacoustics may be a viable method for not only evaluating Mulloway biomass in estuaries, but also various other priority species; especially if individual sizes can be discerned.

8.1. Further development

This project has identified several areas of future development. But because of the rapid expansion of science relevant to this field, prior to commencing new work, another comprehensive literature review may be warranted to ensure that the most current and relevant information is considered.

In future development for the species included in the current study, there is substantial scope to refine eDNA-based methods for population size estimates. More specifically, for Mulloway, where the relationship between eDNA concentration and biomass varied across seasons, there is a clear need to continue data collection efforts. This work may involve conducting eDNA sampling at various depths, during different tide conditions, and across multiple seasons over several years to capture the full scope of environmental variability. Similarly, for Golden Perch, Bony Bream and potentially Murray Cod, further research exploring the intricacies of eDNA sampling under varying environmental conditions could contribute to more accurate biomass estimates and enhance our understanding of ecological dynamics influencing eDNA concentrations. Such work would benefit from the input of an ecological modeller on the project team.

For new species, future work should prioritise aquaria studies, and these should all include an experiment specifically aimed at assessing the shedding rates of individual fish. If the results are favourable, then field trials are clearly warranted. Unfavourable results may require review, although we have shown that this outcome is not necessarily definitive in terms of identifying the suitability of a particular species. And nor should this probably be the case, because intuitively there should always be a positive relationship between the weight of fish and their shed DNA. The difficulty lies in deciphering meaningful utility in the presence of unknown confounding factors. Technology will likely facilitate overcoming such obstacles.

It is possible that eDNA methods of estimating biomass may outperform conventional methods that estimate CPUE. However, achieving such an outcome requires testing eDNA concentrations against absolute population estimates. Beyond closed systems, such estimates can be obtained through capture-mark-recapture methods, although these can be costly, time consuming and may overlook smaller life stages. Nevertheless, such experiments have been successfully conducted in lake environments where strong correlations have been achieved (Spear et al., 2021).

Most studies (including this project) incorporating controlled aquaria experiments generate a range of biomasses by altering the number of similar-sized fish in aquaria. This does not address the potential differences in eDNA production between different-sized individuals. Future work might include allometric scaling to account for potential differences in eDNA production across individuals of different sizes and improve correlations between eDNA concentration and biomass (Spear et al., 2021; Yates et al., 2021).

The benefits of quantifying DNA also extend beyond the scope of the current project. For example, an eDNA-based approach for detecting spawning of Macquarie Perch (*Macquaria australasica*) has been developed. This method assessed the change in the ratio of nuclear to mitochondrial DNA that occurred when the species was spawning (Bylemans et al., 2017). It is recommended to explore similar approaches for other key species, including Mulloway, where the ability to accurately predict spawning aggregations could inform management strategies such as fine-scale closures and enhance stock recovery efforts.

Given the rapid advancement of technology allowing for the extraction of single cells from eDNA samples (Adams et al., 2019), future research could explore the potential of this approach. Collecting and extracting DNA from individual cells might not only provide population-size estimates based on identified individual fish within a sample, but could simultaneously provide valuable insights into effective population size, genetic structure, and diversity.

9. Extension and adoption

As evident from the project outputs (e.g. the FRDC final report, lay summaries and presentations to end users, five published manuscripts, conference abstract and FRDC Fish News article), the outputs of this research (1) identified the key abiotic and biotic factors affecting correlation between eDNA concentration and biomass in controlled and natural conditions, (2) identified the utility of eDNA to estimate the biomass of four fish species and abundance of one, and (3) provided clear direction for future research to enable eDNA estimates to be adopted as a method for monitoring the relative biomass of key species.

The extension and adoption plan objectives were to:

1. provide sufficient information to clearly encapsulate the utility of eDNA for assisting in fish abundance and/or biomass estimates and resource management;
2. ensure that all stakeholders, including state and federal agencies and international NGOs are aware of the research outcomes; and
3. encourage the ongoing development of DNA-based techniques as non-lethal, fishery-independent methods for assessing fish population abundance and biomass.

These objectives were achieved according to the following plan:

1. *Target audience: Key stakeholder groups (recreational and commercial fishers) and the general public.*

   Key message: An initial project brief, and then 12-monthly non-technical summaries of research results were provided.
   Communication/extension: The above project brief and project summaries were shared with NSW DPIRD fisheries managers and stakeholders in the form of lay summaries and presentations (Appendices 10–14) and an FRDC FishNews article in the last quarter of 2024 (Appendix 15). Matt Broadhurst spent 20 days onboard commercial prawn trawlers sampling Mulloway and promoting the project. In addition, the project results were discussed at Mulloway Harvest Strategy Working Group meetings.

2. Target audience: Fisheries managers.

   Key message: Ongoing outcomes of the research and the recommended utility of the approaches.
   Communication/extension: NSW DPIRD Fisheries managers were briefed throughout the project (Appendices 8–10), with state resource managers and scientists provided with copies of the project summary (Appendix 12) and through conversations concerning the applicability of the eDNA approach and what potentially means for future assessments. We provided lay summaries of the five published manuscripts (Appendix 13) and undertook written correspondence to fisheries officers and the fishing industry (Appendices 11 and 12) to explain the work and how it could be implemented for biomass estimates in the future.

3. *Target audience: national and international scientific community (and NGOs)*

Key message: The aims, methodologies and results of experiments.
Communication/extension: Five papers (Appendices 1–5) were published prior to submitting this final report to the FRDC, thus nearly all results were critically reviewed by the scientific community and made publicly available before publication of this FRDC report. The literature review has already been cited >250 times, indicating the global extension of the first phase of the project. The early results of the work were presented to the 1st Australian and New Zealand Environmental DNA (eDNA) Conference: Innovation & Application in 2023 (Appendix 7). The ultimate success of the extension plan will be measured by the eventual implementation of eDNA methods to estimate biomass.

10. Project coverage

The project coverage is detailed in Appendices 1–15.

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12. Appendices

Appendix 1. Rourke, M.L., Fowler, A.M., Hughes, J., Broadhurst, M.K., DiBattista, J.D., Fielder, S., Wilkes Walburn, J. and Furlan, E.M. 2022. Environmental DNA (eDNA) as tool for assessing fish biomass: a review of approaches and future considerations. Env. DNA 4, 9–33. Link to article

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SPECIAL ISSUE ARTICLE

Environmental DNA

WILEY

Environmental DNA (eDNA) as a tool for assessing fish biomass: A review of approaches and future considerations for resource surveys

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Abstract

Environmental DNA (eDNA) has revolutionized our ability to identify the presence and distributions of terrestrial and aquatic organisms. Recent evidence suggests the concentration of eDNA could also provide a rapid, cost-effective indicator of abundance and/or biomass for fisheries stock assessments. Globally, fisheries resources are under immense pressure, and their sustainable harvest requires accurate information on the sizes of fished stocks. However, in many cases the required information remains elusive because of a reliance on imprecise or costly fishery-dependent and independent data. Here, we review the literature describing relationships between eDNA concentrations and fish abundance and/or biomass, as well as key influencing factors, as a precursor to determining the broader utility of eDNA for monitoring fish populations. We reviewed 63 studies published between 2012 and 2020 and found 90% identified positive relationships between eDNA concentrations and the abundance and/or biomass of focal species. Key influencing biotic factors included the taxon examined as well as their body size, distribution, reproduction, and migration. Key abiotic factors mostly comprised hydrological processes affecting the dispersal and persistence of eDNA, especially water flow and temperature, although eDNA collection methods were also influential. The cumulative influence of these different factors likely explains the substantial variability observed in eDNA concentrations, both within and among studies. Nevertheless, there is considerable evidence to support using eDNA as an ancillary tool for assessing fish population abundance and/or biomass across discrete spatio-temporal scales, following preliminary investigations to determine species- and context-specific factors influencing the eDNA abundance/biomass relationship. Advantages of eDNA monitoring relative to other approaches include reduced costs, increased efficiencies, and nonlethal sampling.

KEYWORDS

abundance, biomass, environmental DNA, fishery-independent survey, stock assessment

1 | INTRODUCTION

The global harvest from wild marine and inland fisheries peaked at ~90 million tonnes during the mid-1990s and has fluctuated around this value to the present day (FAO, 2018). While there remains conjecture over the accuracy of these estimates (Branch et al., 2011; Pauly & Zeller, 2017), it is clear that within the same period (and prior to the early 1970s) the proportion of sustainably harvested stocks declined. The most recent estimate suggests only 67% of stocks are sustainably harvested (FAO, 2018). In addition to concerns over the future sustainability of the world's harvested stocks are the impacts to populations of bycatch species, particularly endangered, threatened, and protected (ETP) species (Gray & Kennelly, 2018; Lewison et al., 2004).

Sustainable harvesting requires accurate information on population size, exploitation rates, population connectivity, dynamics, and size structures. However, much of the intrinsic information can be challenging to obtain for many species. Estimating the size (usually biomass) of fished populations, or proxies of size that allow relative temporal trends to be examined, is usually achieved by collating *in situ* fishery-dependent or fishery-independent data as a basis for stock assessments (Beverton & Holt, 1957; Hilborn & Walters, 1992).

Fishery-dependent methods typically involve collecting catch data from vessel logbooks and/or scientific sampling onboard or during landings (Doubleday & Rivard, 1983). While cost-effective and broad scale, such data are subject to numerous biases, including variable fishing effort or efficiency and/or hyperstability (where catch per unit of effort remains stable while the population declines; Dennis et al., 2015). Commercial catches are also a diminishing data source for assessment in some developed nations, owing to declining yields due to fleet consolidations and increased pressure from recreational fisheries that are often poorly assessed themselves (Gray & Kennelly, 2017). Other assessment methods (mostly among developed nations) utilize fishery-independent surveys by deploying similar gears as those fished commercially (Dennis et al., 2015) that are sometimes supplemented by larval sampling (e.g., egg counts for fish, or puerulus surveys for rock lobsters) (Chittleborough & Phillips, 1975; Ospina-Álvarez et al., 2013). These methods can provide high-quality representative data for assessments, but are often costly and logistically demanding. For ETP species, conventional survey methods for stock assessment are generally too invasive and often conflict with animal-welfare legislation. Cost-effective data sources that are free from fisheries operational biases and do not impact populations of declining species will be invaluable for supporting sustainable fisheries worldwide (Gray & Kennelly, 2018).

One alternative approach to conventional survey methods involves genetic analyses through sampling environmental DNA (eDNA) (Kamoroff & Goldberg, 2018; Shelton et al., 2019). All organisms release mucus, feces, urine, gametes, and skin cells which, for aquatic species, result in intra- and extracellular DNA being suspended in the water column and detectable for up to ~60 days (Dejean et al., 2012; Strickler et al., 2015; Thomsen et al., 2012). This eDNA can be collected and used to identify the recent presence and distribution of a species; often at lower costs than traditional survey methods (Jerde, 2019; Lugg et al., 2018; Yamamoto et al., 2016). Alternatively, eDNA metabarcoding using universal primers can allow multiple species to be rapidly and concurrently identified (Berry et al., 2019; McInerney & Rees, 2018; Shaw et al., 2016; Stein et al., 2014; Thomsen et al., 2016; Yamamoto et al., 2017).

Most of the early research on eDNA was constrained to detecting species presence (Cristescu & Hebert, 2018; Deiner et al., 2017; Evans & Lamberti, 2018; Rees et al., 2014; Thomsen & Willerslev, 2015). More recent investigations have examined whether eDNA concentrations correlate with the abundance and/or biomass of aquatic taxa (e.g., fish—Takahara et al. (2012); amphibians—Thomsen et al. (2012); Pilliod et al. (2013); and molluscs—Goldberg et al. (2013)). For some fish species, these relationships have been confirmed in controlled environment (aquaria/mesocosm) studies (Doi et al., 2015; Klymus et al., 2015), but are less clear in natural environments (Spear et al., 2015; Yamamoto et al., 2016; Yates et al., 2019)—reflecting at least two considerations. First, most studies have compared eDNA concentrations to abundance and/or biomass estimated via traditional survey methods, which have their own inherent biases (Lyon et al., 2014). Second, and perhaps more importantly, a plethora of uncontrolled species-specific biotic and abiotic factors may affect eDNA concentrations, and in turn abundance and/or biomass estimates (Deiner et al., 2017; Hansen et al., 2018; Stewart, 2019; Yates et al., 2019). Nevertheless, multiple studies showing correlations between abundance and/or biomass and eDNA concentrations for various taxa justify not only ongoing efforts to improve accuracy and precision, but also a synthesis of existing information to help refine future efforts (Mace et al., 2000; Shelton et al., 2016). Ultimately, consolidation of efforts in this area is required to inform fisheries management and conservation efforts globally (Shelton et al., 2016).

Based on the information above, our primary objective in this synthesis paper was to evaluate the broader utility and limitations of eDNA for quantifying the abundance and/or biomass of fish through a systematic review of the available literature. Specifically, we aimed to (a) collate published peer-reviewed studies and describe biotic and abiotic factors affecting the relationship between eDNA concentrations and abundance and/or biomass, and then (b) use this information to propose future research efforts in this developing field.

2 | METHODS

A search was conducted on the Web of Science for published, peer-reviewed literature (January 2000 to October 2020) with predetermined stem keywords (and variants) including 'eDNA or environmental DNA', 'abundance, or biomass, or quanti*', 'marine or aquatic or freshwater or river* or estuar*'. This initial search identified 631 publications, which were further filtered using 'fish'. All papers referencing fish were manually filtered and only retained if their titles or abstracts were consistent with the stated objective above.

TABLE 1 Alphabetical list of family, scientific and common names, and taxonomic authority and target region of species-specific primers for fish mentioned in the review, with superscripts describing the most recent International Union for the Conservation of Nature (IUCN) Red List global classification: NE, not evaluated; DD, data deficient; LC, least concern; NT, near threatened; VU, vulnerable; and EN, endangered. The number of reviewed studies assessing each species (species-specific markers only) was either one, or as marked in parentheses. ‡The specificity of the oligonucleotides may differ depending on co-occurring species

Family	Accepted Latin name	Taxonomic authority	Common name	Target region and reference for oligonucleotides‡
Acipenseridae	*Acipenser fulvescens* LC	Rafinesque, 1817	Lake sturgeon	COI and CytB (Yusishen et al., 2020)
Anguillidae	*Anguilla japonica* EN (2)	Temminck & Schlegel, 1846	Japanese eel	16S rRNA (Watanabe et al., 2005)
	Anguilla marmorata LC	Quoy & Gaimard, 1824	Giant mottled eel	16S rRNA (Itakura et al., 2020)
Carangidae	*Trachurus japonicus* NT (5)	(Temminck & Schlegel, 1844)	Japanese jack mackerel	CytB (Fukaya et al., 2020; Jo et al., 2017; Takahashi et al., 2020; Yamamoto et al., 2016)
	Micropterus salmoides LC	(Lacepède, 1802)	Largemouth bass	CO1 and ND4 (Perez et al., 2017)
Centrarchidae	*Lepomis macrochirus* LC	Rafinesque, 1819	Bluegill sunfish	CytB (Maruyama et al., 2014)
Cichlidae	*Hemichromis letourneuxi* LC	Sauvage, 1880	African jewelfish	COI (Diaz-Ferguson et al., 2014)
Clupeidae	*Alosa aestivalis* VU	(Mitchill, 1814)	Blueback herring	COI (Plough et al., 2018)
	Alosa pseudoharengus LC	(Wilson, 1811)	Alewife	COI (Plough et al., 2018)
	Clupea harengus LC	Linnaeus, 1758	Atlantic herring	CytB and ND4 (Knudsen et al., 2019)
	Dorosoma cepedianum LC	(Lesueur, 1818)	Gizzard chad	ND3 and ND5 (Perez et al., 2017)
	Sardinops sagax LC	(Jenyns, 1842)	Pacific sardine	mtD-loop (Sassoubre et al., 2016)
Cobitidae	*Misgurnus anguillicaudatus* LC (2)	(Cantor, 1842)	Oriental weatherloach	12S rRNA (Furlan et al., 2016)
Cyprinidae	*Cyprinus carpio* VU (6)	Linnaeus, 1758	Common carp	CytB (Eichmiller et al., 2014; Takahara et al., 2012); 12S rRNA (Furlan & Gleeson, 2016b)
	Hypophthalmichthys molitrix NT (3)	(Valenciennes, 1844)	Silver carp	mtD-loop (Coulter et al., 2013); mtDNA (Farrington et al., 2015)
	Hypophthalmichthys nobilis DD (2)	(Richardson, 1845)	Bighead carp	mtD-loop (Coulter et al., 2013; Erickson et al., 2016)
	Opsariichthys uncirostris NE	(Temminck & Schlegel, 1846)	Three-lips	mtD-loop (Yamanaka et al., 2018)
	Rhinichthys cobitis VU	(Girard, 1856)	Loach minnow	CytB (Dysthe et al., 2016)
Engraulidae	*Engraulis mordax* LC	Girard, 1854	Northern anchovy	mtD-loop (Sassoubre et al., 2016)
	Engraulis japonicus LC	Temminck & Schlegel, 1846	Japanese anchovy	CytB (Ushio et al., 2018)
Gadidae	*Gadus morhua* VU	Linnaeus, 1758	Atlantic cod	CytB and ND4 (Knudsen et al., 2019)
Gobiidae	*Eucyclogobius newberryi* NT	(Girard, 1856)	Tidewater goby	CytB (Schmelzle & Kinziger, 2016)
	Neogobius melanostomus LC	(Pallas, 1814)	Round goby	COI (Nathan et al., 2015)
Labridae	*Halichoeres tenuispinis* LC	(Günther, 1862)	Wrasse	CytB (Takahashi et al., 2020)
Osmeridae	*Thaleichthys pacificus* LC	(Richardson, 1836)	Eulachon	COI (Pochardt et al., 2019)
Oplegnathidae	*Oplegnathus fasciatus* NE	(Temminck and Schlegel, 1844)	Striped knifejaw	CytB (Takahashi et al., 2020)

(Continues)

TABLE 1 (Continued)

Family	Accepted Latin name	Taxonomic authority	Common name	Target region and reference for oligonucleotides[‡]
Percichthyidae	*Macquaria australasica* [EN]	Cuvier, 1830	Macquarie perch	12S rRNA and 18S rRNA (Piggott, 2016)
Percidae	*Perca fluviatilis* [LC]	Linnaeus, 1758	Redfin perch	12S rRNA (Furlan & Gleeson, 2016a)
	Etheostoma flabellare [LC]	Rafinesque, 1819	Fantail darter	CytB (Guivas & Brammell, 2020)
Petromyzontidae	*Petromyzon marinus* [LC] (2)	Linnaeus, 1758	Sea lamprey	COI (Gingera et al., 2016; Gustavson et al., 2015); CytB, ND1 and ND4 (Schloesser et al., 2018)
Plecoglossidae	*Plecoglossus altivelis* [DD]	(Temminck & Schlegel, 1846)	Ayu	CytB (Yamanaka & Minamoto, 2016)
	Plecoglossus altivelis ryukyuensis [EN]	(Temminck & Schlegel, 1846)	Ryukyu ayu	ND5 and COI (Akamatsu et al., 2020)
Pleuronectidae	*Platichthys flesus* [LC]	(Linnaeus, 1758)	European flounder	CytB and ND4 (Knudsen et al., 2019)
	Pleuronectes platessa[LC]	Linnaeus, 1758	European plaice	CytB and ND4 (Knudsen et al., 2019)
Salmonidae	*Oncorhynchus kisutch* [NE]	(Walbaum, 1792)	Coho salmon	COI (Rasmussen Hellberg et al., 2010)
	Oncorhynchus nerka [LC] (2)	(Walbaum, 1792)	Sockeye salmon	COX3 (Tillotson et al., 2018); COI (Rasmussen Hellberg et al., 2010)
	Oncorhynchus tshawytscha [NE]	(Walbaum, 1792)	Chinook salmon	CO3/ND3 (Shelton et al., 2019)
	Parahucho perryi [NE]	(Brevoort, 1856)	Sakhalin taimen	ND2 (Mizumoto et al., 2018)
	Salmo salar [VU]	Linnaeus, 1758	Atlantic salmon	NAD5 and COI (Wood et al., 2020)
	Salmo trutta [LC] (2)	Linnaeus, 1758	Brown trout	COI (Deutschmann et al., 2019); CytB (Capo et al., 2020)
	Salvelinus alpinus [LC] (2)	(Linnaeus, 1758)	Arctic char	CytB (Capo et al., 2020; Rodgers et al., 2018)
	Salvelinus fontinalis [NE] (5)	(Mitchill, 1814)	Brook trout/brook charr	CytB (Jane et al., 2015; Wilcox et al., 2013)
	Salvelinus namaycush [NE]	(Walbaum, 1792)	Lake trout	COI (Lacoursière-Roussel et al., 2016a)
Scombridae	*Scomber scombrus* [LC]	Linnaeus, 1758	Atlantic mackerel	CytB and Nd4 (Knudsen et al., 2019)
	Scomber japonicus [LC]	Houttuyn, 1782	Pacific chub mackerel	COI (Sassoubre et al., 2016)
Sparidae	*Acanthopagrus schlegelii* [LC]	(Bleeker, 1854)	Blackhead seabream	CytB (Takahashi et al., 2020)

The collected literature was also assessed for any additional relevant references within each publication. The key methods, outcomes, and/or limitations of each paper were tabulated, and the most common themes were used to partition topics for this review. The focus was to identify studies that tested the relationship between eDNA concentrations and fish abundance and/or biomass. We identified a relationship as being positive (or negative) when it was explicitly stated by the authors of the publications and objectively supported by hypothesis testing with $p = 0.05$.

3 | RESULTS AND DISCUSSION

In total, 63 papers assessing both freshwater and saltwater fish species satisfied the review criteria, with an almost linear increase in publishing between the first article in 2012 and the most recent in 2020 (Tables 1 and 2; Figure 1). Of note, we failed to find any references to species-specific or metabarcoding approaches based on eDNA analyses in aquatic environments until 2008 (amphibians) and 2012 (amphibians, invertebrates, fish, and mammals), respectively (Ficetola et al., 2008; Thomsen et al., 2012). Most studies were completed in the United States of America (USA; 53%) and Japan (25%) and were biased toward experiments done in the field (rivers, lakes, estuaries, and the sea; 60%) followed by those in controlled environments (aquaria or artificial ponds; 25%). The remaining studies (14%) were conducted across a combination of the field and controlled environments, but all within developed nations (Table 1, Figure 2).

Salmonidae (includes salmon, trouts, chars, freshwater whitefishes, and graylings) was the most assessed family with 16 papers using species-specific detection methods, followed by Cyprinidae (13 papers; includes carps, the true minnows and their relatives) (Table 1). Only three species-specific assessments involved globally listed ETP species (Table 1). Most studies used species-specific detection methods (51 or 81% across 46 species; Tables 1 and 2) rather than metabarcoding methods (12 or 19% across > 200 species; Table 2) to evaluate eDNA concentrations, with the latter restricted to estimating only relative abundance and/or biomass.

Irrespective of the experimental location, only six (10%) of the 63 studies accepted the null hypothesis of no relationship between eDNA and the abundance and/or biomass of focal species (Capo et al., 2019; Deutschmann et al., 2019; Fraija-Fernandez et al., 2020; Hinlo et al., 2018; Knudsen et al., 2019; Perez et al., 2017) (Table 2). While this general outcome is compelling, it is important to acknowledge the possibility of biases whereby researchers are less likely to publish their null or negative results. Nevertheless, among the published studies there was consistent support for the utility of the approach with 46 (90%) and 11 (92%) studies incorporating species-specific or metabarcoding methods, respectively, reporting positive correlations between eDNA concentrations or read counts and abundance and/or biomass (Table 2).

Notwithstanding the clear trend identified, most studies noted at least some uncertainty around absolute (versus relative) abundance or biomass estimates. Such uncertainty was due to the processes affecting the production, degradation, and transport of DNA in the environment (Hansen et al., 2018; Klymus et al., 2015; Wood et al., 2020), as well as the choice of eDNA capture, extraction methods, and primer amplification biases (Eichmiller et al., 2016; Kelly et al., 2014). In some cases, this uncertainty might be considered acceptable (e.g., generating a relative abundance index for temporal comparisons), but in other cases could produce unacceptable biases (e.g., attempting to understand the population size of an ETP species). The biotic and abiotic factors most commonly identified across studies are critically discussed below and used to consider the future utility of eDNA concentrations for estimating abundances and/or biomasses of fish populations.

3.1 | Biotic factors affecting eDNA concentrations

Considerable research has been completed to examine what is perhaps the key biological consideration for estimating abundance/biomass: How the quantity of eDNA in the water is affected by biotic factors directly related to eDNA production by a focal organism (Table 2). Various biotic factors affect eDNA concentrations, with interactive effects frequently identified among both biotic and abiotic influences. Such an outcome is somewhat implicit considering the source for eDNA is the biological organism itself, and so the movements of these molecules reflect intrinsic processes, as well as external influences on those intrinsic processes. The influence of each of these variables is discussed separately below, although the interrelated effects of intrinsic factors on eDNA production mean that isolating individual effects is challenging. Despite this, knowledge of combined effects may be sufficient to correct estimates of abundance or biomass derived from measures of eDNA concentration.

3.1.1 | Intraspecific variation in DNA production

Intraspecific variation in DNA production and shedding rates among individuals experiencing the same environment and exhibiting the same behavior could potentially impact estimates of abundance and/or biomass (Kelly et al., 2014). Several aquaria studies reported large variations in DNA shedding rates, despite using similar-sized individuals of the same species and after eliminating some other confounding influences (Horiuchi et al., 2019; Klymus et al., 2015; Minamoto et al., 2017; Wilcox et al., 2016). For example, Klymus et al. (2015) observed up to a 100-fold variation in day-to-day DNA shedding (interpolated from eDNA in the water) from the same fish under controlled conditions—variability that the authors postulated was due to different sources of the DNA, including tissues, cells, and fecal debris that were unlikely to have be evenly dispersed in the water column. In another example, the greatest DNA shedding rates during experimental trials using tanks housing round gobies (*Neogobius melanostomus*) came from a tank with a single fish versus

TABLE 2 Spatio-temporal summary of peer-reviewed literature published between January 2000 and October 2020 describing methods for assessing null hypotheses associated with the utility of eDNA, and key limiting factors, for quantifying the abundance and/or biomass of fish. C, Controlled environment; L, lake; R, river; E, estuary, S, sea; Sp, species-specific; Met, metabarcoding; and significant (+) positive or (–) negative effect if null hypothesis was rejected (continuous variables only), $p < 0.05$. Refer to Table 1 for the Latin names. *Key null hypothesis was interpreted where not clearly stated

Country	Species	Key null hypothesis (*Ho*)*	Water volume collected/filtered and DNA extraction method
Japan[C, L]	Common carp [Sp]	Difference between eDNA concentrations and water temperature and known biomass	0.02 L centrifuged into pellet, with DNA extracted using a Qiagen DNeasy Blood and Tissue Kit (aquaria exp). 2.0 L through a 3.0 or 0.8-μm polycarbonate filter, with DNA extracted as above (mesocosm exp)
USA[C, R]	African jewelfish[Sp]	Difference between eDNA concentrations and known abundance	1.0 L through a 0.45-μm cellulose nitrate filter, with DNA extracted using the Rapid Water DNA Isolation Kit
USA[C, L]	Common carp [Sp]	Difference between eDNA concentrations and mark–recapture and telemetry for estimating distribution	0.2 L through 1.5-mm glass microfiber filters, with DNA extracted using the QIAamp DNA Stool Mini Kit
USA[C]	Confined assemblage of 12 marine species[Met]	Difference between eDNA metabarcoding reads and known species abundance	1.0 L through 0.22-μm membrane filters, with DNA extracted using the Qiagen DNeasy Blood and Tissue Kit
USA[C]	Round goby[Sp]	Difference between eDNA concentrations derived via PCR, qPCR and ddPCR for known abundance/biomass	2.0 L through 1.5-μm glass filter papers, with DNA extracted using a MoBio PowerWater DNA Isolation Kit
Japan[C]	Bluegill sunfish[Sp]	Difference between eDNA concentrations and life stage and known abundance	0.015 L centrifuged into pellet, with DNA extracted using a Qiagen DNeasy Blood and Tissue Kit
Japan[C]	Common carp[Sp]	Difference between eDNA concentrations derived via qPCR and ddPCR and known abundance/biomass.	0.015 L centrifuged into pellet, with DNA extracted using a Qiagen DNeasy Blood and Tissue Kit
USA[R]	Brook trout[Sp]	Difference between eDNA concentrations and sampling distance, water flow and known biomass	6.0 L through a 1.5-μm glass fiber filter, with DNA extracted using the MoBio PowerWater DNA Isolation Kit
USA[C]	Silver carp and bighead carp[Sp]	Difference between eDNA concentrations and temperature, diet, and known biomass	0.05 L centrifuged into pellet, with DNA extracted using a phenol–chloroform method
USA[C]	Common carp[Sp]	Difference between eDNA concentration and capture methods (precipitation, centrifugation, and filtration) and six commercially available DNA extraction kits.	1.0 L with eDNA captured via precipitation, centrifugation or filtration, with DNA extracted using the QIAamp DNA Stool Mini Kit. 1.0 L through 0.6-um polycarbonate membrane filters, with DNA extracted by one of six commercial kits.
USA[R]	Silver carp and bighead carp[Sp]	Difference between eDNA concentrations and fish movement, water discharge and egg densities	0.05 L centrifuged into pellet, with DNA extracted using the Qiagen DNeasy Blood and Tissue Kit
USA[C]	Eight fish and one amphibian species[Met]	Difference between eDNA metabarcoding reads and known abundance/biomass	0.25 L through 1.2-um polycarbonate membrane filters, DNA extracted using a CTAB method
UK[L]	Freshwater fish assemblages in three lakes[Met]	Difference between eDNA metabarcoding reads and gill-netting surveys for estimating abundance	2.0 L through a 0.4-μm cellulose nitrate membrane filter, with DNA extracted using the PowerWater DNA Isolation Kit
Canada[L]	Lake trout[Sp]	Difference between eDNA concentrations and gillnet surveys for estimating abundance/biomass	1.0 L through a 1.2-μm glass microfiber filter, with DNA extracted using QIAshredder and Qiagen DNeasy Blood and Tissue Kit

Assay and target region	eDNA quantification method	Biotic/abiotic factors assessed	Reference
qPCR TaqMan assay of the CytB gene	Standard curves based on serial dilutions of DNA	eDNA capture method, water temperature in aquaria (no effect), water temperature in the lake (+) and biomass (+)	Takahara et al. (2012)
qPCR TaqMan assay of the COI gene	Standard curves based on serial dilutions of DNA and Ct values used as a proxy for DNA	Abundance (+)	Diaz-Ferguson et al. (2014)
qPCR TaqMan assay of the CytB gene	Standard curves based on serial dilutions of DNA	High-use versus low-use areas versus concentration of eDNA (+) and sampling depth	Eichmiller et al. (2014)
Metabarcoding of the 12S rRNA gene, Illumina MiSeq platform	eDNA sequence read abundance used as a proxy for relative fish abundance	Rank biomass abundance of bony fish versus rank DNA sequence abundance (+)	Kelly et al. (2014)
PCR, qPCR and dPCR TaqMan assays of the COI gene	Standard curves (qPCR), and QuantaSoft software (ddPCR)	qPCR/ddPCR accuracy, analysis time/cost and biomass (+)	Nathan et al. (2014)
qPCR TaqMan assay of the CytB gene	Standard curves based on serial dilutions of DNA	eDNA shedding rate with life stage (adult/juvenile) and abundance (+)	Maruyama et al. (2014)
qPCR TaqMan assay of the CytB gene	Standard curves based on serial dilutions of DNA (qPCR), Bio-Rad QuantaSoft software used to quantify DNA (ddPCR)	Biomass (+), abundance (+) and qPCR/ddPCR accuracy	Doi et al. (2015)
qPCR TaqMan assay of the CytB gene	Standard curves based on serial dilutions of DNA	eDNA transport distance (+/−), flow (+/−), biomass (+) and PCR inhibition	Jane et al. (2015)
qPCR TaqMan assay of the mtD-loop region	Standard curves based on serial dilutions of DNA	Biomass (+), diet (+), water temperature (no effect) and eDNA shedding rates	Klymus et al. (2015)
qPCR TaqMan assay of the CytB gene	Standard curves based on serial dilutions of DNA	eDNA capture method, type of extraction kit and biomass (+)	Eichmiller et al. (2016)
qPCR Taqman assay of the mtD-loop region	Standard curves based on serial dilutions of DNA	Telemetry counts (+), river discharge (+), egg densities (no effect) and relative abundance (+)	Erickson et al. (2016)
Metabarcoding of the CytB, 12S and 16S genes, Illumina MiSeq platform	eDNA sequence read abundance used as a proxy for relative fish abundance/biomass	Species detection rate (+), eDNA read abundance and species abundance and biomass (+)	Evans et al. (2016)
Metabarcoding of the 12S rRNA and CytB genes, Illumina MiSeq platform	eDNA sequence read abundance used as a proxy for relative fish abundance	Species detection rates (+), eDNA read count and site occupancy versus rank abundance or biomass (+)	Hänfling et al. (2016)
qPCR Taqman assay of the COI gene	Standard curves based on serial dilutions of DNA	Water temperature, dissolved oxygen, pH and turbidity (no effect), abundance (+) and biomass (+)	Lacoursière-Roussel et al. (2016a)

(Continues)

TABLE 2 (Continued)

Country	Species	Key null hypothesis (*Ho*)*	Water volume collected/filtered and DNA extraction method
Canada[C]	Brook charr[Sp]	Difference between eDNA concentrations and water temperature, capture method, and known abundance/biomass	1.0 L through one of five filters (0.2 and 0.45-µm mixed cellulose ester, and 0.7, 1.2 and 3.0-µm glass microfiber), with DNA extracted using salt extraction method
Australia[C]	Macquarie perch[Sp]	Difference between eDNA concentrations and sampling methods, extraction methods, marker choice, amplicon size and dilutions of DNA as a proxy for biomass	0.25 L though (1) 0.45-µm cellulose nitrate filter and DNeasy Blood and Tissue Kit, (2) 0.45-µm cellulose nitrate filter and Phenol-Chloroform Isoamyl or (3) sodium acetate and ethanol precipitation and the Qiagen DNeasy Blood Tissue Kit
USA[C]	Northern anchovy, Pacific sardine and, Pacific chub mackerel[Sp]	Difference in eDNA shedding and decay rates	0.25–0.50 L through 0.2-µm polycarbonate filters, with DNA extracted using the Qiagen DNeasy Blood Tissue Kit
USA[E]	Tidewater goby[Sp]	Difference between eDNA concentrations and seining for species detection and estimating abundance	2.0 L through a 3.0 µm polycarbonate, track-etched filter, with DNA extracted using the Qiagen DNeasy Blood and Tissue Kit and QIAshredder
Greenland[S]	Marine fish assemblage[Met]	Difference between eDNA metabarcoding reads and bottom-trawl survey methods for estimating abundance	1.5 L through a 0.45-µm nylon filter, with DNA extracted using bead beating and the Qiagen DNeasy Blood and Tissue Kit
USA[C, R]	Brook trout[Sp]	Difference between eDNA concentrations and electrofishing for estimating detection probabilities/abundance. Difference in rates of eDNA shedding and persistence	1.0–6.0 L through 1.5-µm pore glass filters, with DNA extracted using QIAshredder and Qiagen Blood and Tissue DNeasy Kit
Japan[S]	Japanese jack mackerel[Sp]	Difference between eDNA concentrations and echo-sounder surveys for estimating biomass	1.0 L through 0.7-µm pore glass filters and DNA extracted using the Qiagen DNeasy Blood and Tissue Kit
USA[R]	Brook trout[Sp]	Difference between eDNA concentrations and electrofishing surveys for estimating abundance	Up to 6.0 L through 1.5-µm glass filters and several grams of sediment stored in plastic bags, with DNA was extracted using MoBio PowerWater and PowerSoil DNA Isolation Kits, respectively
Japan[R]	Ayu[Sp]	Difference between eDNA concentrations and snorkeling surveys for estimating abundance/biomass	1.0 L filtered through 0.7-µm glass filters, with DNA extracted using a Qiagen DNeasy Blood and Tissue Kit
Australia[R]	Common carp, redfin perch and oriental weatherloach[Sp]	Difference between eDNA concentrations and fyke netting for estimating abundance	2.0 L through 1.2-µm glass fiber filters, with DNA extracted using the MoBio PowerWater DNA Isolation Kit
Japan[C, S]	Japanese jack mackerel[Sp]	Difference between the decay rates of long and short eDNA fragments. Difference between eDNA concentration and acoustic surveys for estimating biomass	1.0 L filtered through 0.7-µm pore glass filters, with DNA extracted using a Qiagen DNeasy Blood and Tissue Kit
USA[L]	Arctic char[Sp]	Difference between eDNA concentrations and mark–recapture for estimations of abundance/biomass. Difference between seasons and sampling depth	5.0 L through 10-µm polyamide filters, with DNA extracted using Qiagen DNeasy Blood and Tissue Kit
USA[L]	Largemouth bass and gizzard chad[Sp]	Difference between eDNA concentrations and electrofishing/gill netting for estimating species presence, relative abundance and biomass	0.015 L centrifuged into pellet, with DNA extracted using the IBI gMAX Mini Genomic DNA Kit

Assay and target region	eDNA quantification method	Biotic/abiotic factors assessed	Reference
qPCR Taqman assay of the CytB gene	Standard curves based on serial dilutions of DNA	Water temperature (+), eDNA capture method and biomass (+)	Lacoursière-Roussel et al. (2016b)
PCR and qPCR SYBR Green assay of the 12S rRNA and 18S rRNA genes	Standard curves based on serial dilutions of DNA	Sampling and extraction methods, PCR strategy, amplicon size, marker choice and biomass (+)	Piggott (2016)
qPCR Taqman assay of the mtD-loop and COI genes	Standard curves based on serial dilutions of DNA	eDNA shedding (+) and decay rates (+), eDNA size fractionation and abundance (+)	Sassoubre et al. (2016)
qPCR TaqMan assay for the CytB gene	Standard curves based on serial dilutions of DNA	Species detection rates (+), abundance (+) and salinity (−)	Schmelzle and Kinziger (2016)
Metabarcoding of the 12S rRNA gene, Illumina MiSeq platform	eDNA sequence read abundance used as a proxy for relative fish abundance	Species detection rates, relative eDNA read abundance versus relative abundance (+) and relative biomass (+) and depth	Thomsen et al. (2016)
qPCR Taqman assay of the CytB gene	Standard curves based on serial dilutions of DNA	Rate of eDNA production, eDNA transport distance, detection probability (+) and abundance (+)	Wilcox et al. (2016)
qPCR Taqman assay of the CytB gene	Standard curves based on serial dilutions of DNA	Sampling location (surface/bottom), species distribution and biomass (+)	Yamamoto et al. (2016)
qPCR TaqMan assay of the CytB gene	Standard curves based on serial dilutions of DNA	Species detection rate (+), abundance (+), biomass (+) and eDNA substrate (water/sediment)	Baldigo et al. (2017)
qPCR TaqMan assay of the CytB gene	Standard curves based on serial dilutions of DNA	Abundance (+), biomass (+) and season	Doi et al. (2017)
qPCR TaqMan assay of the 12S rRNA gene	Standard curves based on serial dilutions of DNA	Species detection rate (+), season, sampling location (surface/subsurface), abundance of carp (no effect), abundance of redfin perch and oriental weatherloach (+)	Hinlo et al. (2017a)
qPCR TaqMan assay of the CytB gene	Standard curves based on serial dilutions of DNA	eDNA decay rate, fragment length (+) and biomass (long fragment +)	Jo et al. (2017)
qPCR TaqMan assay of the CytB gene	Standard curves based on serial dilutions of DNA	Abundance (+), biomass (+), depth (±) and season	Klobucar et al. (2017)
qPCR TaqMan assay of the COI, ND4, ND3 and ND5 genes	Standard curves based on serial dilutions of DNA	Season, relative abundance (no effect) and biomass (no effect)	Perez et al. (2017)

(Continues)

TABLE 2 (Continued)

Country	Species	Key null hypothesis (*Ho*)*	Water volume collected/filtered and DNA extraction method
USA[E]	Estuarine fish assemblage[Met]	Difference between eDNA metabarcoding reads and traditional surveys for estimating abundance	1.0 L through a 0.45-μm polyamide filter, with DNA extracted using MoBio Powersoil Kit
USA[C, R]	Common carp[Sp]	Difference in statistical models accounting for factors influencing biomass estimates from eDNA concentration data	0.015 L centrifuged into pellet, with DNA extracted from pellet using the Qiagen DNeasy Blood and Tissue Kit
Australia[R]	Oriental weatherloach[Sp]	Difference between eDNA concentrations and fyke netting/electrofishing for estimating abundance	2.0 L through 1.2-μm glass fiber filter, with DNA extracted using a modified Qiagen DNeasy Kit
USA[L]	Brook trout[Sp]	Difference in eDNA concentrations between where fish had been eradicated versus where fish remained, using gillnets for estimating abundance/biomass	0.25 L through 1.2-μm polycarbonate filter membrane, with DNA extracted using aQIAshredder and Qiagen DNeasy Blood and Tissue Kit
UK[C]	Freshwater fish assemblage[Met]	Difference between eDNA metabarcoding reads and known species presence and abundance/biomass	0.3 L through one of six filtration types, with DNA extracted using the MoBio PowerWater DNA Isolation Kit
Japan[R]	Three-lips[Sp]	Difference between eDNA concentrations and visual sampling for estimating abundance	0.5 L through a 0.7-μm glass filter membrane, with DNA extracted using a Qiagen DNeasy Blood and Tissue DNA Kit
Japan[C]	Sakhalin taimen[Sp]	Difference between eDNA concentration and known biomass	1.0 L through 0.7-μm glass-membrane filters, with DNA extracted using a slightly modified Qiagen DNeasy Blood and Tissue Kit.
USA[C, L]	Round goby[Sp]	Difference between eDNA concentrations and seining/traps/mark–recapture for estimating abundance. Difference in rates of eDNA shedding and decay	0.05–1 L through either 1.5- or 0.22-μm nitrocellulose filters, with DNA extracted using a slightly modified MoBio PowerWater Kit
USA[R]	Alewife and blueback herring[Sp]	Difference between eDNA concentration and visual surveys/ichthyoplankton sampling for presence and estimating abundance	~0.8 L through a 1.0-μm cellulose nitrate filter, with DNA extracted using a slightly modified Omega Biotek EZNA Water Kit
France/ Switzerland[R]	Freshwater fish assemblage[Met]	Difference between eDNA concentrations and electrofishing for estimating relative abundances	30.0 L through a 0.45-μm cross-flow filtration capsule, with DNA extracted using a combination of the Qiagen DNeasy Blood and Tissue Extraction Kit and the NucleoSpin Soil Kit
USA[C]	Sea lamprey[Sp]	Relationship between eDNA (concentration and detection probability) and known abundances/biomass	1.0 L through a 1.5-μm glass microfiber filter, with DNA extracted using the gMax Mini Genomic Extraction Kit
USA[R]	Sockeye salmon[Sp]	Difference between eDNA concentrations and visual counts for estimating abundances	1.0 L through a 0.45- μm cellulose nitrate filter, with DNA extracted using Qiagen DNeasy Blood and Tissue Kit
Ireland[R]	Sea lamprey[Sp]	Difference between eDNA concentrations and visual surveys of individuals/nests for estimating abundance	2.0 L through a 0.45- μm cellulose nitrate filter, with DNA extracted using a Chelex Chelating resin
Sweden[L]	Brown trout and Arctic char[Sp]	Difference between eDNA concentrations and standardized gillnet surveys and relationships with environmental parameters	0.75–1 L through 1.2 μm glass fiber and 0.45 μm mixed cellulose ester filters, with DNA extracted using the DNeasy Blood and Tissue Kit
USA[R]	Silver carp[Sp]	Difference between eDNA concentrations and hydroacoustic and electrofishing/ gillnet surveys for estimating abundance/ biomass	2.0 L through a 1.5-mm glass microfiber filter, with DNA extracted using a FastDNA Spin Kit

Assay and target region	eDNA quantification method	Biotic/abiotic factors assessed	Reference
Metabarcoding of the 12S rRNA gene, Illumina MiSeq platform	eDNA sequence read abundance used as a proxy for relative abundance estimates	Seasonal abundance (+), eDNA read abundance versus relative abundance (+), and habitat preference	Stoeckle et al. (2017)
ddPCR TaqMan assay of the CytB gene	Bio-Rad QuantaSoft software used to quantify DNA	Statistical model choice and biomass (+)	Chambert et al. (2018)
qPCR TaqMan assay for the 12S rRNA gene	Standard curves based on serial dilutions of DNA	Abundance (no effect)	Hinlo et al. (2018)
qPCR TaqMan assay of the CytB gene	Standard curves based on serial dilutions of DNA	Abundance (+) and biomass (+)	Kamoroff and Goldberg (2018)
Metabarcoding of the 12S rRNA, Illumina MiSeq platform	Species detection rates based on the relative read counts as a proxy for abundance estimates	eDNA capture method, species detection rates, relative abundance (+) and relative biomass (+)	Li et al. (2018)
qPCR TaqMan assay of the mtD-loop region	Standard curves based on serial dilutions of DNA	Abundance (+), eDNA transport distance (no effect) and seasonal changes (+)	Maruyama et al. (2018)
qPCR TaqMan assay for the ND2 gene	Standard curves based on serial dilutions of DNA	Aquarium size, fish age, body size and biomass (+)	Mizumoto et al. (2018)
qPCR TaqMan assay of the COI gene	Standard curves based on serial dilutions of DNA	Species detection rate (+), abundance (+) and DNA shedding and decay rate	Nevers et al. (2018)
qPCR TaqMan assay for the COI gene	Standard curves based on serial dilutions of DNA	Species detection rate (+) and abundance (+)	Plough et al. (2018)
Metabarcoding of the 12S rRNA gene, Illumina HiSeq platform	Species detection rates based on the relative read counts as a proxy for abundance estimates	Species detection rate and relative abundance (+)	Pont et al. (2018)
qPCR TaqMan assay of the COI, CytB, ND1 and ND4 genes	Standard curves based on serial dilutions of DNA	Fish life stage, abundance (+) and biomass (+)	Schloesser et al. (2018)
qPCR TaqMan assay of the COX3 gene	Standard curves based on serial dilutions of DNA	eDNA transport distance (+), water temperature (minor +) and abundance (+)	Tillotson et al. (2018)
qPCR TaqMan assay of the COI gene	Standard curves based on serial dilutions of DNA	Seasonal movement, spawning and abundance (+)	Bracken et al. (2019)
ddPCR assay of the CytB gene	Divided the mean number of positive droplets by the volume used in ddPCR and multiplied by the total volume of the DNA extract	Biomass, abundance, dissolve organic carbon and temperature (no effect)	Capo et al. (2019)
qPCR TaqMan assay of mitochondrial genes	Standard curves based on serial dilutions of DNA	Abundance (+) and biomass (+)	Coulter et al. (2019)

(Continues)

TABLE 2 (Continued)

Country	Species	Key null hypothesis (*Ho*)*	Water volume collected/filtered and DNA extraction method
Germany[C, R]	Brown trout[Sp]	Difference between eDNA concentrations and known biomass. Difference between eDNA concentrations and distance	1.0 L through 0.45-μm nitrocellulose filters, with DNA extracted using the Qiagen DNeasy Blood and Tissue Kit
Japan[C]	Japanese jack mackerel[Sp]	Difference between eDNA concentration and known biomass	1.0 L through a 0.7- μm glass filter, with DNA extracted using a Qiagen DNeasy Blood and Tissue Kit
Japan[R]	Japanese eel[Sp]	Difference between eDNA concentrations and electrofishing for estimating species presence and abundance/biomass	1 L through a 0.7-μM glass filter, with DNA extracted using a Qiagen DNeasy Blood and Tissue Kit
Denmark[S]	Atlantic herring, Atlantic cod, European flounder, European plaice and Atlantic mackerel[Sp]	Difference between eDNA concentrations and benthic trawls for estimating abundance/biomass	1.5 L through a 0.22-μm Sterivex filter, with DNA extracted using a Qiagen DNeasy Blood andTissue Kit
USA[R]	Sockeye salmon and coho salmon[Sp]	Difference between eDNA concentrations and daily human count data for estimating abundances	1.0 L through a 0.45-μm cellulose nitrate filter, with DNA extracted using a Qiagen DNeasy Blood and Tissue Kit
UK[L]	Freshwater assemblages in 14 lakes[Met]	Difference between eDNA metabarcoding reads and historical information for estimating relative abundances	2.0 L through a 0.45-μm mixed cellulose acetate and nitrate filter, with DNA extracted using a Qiagen DNeasy PowerWater Kit
USA[E]	Eulachon[Sp]	Difference between eDNA concentrations and mark–recapture for estimating abundance	1.0 L through a 0.45-μm cellulose nitrate filter, with DNA extracted using the Qiagen DNeasy Blood and Tissue Kit
USA[R]	Loach minnow[Sp]	Difference between eDNA concentrations and electrofishing/seining for estimating species presence and biomass	5.0 L through a 1.5-μm pore-size glass fiber filter, with DNA extracted using a slightly modified Qiagen DNeasy Blood and GTissue Kit
Faroe Islands[S]	Atlantic cod[Sp]	Difference between eDNA concentrations and benthic trawls for estimating biomass	1.5 L through a 0.2-μm Sterivex filter, with DNA extracted using a modified protocol of the Qiagen DNeasy Blood and Tissue Kit
USA[L]	Freshwater assemblages in eight lakes[Met]	Difference between eDNA metabarcoding reads and traditional methods for estimating species presence and relative abundances	1.0 L through a 0.45 μm nitrocellulose filter, DNA was extracted using a Qiagen QIAshredder and the Qiagen DNeasy Blood and Tissue Kit
USA[E]	Chinook salmon[Sp]	Difference between eDNA concentrations and beach seining for estimating abundance/biomass	1.0 L through a 0.45-μm cellulose acetate membrane, with DNA extracted using a chloroform:isoamyl alcohol method
Japan[R]	Ryukyu ayu[Sp]	Difference between eDNA concentration and snorkeling surveys for estimating abundance	1.0 L through a 0.7-μm glass fiber filter, with DNA extracted using the Qiagen DNeasy Blood and Tissue Kit
UK[C]	Freshwater fish assemblage[Met]	Difference between eDNA metabarcoding reads and known species presence and abundance/biomass and differences between filter types	1.0 L through 0.45-μm mixed cellulose ester filters or 0.35 L through 0.45-μm Sterivex filter, with DNA extracted using the Mu-DNA protocol for water samples
Spain[S]	Marine fish assemblage [Met]	Difference between eDNA metabarcoding reads and biomass estimated from trawling	5 L through Sterivex 0.45-μm enclosed filters, DNA extracted using the DNeasy Blood and Tissue Kit
Japan[S]	Japanese jack mackerel[Sp]	Difference between eDNA concentration and echo-sounder abundance	1.0 L through 0.7-μm glass microfiber filter, with DNA extracted using the DNeasy Blood and Tissue Kit
France[R]	Freshwater fish assemblage[Met]	Difference between eDNA metabarcoding read count and electrofishing relative abundance	30 L through 0.45-μm VigiDNA cross-flow filtration capsule, with DNA extracted using the DNeasy Blood and Tissue Kit
USA[C]	Fantail darter[Sp]	Difference between eDNA concentration and known biomass and difference between single versus. multiple (3) filters	1.2 L through 0.7-μm glass fiber filter, with DNA extracted using the DNeasy Blood and Tissue kit

Assay and target region	eDNA quantification method	Biotic/abiotic factors assessed	Reference
qPCR TaqMan assay of the COI gene	Standard curves based on serial dilutions of DNA	eDNA transport distance and biomass (no effect)	Deutschmann et al. (2019)
qPCR TaqMan assay of the CytB gene	Standard curves based on serial dilutions of DNA	Biomass (+)	Horiuchi et al. (2019)
qPCR TaqMan assay of the 16S rRNA gene	Standard curves based on serial dilutions of DNA	Species detection rate (+), biomass (+) and abundance (+)	Itakura et al. (2019)
qPCR TaqMan assay of the CytB and Nd4 genes	Standard curves based on serial dilutions of DNA	Species distribution (+) and biomass (no effect)	Knudsen et al. (2019)
qPCR TaqMan assay of the COI gene	Standard curves based on serial dilutions of DNA	Flow, water temperature, fish life stage and abundance (+)	Levi et al. (2019)
Metabarcoding of the 12S rRNA and CytB gene, Illumina MiSeq platform	Site occupancy based on the relative read counts as a proxy for abundance estimates	Species distribution (+) and relative abundance (+)	Li et al. (2019)
ddPCR assay of the COI gene	Bio-Rad QuantaSoft software used to quantify DNA	Flow and biomass (+)	Pochardt et al. (2019)
qPCR TaqMan assay of the CytB gene	Standard curves based on serial dilutions of DNA	Species detection rate (+), eDNA persistence rate and biomass (+)	Robinson et al. (2019)
qPCR TaqMan assay of the mtD-loop region	Standard curves based on serial dilutions of DNA	Abundance (+) and biomass (+)	Salter et al. (2019)
Metabarcoding of the 12S rRNA and 16S rRNA genes, Illumina MiSeq platform	Species detection rates based on the relative read counts as a proxy for abundance estimates	Sampling effort, species detection rate (+) and relative abundance (+)	Sard et al. (2019)
qPCR TaqMan assay of the CO3/ND3 gene	Standard curves based on serial dilutions of DNA	Spatial scale (+), biomass (+) and abundance (+)	Shelton et al. (2019)
qPCR TaqMan assay of the ND4 gene	Standard curves based on serial dilutions of DNA	Species detection rate (+) and biomass (+)	Akamatsu et al. (2020)
Metabarcoding of the 12S rRNA and 16S rRNA genes, Illumina MiSeq platform	Spearman's rank correlation coefficient to calculate correlation between biomass/abundance and average read counts and site occupancy	Biomass (+), abundance (+) and filter types and biomass/abundance (no effect)	Di Muri et al. (2020)
Metabarcoding of the 12S rRNA gene, Illumina MiSeq platform	Pearson correlation coefficient to calculate correlation between the number of reads and biomass	Biomass (no effect)	Fraija-Fernandez et al. (2020)
qPCR TaqMan assay of the CytB gene	Standard curves based on serial dilutions of DNA	Abundance (+)	Fukaya et al. (2020)
Metabarcoding of the 12S rRNA gene, Illumina HiSeq platform	Spearman's rank order correlation to calculate correlation between electrofishing relative abundance and relative number of reads per species	Relative abundance (+)	Goutte et al. (2020)
qPCR TaqMan assay of the CytB gene	Standard curves based on serial dilutions of DNA	Biomass (+) and number of filters (+)	Guivas and Brammell (2020)

(Continues)

TABLE 2 (Continued)

Country	Species	Key null hypothesis (*Ho*)*	Water volume collected/filtered and DNA extraction method
Japan and Taiwan[R]	Giant mottled eel and Japanese eel[Sp]	Difference between eDNA concentration and electrofishing biomass and abundance	1.0 L through 0.7-μm glass fiber filters, with DNA extracted using the DNeasy Blood and Tissue Kit
Japan[S]	Blackhead seabream, Japanese anchovy, wrasse, stiped knifejaw and Japanese jack mackerel[Sp]	Difference between eDNA concentrations and visual (SCUBA) biomass estimates and differences between filter types	1.0 L through 0.45 -μm Sterivex filter and 1 L through 0.7-μm glass fiber filters, with DNA extracted using the DNeasy Blood and Tissue Kit
USA[R]	Atlantic salmon[Sp]	Difference between eDNA concentrations/ detection rates and downstream distance	1.0 L through a 1.5-μm glass fiber filter, with DNA extracted using the Qiagen DNeasy Spin Column Kit
Canada[C, R]	Lake sturgeon[Sp]	Difference between eDNA concentration and relative abundance/species presence	1.0.0–2 L through 1.5-μm glass fiber filter, with DNA extracted using the Qiagen DNeasy Blood and Tissue Kit

those containing three fish, although fish sexes were not determined and could have affected shedding rates (Nevers et al., 2018). Additional considerations here are possible confounding effects of animal stress, size and/or basal metabolism.

3.1.2 | Stress

Stress may account for considerable intraspecific variation in DNA shedding, especially in aquaria. In some studies, eDNA concentrations have been greatest immediately after fish were first introduced into tanks. The elevated eDNA resulted from increased activity of individuals is presumably associated with physiological stress during the acclimation period, with a subsequent decline in concentrations recorded as individuals became accustomed to confinement (Maruyama et al., 2014; Nevers et al., 2018; Sassoubre et al., 2016; Takahara et al., 2012). To minimize the confounding effects of fish stress on the outcomes of future aquaria experiments, pilot work should always be undertaken to determine when eDNA concentrations plateau following fish introduction into experimental tanks.

3.1.3 | Metabolism and size

The metabolic rate describes the energy expenditure of an organism while it is at rest and varies with body size and temperature (Gillooly et al., 2001). Given that temperature variably affects many physiological, ecological, and biological processes via complex interactions among fish (discussed below), metabolic rate likely affects eDNA shedding by species or individuals, potentially influencing subsequent interpretations of eDNA quantification. For example, Lacoursière-Roussel et al. (2016b) hypothesized that increased eDNA concentrations at higher temperatures were due to an increased metabolic rate leading to shedding of more epidermal cells and other secretions (feces and urine). Thus, to avoid overestimating fish abundance and/or biomass from eDNA concentrations, temperature must be considered.

In many teleost fishes, their metabolic rate varies with life stage and is therefore directly linked to body size (Post & Lee, 1996). Consequently, different-sized individuals of the same species can exhibit different DNA shedding rates (Klymus et al., 2015; Maruyama et al., 2014; Wilcox et al., 2016), potentially influencing the interpretation of eDNA quantification for some species. Small individuals have a relatively greater metabolic rate per unit bodyweight versus larger individuals (Chabot et al., 2016), and therefore may shed relatively more DNA compared to a single large fish. For example, during tank trials, Maruyama et al. (2014) observed that the DNA release rates by individual bluegill sunfish (*Lepomis macrochirus*) were three or four times greater in adults than juveniles, but when scaled to body size, juvenile release rates were four times greater owing to the ontogenetic decrease in metabolic activity. Consequently, attempting to estimate a population's abundance or biomass from eDNA concentrations, without knowing size distributions, could potentially give misleading estimates if size-based variation in DNA shedding rates was substantial. In such situations, concomitant knowledge of the size and age structures of the focal species within a sampling area, derived via ancillary fishery-dependent or fishery-independent techniques, is therefore essential (Hansen et al., 2018). An implicit requirement here is also to determine the magnitude of differences (if any) in DNA shedding rates according to the size or age of the fish species.

3.1.4 | Distribution and density

The generally patchy distributions of fish will result in eDNA concentrations that vary across space and time (Eichmiller et al., 2014; Itakura et al., 2019; Takahara et al., 2012). Such variation may affect the accuracy of subsequent abundance or biomass estimates, particularly if sampling is not done across appropriate spatio-temporal

Assay and target region	eDNA quantification method	Biotic/abiotic factors assessed	Reference
qPCR TaqMan assay of the 16S rRNA gene	Standard curves based on serial dilutions of DNA	Abundance (+), biomass (+), spatial distribution of eDNA concentration (Japanese eel only) (–)	Itakura et al. (2020)
qPCR TaqMan assay of the CytB gene	Standard curves based on serial dilutions of DNA	Biomass (+) and filter types (no effect)	Takahashi et al. (2020)
qPCR TaqMan assay of the ND5 and COI genes	Standard curves based on serial dilutions of DNA	eDNA transport distance, optimal sampling distance, assay type and biomass (+)	Wood et al., 2020(Wood et al., 2020)
qPCR TaqMan assay of the COI and CytB gene	Ct values used as a proxy DNA concentration	Biomass (+)	(Yusishen et al., 2020)

scales and/or insufficient water is collected to enable replication (Furlan et al., 2016; Moyer et al., 2014). For example, when common carp (*Cyprinus carpio*) were heterogeneously distributed across lakes in the upper Mississippi River basin, their eDNA was occasionally detectable and only loosely associated with moderately dense groups of fish (Ghosal et al., 2018). However, when fish were attracted to a single site (using bait), a doubling of fish density resulted in a 500-fold increase in eDNA concentrations.

In another study in a temperate freshwater lake in the northern USA, Eichmiller et al. (2014) noted that common carp eDNA concentrations were over seven times greater in areas with a high frequency of carp habitation than areas with a low habitation frequency. Specifically, eDNA was detected in 100% of water samples from areas of high use by common carp compared with only 63% of samples from the low-use areas (Eichmiller et al., 2014). Given the strength of this latter relationship and the known patchy distribution of eDNA in the environment, the authors concluded that any sampling methods and data interpretation should be primarily informed by existing fish distributions because the probability of detecting a target organism may drastically decline within tens of meters.

There was not always a consistent linear relationship between fish density and eDNA concentration (Coulter et al., 2019; Doi et al., 2017; Ghosal et al., 2018). As one example, both the numerical and biomass densities of silver carp (*Hypophthalmichthys molitrix*) in a river system were positively correlated with eDNA concentrations and detection rates at low densities, but plateaued at greater densities (Coulter et al., 2019). Consequently, reliable population estimates from eDNA concentrations for this species are currently restricted to areas with low densities of fish.

3.1.5 | Feeding and diet

Feeding and diet can significantly affect DNA shedding rates. Ghosal et al. (2018) demonstrated that actively feeding common carp in a lake produced substantially more eDNA than less actively feeding conspecifics. Similarly, among aquaria-housed bighead carp (*Hypophthalmichthys nobilis*) the amount of DNA shed was up to tenfold greater in fed than nonfed individuals (Klymus et al., 2015). Klymus et al. (2015) also hypothesized that a diet of textured crustacean food would slough more epithelial cells from the gut and thus increase DNA shedding rates versus a diet consisting of soft textured algae; however, fish fed algal diets had greater shedding rates, which the authors attributed to a higher feeding rate. This latter study implies that both diet composition and the frequency of feeding may influence DNA shedding and thus eDNA concentrations in the water.

3.1.6 | Reproduction and migration

The reviewed studies also show that eDNA concentrations in aquatic systems depend on the life stages of the fish inhabiting that body of water and any spawning activity and/or movements/migration (Table 2). Spawning and associated migrations, which are typically seasonal, evoke fluctuations in eDNA concentrations due to the communal release of gametes, increased activity and congregation of often large numbers of adults and the presence of juvenile fish (Bracken et al., 2019; Bylemans et al., 2017; Kamoroff & Goldberg, 2018; Maruyama et al., 2018; Nevers et al., 2018; Plough et al., 2018; Schloesser et al., 2018; Spear et al., 2015). For example, Bracken et al. (2019) observed the concentration of eDNA in river water increased during the spawning season of sea lamprey (*Petromyzon marinus*), simply attributed to more adult fish (including the carcasses of those that died after spawning) and gametes. Another study demonstrated the ratio of nuclear-to-mitochondrial eDNA from Macquarie perch (Macquaria australasica) was greatest during their spawning season (Bylemans et al., 2017). One simple way of limiting the impact that spawning can have on baseline levels of eDNA is to sample outside spawning seasons.

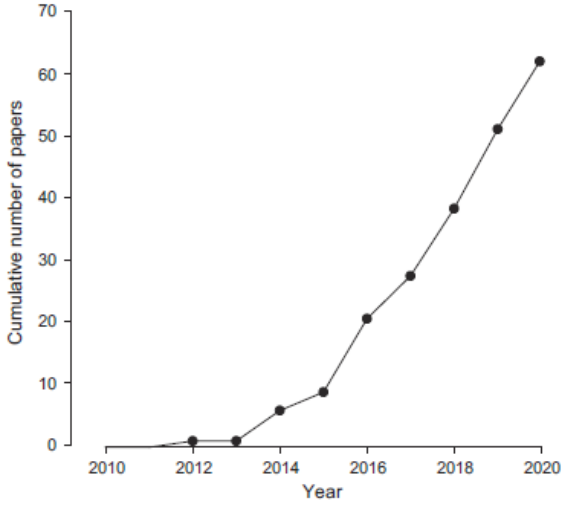

FIGURE 1 Cumulative number of articles published in the primary literature from 2012 to October 2020 assessing the utility of environmental DNA for estimating the abundance and/or biomass of fish across all environments (i.e., controlled and wild)

3.2 | Abiotic factors affecting eDNA concentrations

Six key abiotic factors were commonly identified as affecting estimates of abundance or biomass from eDNA concentrations (Table 2). Further, nearly all abiotic factors have complex interactive or confounding effects that encapsulate influences on the general ecology and biology of species, which then affect DNA shedding rates and/or volume (as discussed above). Here, we limit our discussion to the direct effects of abiotic factors on eDNA concentrations.

3.2.1 | Water flow

One of the most important abiotic factors affecting eDNA concentrations is regional water flow (Harrison et al., 2019). By dispersing eDNA away from its source, water flow influences the spatial scale over which abundance and/or biomass can be meaningfully estimated, and affects numerous other factors that influence eDNA production, degradation, dilution, and deposition (Fukaya et al., 2020; Hansen et al., 2018; Itakura et al., 2020; Pont et al., 2018; Thomsen et al., 2016). Due to the challenges involved, few studies have directly examined the effects of water flow on eDNA concentrations, but those that have support complex system-specific responses that require further investigation (Hinlo et al., 2018; Laporte et al., 2020; Robinson et al., 2019; Wilcox et al., 2016). For example, using cage introductions of brook trout (*Salvelinus fontinalis*) in lotic systems, Jane et al. (2015) noted the relationship between water flow and eDNA copy number varied depending on system flow rates. Specifically, the eDNA copy number was highest immediately downstream of the caged trout in low flow scenarios, but relatively constant at the most upstream and downstream locations during high flow scenarios (Jane et al., 2015).

The potential for nonlinear relationships between flow and eDNA concentrations with distance from a point source is also apparent, with a peak in concentration observed 70 m downstream of caged Atlantic salmon (*Salmo salar*) (Wood et al., 2020) and 50 to 70 m downstream of Japanese eel (*Anguilla japonica*) capture sites (Itakura et al., 2020). The recognized influence of flow on eDNA concentrations led to the recent development of an "eDNA rate" for Pacific salmon (*Oncorhynchus* spp.) in lotic systems—a correction metric that combines eDNA concentrations with flow velocities to improve abundance estimates (Levi et al., 2019). Systems influenced by tidal flow or influxes from tide-dominated estuaries also pose challenges to estimate abundance or biomass. As one solution, Pochardt et al. (2020) used a flow-corrected eDNA rate to predict the abundance of eulachon (*Thaleichthys pacificus*) at the mouth of a river adjacent to an estuary as determined through mark–recapture population estimates. The eDNA concentration, combined with sampling at low tide to minimize the effects of dilution or intrusion of eDNA from the tidal flow, resulted in the authors successfully predicting eulachon abundance (Pochardt et al., 2020).

Despite the potential for broad and complex dispersal patterns of eDNA, the reviewed studies imply effective spatial scales of abundance and/or biomass estimation for fish may be considerably smaller than previously considered (Table 2). In lotic systems, studies either identified a relatively rapid decline of eDNA concentrations beyond their source (Deutschmann et al., 2019; Shogren et al., 2017; Wood et al., 2020), or found no evidence of downstream eDNA accumulation (Maruyama et al., 2018; Tillotson et al., 2018). The latter might be expected if production and transport exceeded degradation and deposition (Tillotson et al., 2018).

In coastal marine systems, slower and multi-directional flows may further narrow the effective spatial scale of abundance and/ or biomass estimation. For example, the best-fit model between acoustic signal intensity and eDNA concentrations of Japanese jack mackerel (*Trachurus japonicus*) in the Sea of Japan was within 10 to 150 m of the eDNA collection point, implying eDNA concentrations likely reflected biomass within 150 m (Yamamoto et al., 2016) (Table 2). Similarly, most detections of striped jack (*Pseudocaranx dentex*) were within 30 m of holding cages at the same study location, with no effect of flow direction on eDNA detections, although eDNA concentrations did decrease with distance from the cages (Murakami et al., 2019) (Table 2).

3.2.2 | Water temperature

Water temperature affects many aspects of the ecology and biology of aquatic species (Person-Le Ruyet et al., 2004; Takahara et al., 2011), and consequently has interactive and/or divergent effects on eDNA concentrations (mostly through shedding, but also decay), which makes drawing conclusions beyond broad trends difficult in the absence of ancillary data on species-specific physiological requirements (Lacoursière-Roussel et al., 2016b). As one example, Takahara et al. (2012) found no

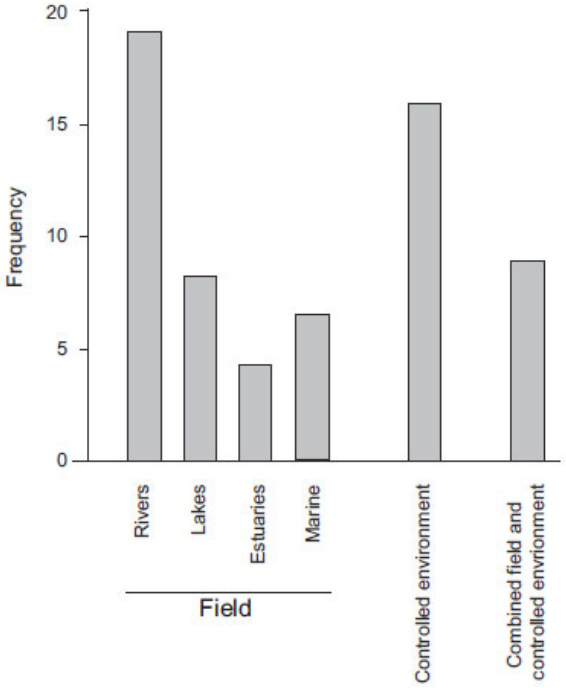

FIGURE 2 The percentage of environmental DNA studies completed between 2012 and October 2020 assessing the abundance and/or biomass of fish in each study environment

effect of three different temperatures (7, 15 and 25°C) on common carp eDNA concentrations in aquaria. In contrast, another aquaria-based study using five densities of brook charr (*Salvelinus fontinalis*) exposed to two temperatures (7 and 14°C) reported a significant effect of temperature on eDNA concentration, with more DNA being released at higher temperatures and a better relationship between abundance and biomass at the higher temperature (Lacoursière-Roussel et al., 2016b). Such divergent effects at least partially reflect species-specific tolerance ranges and metabolic rates affecting shedding, and also any subsequent eDNA decay once shed from the fish.

Field studies similarly report contrasting effects of temperature on eDNA concentrations. In one relevant study, water sampling from a freshwater lagoon in winter revealed a strong correlation between water temperature and the concentration of common carp eDNA (Takahara et al., 2012). Warmer areas of the lagoon had greater concentrations of eDNA potentially because the fish were seeking optimal water temperatures to maintain their metabolism. In contrast, another study of 12 Canadian freshwater lakes found that lake trout (*Salvelinus namaycush*) eDNA concentrations did not vary with temperature; however, the temperatures were all low (<15°C), and it was hypothesized conditions were conducive to fish being variably distributed throughout the water column and thus randomly shedding DNA (Lacoursière-Roussel et al., 2016a).

Further, it is important to note that any effects of water temperature likely reflect broader seasonal influences, which can affect both the detection probability and the concentration of eDNA present in the environment due to the activity levels of the fish (Furlan et al., 2016). For example, Doi et al. (2017) collected water samples from Japanese rivers in spring, summer, and autumn and measured the eDNA concentration of ayu (*Plecoglossus altivelis*). The relationships between eDNA concentrations and estimated biomass of ayu were similar across seasons, likely because the fish did not migrate and were highly active. These studies serve to illustrate potential issues with extrapolating the effects of temperature between species (even those occupying similar habitats) and underscore the need to adequately understand the biology of the focal species and the factors affecting their likely distributions.

3.2.3 | Water depth

Although not assessed at the same level of detail as horizontal flow or temperature, the eDNA sampling depth can clearly bias estimates of fish abundance or biomass, reflecting not only species-specific vertical distributions and habitat preferences, but also the absolute depth of the system (Diaz-Ferguson et al., 2014; Eichmiller et al., 2014; Hinlo et al., 2017a; Klobucar et al., 2017; Moyer et al., 2014). For example, Hinlo et al. (2017a) compared eDNA concentrations from Oriental weatherloach (*Misgurnus anguillicaudatus*) between the surface and subsurface of an Australian river and found few differences, although they attributed the homogeneity to the shallow depths of the surveyed river and the strong effects of vertical mixing. In contrast, findings from lakes have been more variable, with eDNA concentrations throughout the water column correlated to stratification and/or preferred depth distributions for each species (Eichmiller et al., 2014; Klobucar et al., 2017; Moyer et al., 2014). Such effects were illustrated by Hänfling et al. (2016) who observed that eDNA detection of multiple fish species in UK freshwater lakes reflected species-specific depth preferences, with deep-dwelling species like Arctic charr (*Salvelinus alpinus*) only recorded at the deepest sampling points (Hänfling et al., 2016).

A few investigations within the marine environment have also identified depth-specific variations in fish eDNA concentrations (Murakami et al., 2019; Yamamoto et al., 2016). In particular, despite a strong relationship between surface eDNA concentrations and acoustic intensity for Japanese jack mackerel, Yamamoto et al. (2016) noted surface and bottom water eDNA concentrations were not correlated, and bottom samples were frequently at the lower bound of eDNA concentration detectability. Considering the observed variation with horizontal flow, greater focus should be directed toward assessing the contributing effects of depth on eDNA concentration.

3.2.4 | Environmental DNA decay

Among the reviewed papers, it was clear that the decay of eDNA variably impacts estimates of abundance and biomass (Coulter et al., 2019; Jo et al., 2017; Nevers et al., 2018; Sassoubre et al., 2016). Decay may be beneficial to some extent by restricting

eDNA concentrations over ecologically relevant scales (Nevers et al., 2018; Sassoubre et al., 2016), or detrimental, in cases where low production and/or rapid decay could potentially prevent establishing a relationship with abundance or biomass (Perez et al., 2017).

Decay rate depends on whether the eDNA is intra- or extracellular and is potentially influenced by several biotic and abiotic factors, including salinity, water temperature, sunlight, pH, microbial activity, and enzymatic digestion (Andruszkiewicz et al., 2017; Barnes & Turner, 2016; Collins et al., 2018; Dejean et al., 2012; Hansen et al., 2018; Sassoubre et al., 2016; Schmelzle & Kinziger, 2016). The rate of eDNA decay may be faster in marine than freshwater environments (Sassoubre et al., 2016). Indeed, Schmelzle and Kinziger (2016) reported that for every unit increase in salinity within the marine environment, eDNA concentrations reduced by 0.07 standard deviations from the mean, thus affecting abundance estimates of tidewater goby (*Eucyclogobius newberryi*).

As stated above, water temperature variably affects eDNA decay, which appears to depend on the species and the environment. At a broader level, there are likely to be seasonal effects, simply because ultraviolet radiation directly degrades DNA (Lindahl, 1993). Nevertheless, studies in aquatic systems suggest that sunlight has a limited influence, probably because other confounding effects such as depth or pH play much larger roles (Andruszkiewicz et al., 2017; Merkes et al., 2014; Strickler et al., 2015). More research under controlled conditions is warranted to assess the influence of sunlight on eDNA decay.

3.2.5 | Methods of capturing, extracting, and amplifying eDNA

Irrespective of the various factors affecting the concentration of eDNA in an aquatic system, subsequently estimating a population's abundance or biomass is contingent on the repeatability of the eDNA concentration estimate. This estimate is the product of the compounding effects of many processes including the substrate used (water/sediment) and method of eDNA capture, extraction, storage, eDNA primer design and validation, and PCR amplification (Baldigo et al., 2017; Eichmiller et al., 2016; Furlan et al., 2016; Hinlo et al., 2017b; Jane et al., 2015; Knudsen et al., 2019; Kumar et al., 2020; Nathan et al., 2014; Thalinger et al., 2020; Thomsen et al., 2016; Yusishen et al., 2020). In the reviewed papers, most studies used a filtration method (e.g., glass fiber filters, mixed cellulose ester filters) to capture eDNA. Only a few studies (predominantly in controlled environments) used alternative methods such as centrifugation and precipitation, most likely because the latter methods utilize substantially smaller volumes of water and thus may be more suited to controlled environments where eDNA concentrations can be relatively high (Eichmiller et al., 2016; Erickson et al., 2016; Klymus et al., 2015; Maruyama et al., 2014; Piggott, 2016).

Only a few studies have directly compared different eDNA capture and extraction methods and their effects on abundance or biomass estimates. Lacoursière-Roussel et al. (2016b) found that five different filters combined with water temperature could affect the strength of the relationship between eDNA concentration and abundance or biomass estimates of brook charr in aquaria. In another aquaria experiment, Eichmiller et al. (2016) evaluated the ability of three eDNA capture methods and six commercial DNA extraction kits to detect and quantify common carp biomass. Their results indicated the choice of capture and extraction methods could affect results. In contrast, in a study of Macquarie perch in an artificial pond, Piggott (2016) tested the effect of three eDNA capture - (filtration and precipitation) - and extraction methods - (Qiagen DNeasy Kit and phenol–chloroform–isoamyl) - on simulated biomass estimates (dam water spiked into the bore water that supplied the dam). All methods produced a strong positive correlation between eDNA concentration and biomass. Similarly, Takahashi et al. (2020) observed a strong correlation between eDNA concentration quantified from open filtration (glass filter) to enclosed filtration (Sterivex) and both of these concentrations were correlated with visually estimated biomasses of five marine fish—although different extraction methods were not assessed. While several studies have assessed the effects of filter type on eDNA concentrations, only one study has investigated the impact of dividing a sample among multiple filters (Guivas & Brammell, 2020). In that study, using either a single versus three filters produced positive correlations with biomass, although multiple filters resulted in a stronger relationship.

For species-specific studies, the vast majority have used qPCR to estimate eDNA concentrations with only three studies utilizing ddPCR (Capo et al., 2019; Doi et al., 2015; Nathan et al., 2014), possibly due to the higher relative cost of the latter instrument. Nathan et al. (2014) conducted mesocosm experiments to compare the performance of three PCR platforms (PCR, qPCR, and ddPCR) for estimating the concentration of round goby eDNA in the water. There was no statistical difference in the estimates of eDNA concentration between qPCR and ddPCR. In contrast, mesocosm experiments with common carp revealed eDNA concentrations determined by ddPCR better estimated absolute abundance and biomass than those determined by qPCR (Doi et al., 2015). While there are few studies evaluating fish eDNA concentrations using different PCR platforms, the available data suggest ddPCR has an advantage over qPCR. Three reasons for this preference are that it (a) does not require a standard curve (which can be compromised by pipetting error); (b) is more accurate for quantifying DNA at low concentrations; and (c) is becoming more cost-effective and thus may be more routinely used in the future (Doi et al., 2015; Nathan et al., 2014).

The success of PCR can be affected by the presence of PCR inhibitors in the sample that persist through the eDNA extraction stage (Akamatsu et al., 2020; Sassoubre et al., 2016). As one relevant example, in an experiment quantifying eDNA concentrations downstream of caged brook trout in a flowing stream in Massachusetts, USA, from midsummer to late autumn, eDNA was consistently detected (Jane et al., 2015). However, the eDNA signal became negligible to absent during autumn (which coincided with an increase in leaf litter) unless an inhibitor-reducing extraction method was used (Jane et al., 2015). This result implies inhibition can potentially mask

high eDNA concentrations, and profoundly affect the detectability and interpretation of abundance and/or biomass estimates (Jane et al., 2015).

Of the 63 reviewed papers, only 12 used metabarcoding to estimate relative abundance and/or biomass. Among these, four were carried out in controlled environments (Di Muri et al., 2020; Evans et al., 2016; Kelly et al., 2014; Li et al., 2018) and the remainder in natural environments (Fraija-Fernandez et al., 2020; Goutte et al., 2020; Hanfling et al., 2016; Li et al., 2019; Pont et al., 2018; Sard et al., 2019; Stoeckle et al., 2017; Thomsen et al., 2016). Nearly all metabarcoding studies (except Fraija-Fernandez et al., 2020) have reported positive correlations between eDNA read counts and relative abundance and/or biomass, supporting its potential utility as a cost-effective and accurate relative quantification method beyond simply identifying species presence.

The few assessed metabarcoding approaches also appear less affected by the filter types or extraction methods. In one study of artificial ponds, Li et al. (2018) reported comparable correlations between eDNA read counts and biomass across six different combinations of filter types (mixed cellulose ester and Sterivex-HV PVDF units), pore sizes and extraction methods (DNeasy Blood & Tissue Kit, PowerWater Kit and PowerWater Sterivex Kit). A more recent metabarcoding study of artificial ponds with known data on abundance and biomass also had consistent read counts from samples filtered using mixed cellulose ester and Sterivex filters (Di Muri et al., 2020). Nevertheless, an important consideration with metabarcoding is the additional primer-specificity biases that it introduces (Kelly et al., 2014).

3.2.6 | Spatial and temporal scales represented by eDNA sampling

It is clear from this review that eDNA concentrations in an aquatic environment vary considerably in space and time, which needs to be considered when designing a sampling regime to ensure accurate estimates of abundance or biomass (Hanfling et al., 2016; Klobucar et al., 2017; Salter et al., 2019; Shelton et al., 2019). This caveat was illustrated by Takahara et al. (2012) during aquaria trials with common carp which demonstrated eDNA concentrations peaked on the third day after fish were added to the tank before reaching equilibrium on the sixth day. Ideally, aquaria trials seeking to compare eDNA concentrations to abundance or biomass should first determine the most appropriate time for sampling after fish have been introduced into a tank. In natural populations, eDNA distributions can be heterogeneous and abiotic mechanisms that remove eDNA, such as decay, sedimentation or water flow, can be rapid (Eichmiller et al., 2014; Jane et al., 2015; Wilcox et al., 2016).

While there is considerable spatio-temporal variability in the movement or dispersal of eDNA (and therefore concentrations) within experiments, an important point here is that fishing gear-based methods of estimating fish abundance or biomass are constrained by single points in space and time (i.e., where and when the gear is deployed). The detectability of eDNA across space and time might therefore provide relatively more information. In support of this statement, Shelton et al. (2019) showed that while eDNA and seine nets provided the same quantitative information for chinook salmon (*Oncorhynchus tshawytscha*) in terms of abundance indices reflecting seasonal migrations, the methods were less correlated when compared across sites, suggesting each approach provides different information about a heterogeneously distributed species. Indeed, seine nets only catch fish if they are present at the target site, whereas DNA is shed into the surrounding water where it is mixed and can persist for some time after the fish have moved on, but in some cases, may not be correlated with abundance. This effect can result in a smoother distribution (in time and space) of eDNA versus the presence of the fish themselves (Shelton et al., 2019).

4 | CONCLUSIONS AND FUTURE DIRECTIONS

It is well established that eDNA can provide valuable information on the spatio-temporal distributions of marine and freshwater fish and can be used to detect the presence of ETP or invasive species, but its utility to quantify the abundance and/or biomass of animals in the field has remained equivocal. Clarifying the efficacy of a quantitative eDNA approach and understanding its utility as an ancillary tool within conventional stock assessments (incorporating data on biology, mortality, recruitment, etc. Quetglas et al., 2017) would be an important step toward developing a cost-effective means for monitoring regional fisheries of both sessile and migratory species.

In this review, we have critically evaluated whether eDNA concentrations (i.e., number of DNA sequences) or read counts as quantified by qPCR, ddPCR or metabarcoding were correlated with the absolute or relative abundance or biomass of fish. We accomplished this evaluation by sourcing studies from the available literature that met select criteria and identified points of congruence. We acknowledge the potential for publication bias, whereby some null results are less likely to be presented. Nevertheless, we note the overwhelmingly positive evidence in support of the development of this quantitative approach, with 90% of the sourced studies demonstrating positive correlations between detectable DNA in the environment and abundance/biomass of the focal species. Variation around this positive correlation was attributed to key biotic (taxon, life history, diet, metabolism, and behavior) and abiotic (water flow, temperature, and capture method) factors. Species-specific assays were more frequently used than metabarcoding assays for quantifying eDNA, and recent research suggests that the former may be more effective in this regard for fish (Bylemans et al., 2019).

Considering the reviewed information, in addition to initial laboratory considerations that include in silico primer development and validation using source-voucher tissues, we suggest focusing on understanding the influences of the biotic and abiotic factors listed above on eDNA concentrations within and among key

species to guide the refinement of future efforts. Indeed, to control for the heterogeneous dispersal of eDNA in the environment, we suggest study designs that include sampling replicates across multiple spatio-temporal scales. It is especially imperative that future studies investigate any potential relationships between eDNA concentrations and abundance or biomass in the field to verify the technique is appropriate for species of interest.

To account for differences in life history and metabolism, complimentary knowledge of the size and age structures of the focal species within a sampling area is important, unless the goal is to explicitly detect variation in eDNA concentrations through time as a proxy for spawning activity or seasonal migrations. However, studies should not be initiated without *a priori* ecological information, unless there is a strong incentive to do so (e.g., EPT species). The diet and behavior of the focal species may also bias eDNA concentrations in the field, but temporally standardized sampling could help to mitigate this problem. Moreover, the effects of abiotic factors such as water flow, depth, and temperature can be partially controlled for by collecting detailed knowledge of the hydrodynamics of the sampling site, vertical stratification of the focal species and historical temperature ranges, in addition to standardizing the time of sampling to account for tides and seasonal changes. Hierarchical sampling designs will be essential for identifying and partitioning spatio-temporal variation in eDNA concentrations, and ensuring such variability is accounted for when estimating abundance or biomass (Hanfling et al., 2016). Newer modeling-based approaches developed in aquatic settings may also compliment such initiatives (Chambert et al., 2018).

Irrespective of the approach, the collection method (i.e., type and pore size of filter, volume of water processed, and preservation method) should be standardized to facilitate comparisons between sampling sites and time points, and where possible, between studies. Based on the accumulated evidence, we therefore recommend developing eDNA as a complimentary (nonlethal) tool for assessing population abundance and/or biomass across different species of fish in both freshwater and marine environments, provided key biotic and abiotic factors are identified, considered, and, where possible, controlled for. With further development, eDNA may provide an effective fishery-independent method for assessing targeted stocks, while avoiding many of the potential biases associated with traditional fishery-dependent methods.

Data Archiving Statement: Data sharing is not applicable to this article because no data were created or analyzed outside of those summarized in the paper.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

MLR, AMF, JMH, MKB, JDB, and SF conceived and designed the research. MLR, AMF, JMH, MKB, JDB, SF, and JWW performed the literature review. MKB produced the figures. MLR, AMF, JMH, MKB, JDB, SF, JWW, and EMF wrote and edited the review.

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SHORT COMMUNICATION

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Robust environmental DNA assay development and validation: A case study with two vulnerable Australian fish

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Abstract

1. Analysis of environmental (e)DNA can facilitate an understanding of the presence and distribution of aquatic species. However, eDNA detection using quantitative PCR requires validated and standardized species-specific assay designs.
2. This study presents two eDNA assays to detect Murray cod, *Maccullochella peelii*, and mulloway, *Argyrosomus japonicus* (two ecologically vulnerable Australian species), based on small fragments of the mitochondrial 12S ribosomal RNA gene. A comprehensive description of species-specific assay development, from assay design to testing *in silico*, *in vitro* and *in situ*, has been included to guide effective assay design and validation in future studies.
3. The results indicate that the assay was species specific for *M. peelii* within its natural distribution. However, the assay also amplified genomic DNA from two allopatric and endangered congeners (*Maccullochella ikei* and *Maccullochella mariensis*), thus potentially facilitating their eDNA detection elsewhere. In contrast, the *A. japonicus* assay was highly species specific with no amplification among close relatives. Both target-species assays are highly sensitive to as few as four and 10 copies per PCR reaction, respectively.
4. This study has demonstrated that the assays assessed are effective tools for detecting the targeted species *in situ* from environmental DNA samples, which will assist efforts to conserve and manage their stocks.

KEYWORDS
detection, eDNA, endangered species, fishery-independent survey, quantitative PCR, species-specific

1 | INTRODUCTION

In the past two decades, the analysis of environmental DNA (eDNA) has facilitated an understanding of the presence and distribution of flora and fauna (Thomsen & Willerslev, 2015; Mathieu et al., 2020), especially aquatic species (Rees et al., 2014). Within aquatic ecosystems, eDNA can complement traditional survey methods (Hinlo et al., 2018; Piggott et al., 2021), which in some cases disturb habitats, are always selective and can preclude the detection of elusive species. The effectiveness of eDNA in detecting priority organisms (Thomas et al., 2020; Piggott et al., 2021), particularly at low population densities (Furlan et al., 2019), has made it a valuable addition to the conservation ecologist's toolkit.

Species-specific approaches to eDNA analyses, usually conducted via quantitative PCR (qPCR), are highly effective for determining presence/absence records (Deiner et al., 2017; Evans &

Lamberti, 2018) and there is increasing evidence of their utility for estimating abundance and/or biomass (Takahara et al., 2012; Rourke et al., 2021). Inherent prerequisites to species-specific detections using eDNA are a validated assay design and efficient amplification (MacDonald & Sarre, 2017; Langlois et al., 2021; Thalinger et al., 2021), which need to be conducted for each species investigated. Such approaches require standardization and repeat-analysis guidelines, and should have a validated and robust assay (Thalinger et al., 2021).

Although there are likely to be future benefits in validating assay design and amplification among all species underpinning aquatic resources within any jurisdiction, limitations to logistics and funding mandate some prioritization. As a starting point, those species listed as endangered, threatened, protected or otherwise vulnerable might be given preference for any new research programme embarking on assessing the utility of eDNA as a complementary tool for resource assessments. Two such Australian species are the freshwater Murray cod, *Maccullochella peelii* and euryhaline mulloway, *Argyrosomus japonicus*. Both species are large (> 1.5 m and 75 kg) and highly prized by recreational fishers. *Argyrosomus japoinius* also forms the basis of important commercial fisheries throughout New South Wales (NSW), Australia (Earl et al., 2020). Owing to overfishing or declines in abundance across their natural range (especially *M. peelii*, which is restricted to the Murray–Darling Basin) both species are managed under recovery plans, encompassing efforts at defining and supporting populations. However, concerns over the low abundances of *M. peelii* and *A. japonicus* combined with their broad spatial distributions render population monitoring via traditional methods inefficient, expensive and potentially unethical (when lethal sampling is required).

Species-specific eDNA approaches may offer an alternative means to rapidly survey the spatio-temporal distributions and potentially estimate population sizes of *M. peelii* and *A. japonicus* – depending on the reliability of a positive relationship between eDNA concentration and fish biomass (Rourke et al., 2021). Like all candidate species, however, there is a need first to develop species-specific qPCR assays. This article describes the steps followed to design (*in silico*) and validate (*in vitro*) species-specific assays for *M. peelii* and *A. japonicus*, and to assess (*in situ*) whether individuals could be detected using eDNA to support future monitoring.

2 | METHODS

2.1 | In silico assay design

Species-specific qPCR assays were designed for *M. peeli* and *A. japonicus* based on a small fragment of the mitochondrial 12S ribosomal RNA (hereafter 12S rRNA) gene. This gene has been widely used in previous species-specific assay development because it presents reasonable inter- and intra-specific variation among fish and has sufficient representation in online reference databases (Benson et al., 2005; Rourke et al., 2021). In-house species reference sequences were generated by sourcing fin clips from *M. peelii* ($n = 5$) caught in the Murray–Darling Basin and *A. japonicus* ($n = 5$) from the Shoalhaven River, NSW. Fin clips ($n = 3$) were also sourced from a co-occurring sciaenid, *Atractoscion atelodus* (teraglin) from the central coast of NSW, a species for which there was no available sequence data in the National Centre for Biotechnology Information (NCBI) GenBank database (Benson et al., 2005).

Genomic DNA from the fin clips was extracted using the PureLink™ Genomic DNA Mini Kit (Invitrogen, CA, USA) according to the manufacturer's instructions. A 390 bp region of 12S rRNA was amplified as described by Furlan & Gleeson (2016) but performed on a QuantStudio3 PCR System (Applied Biosystems, CA, USA). The PCR products were purified using the PureLink™ PCR Purification Kit (Invitrogen) and then sent to the Australian Genome Research Facility (AGRF) in Sydney, Australia for forward and reverse Sanger sequencing using a 3730xl DNA analyzer.

Trimmed and edited in-house reference sequences from *M. peelii* and *A. japonicus* were imported into Geneious Prime version 2020.0.5 (https://www.geneious.com) and aligned to all other 12S rRNA sequences recorded for both species downloaded from the NCBI database (Table S1). *Maccullochella peelii* sequences were aligned to all species within the Percichthyidae known to occur in Australia ($n = 17$), except *Guyu wujalwujalensis* (Table S1, Hardy et al., 2011). *Argyrosomus japonicus* sequences were aligned to all closely related species restricted to eastern and southern Australia (nine species from the Sciaenidae, Table S1). In addition, all sequences generated in-house were uploaded to the NCBI database under accession numbers MZ766144 to MZ766156.

Primers and probes were manually designed in Geneious Prime by maximizing the number of base pair mismatches with all other known co-occurring species with available sequence data. Annealing temperature (T_m), GC content and secondary structure were checked using the in-built Primer3 software in Geneious Prime to ensure low homo- and hetero-dimerization potential. The primer/probe assays were then tested for specificity and cross-amplification to non-target species *in silico* using Primer-BLAST (Ye et al., 2012; https://www.ncbi.nlm.nih.gov/tools/primer-blast/).

2.2 | *In vitro* assay specificity and sensitivity

Primer/probe assays (Table 1) were tested for amplification specificity with DNA sourced from tissue of the target and closely related co-occurring species as described above (Table S1). Quantitative PCR was carried out in triplicate 20 µl reactions, comprising 10 µl TaqMan™ Environmental Master Mix 2.0 (Applied Biosystems), 1 µl TaqMan™ Gene Expression assay 20× (Applied Biosystems), 7 µl DNase/RNase-free water and 2 µl of genomic DNA from up to three individuals of each species. The PCR cycling was conducted on a QuantStudio3 PCR System (Applied Biosystems) with thermal cycling conditions set at 50 °C (2 min) and 95 °C (10 min), followed by 50 cycles of 95 °C (15 s) and 60 °C (60 s). Any non-target positive amplification was sequenced at the AGRF (as detailed above) to determine its origins.

TABLE 1 Primer and hydrolysis probe sequences designed to amplify a short region of the 12S rRNA gene in Murray cod, *Maccullochella peelii*, and mulloway, *Argyrosomus japonicus*

Target species	Label	Sequence (5′–3′)	Length (bp)
M. peelii	M.pee_12S_F	CCCTTGTTCCACCAGCCTA	19
	M.pee_12S_R	GTTCTGGGTTGTACCAATTATGCT	24
	M.pee_12S_probe	(FAM)-CCAGCTTACCCTGTGAAGGACCTA-(MGB)	24
	M.pee_12S_synth_oligo[a]	CCCTTGTTCCACCAGCCTATATACCGACGGCGCC AGCTTACCCTGTGAAGGACCTATAGTAAGCATAA TTGGTACAACCCAGAAC	85
A. japonicus	A.jap_12S_F	CTCACCCTTCTTTGTTTCCC	20
	A.jap_12S_R	CATCATTCGTTTTCTCTGTGTC	22
	A.jap_12S_probe	(FAM)-CCGTCGTCAGCTTACCCTGTG-(MGB)	21
	A.jap_12S_synth_oligo[a]	CTCACCCTTCTTTGTTTCCCCCGCCTATATACCGC CGTCGTCAGCTTACCCTGTGAAGGACTTATAGTA AGCAAAATTGGTACAACCCTAAACGCCAGGTCG AGGTGATACGCTAGGAAGGGGAAGAAATGGGCT ACATTCTCTGACACAGAGAAAACGAATGATG	166

[a]The synthetic oligonucleotide is given with the 8 bp reverse complement sequence underlined.

TABLE 2 Species-specific assay sensitivity for quantitative PCR (qPCR) detection of Murray cod, *Maccullochella peelii*, and mulloway, *Argyrosomus japonicus*. Modelled limit of detection (LOD) and modelled limit of quantification (LOQ) in copies per reaction

Target species	Assay	Sensitivity		Modelled LOD	Modelled LOQ	Annealing temperature
M. peelii	M.pee_12S_assay	Efficiency (%)	96.69	4	32	59 °C
		R^2	0.998			
		Slope	−3.404			
A. japonicus	A.jap_12S_assay	Efficiency (%)	95.39	9.4	70	56 °C
		R^2	0.998			
		Slope	−3.437			

The efficiency of each assay was tested using synthetic oligonucleotides (gBlock™ Gene Fragments; Integrated DNA Technologies) of the target species' 12S rRNA gene. All synthetic oligonucleotides were designed to contain a modified sequence consisting of an 8 bp segment as a reverse complement of the original sequence to facilitate identification of the synthetic oligonucleotide via Sanger sequencing. Synthetic oligonucleotides were diluted in tRNA buffer (1:250 diluted in DNase/RNase free water; Sigma-Aldrich, MO, USA) by a factor of 10 to generate concentrations between 10^{-2} and 10^{-8} ng μl^{-1} (*M. peeli* 20,412,514.4 and 20.4 copies μl^{-1}; *A. japonicus* 16,470,025.1 and 16.4 copies μl^{-1}).

The optimum annealing temperature for each assay was determined using a temperature gradient qPCR. Three temperatures based around the theoretical T_m of the primers were tested (M.pee_12S_assay, 58 °C, 59 °C, 60 °C; A.jap_12S_assay, 55 °C, 56 °C, 57 °C). Three 20 µl qPCR replicates, with 2 µl of each concentration of the synthetic oligonucleotides serving as the template, were used to create a standard curve at each temperature. The optimal annealing temperature was identified as the temperature with reaction efficiency closest to 100%. A calibration curve was generated using eight 20 µl qPCR replicates, with 2 µl of each concentration of the synthetic oligonucleotides serving as the template. The limits of detection (LOD) and quantification (LOQ) of each assay were further assessed by diluting the synthetic oligonucleotides to 50, 30, 10, 5, 2 and 1 copies μl^{-1}. The assay calibration and LOD/LOQ tests were carried out using qPCR reactions and conditions as described above, and at the optimized annealing temperature for each assay (Table 2).

The reaction efficiency and R^2 for the standard curve were calculated using Quantstudio™ Design & Analysis Software version 1.5.1. The LOD and LOQ were modelled in R (R Core Team, 2020), using the curve-fitting method and R script, described and published by Klymus et al. (2020). The standardized definitions described by Klymus et al. (2020), where the LOD is the lowest standard concentration at which 95% of technical replicates amplify and the LOQ is the lowest standard concentration for which the coefficient of variation (CV) value is <35%, were applied here. Quality controls used to identify contamination issues included negative DNA extraction controls and no template PCR controls (NTC PCR), with tissue-derived DNA from the target species acting as positive controls.

2.3 | *In situ* field validation of species-specific qPCR assays

Assay performance was evaluated *in situ* on eDNA samples collected at freshwater and estuarine sites, with and without historical catch records for each species (NSW DPI Fisheries unpublished data). Three sites were identified on the Murrumbidgee River, NSW (Buckingbong Boat Ramp, Narrandera Boat Ramp and Port Phillip Trail) for the *M. peelii* assay and three sites in the Hawkesbury River catchment, NSW (Cogra Bay, Spencer Point and Mountain Avenue) for the *A. japonicus* assay (Figure 1). In each system, two sites had known populations of the target species and one control site was chosen based on the target species being absent according to historical records.

Eight surface water samples (depth 5–10 cm) were filtered at each site using the Smith–Root eDNA sampler™ (WA, USA), which used a mobile computer-controlled pump to standardize *in situ* sample filtration and employed preloaded filters in an inline filter housing to minimize contamination (Thomas et al., 2020). Of the eight samples, three were collected from each shoreline ($n = 6$) and two from the channel ($n = 2$). Samples within each site were separated by at least 50 m and encompassed different habitat types. Owing to variable turbidity, water volumes between 0.5 and 2.0 L were filtered through a 5 µm polyethersulfone filter enclosed in the sterile filter housing (Smith–Root) at 1.0 L min^{-1} and with a maximum pressure of 10 psi. An equipment control (EC) comprising 1.0 L of sterile water was filtered at each site before the field sampling. Filter papers were immediately removed using sterile forceps, preserved in 100% ethanol, transported to the laboratory and stored at 4 °C.

All further laboratory processing of filters to isolate and amplify DNA occurred in a purpose-built facility at the Narrandera Fisheries Centre, Australia. This facility includes three separate UV-sterile rooms each with a PCR hood (AirClean Systems, NC, USA) for DNA extraction, PCR preparation and DNA template loading. The DNA was extracted from the filter papers (within 4 weeks of collection) using the PureLink™ Genomic DNA Mini Kit (Invitrogen) with a modified protocol. Each filter paper was halved and placed into two 2.0 ml tubes using sterile scissors and forceps prior to cell lysis with 500 µl of Genomic Digestion Buffer and 20 µl Proteinase K for 1 h at 55 °C. The filter paper was then removed, placed into a QIAshredder™ tube (Qiagen, Germany) and spun down to recover all lysate. Recovered aliquots were returned to the lysate in each tube, before 400 µl of Genomic Binding/Lysis Buffer and 400 µl of 100% ethanol were added. The lysate was then loaded into spin columns and the protocol followed according to the manufacturer's instructions. Final eDNA extracts were eluted in 100 µl of Genomic Elution Buffer.

FIGURE 1 Site locations and eDNA results from the field validation of each targeted assay assessing Murray cod, *Maccullochella peelii*, and mulloway, *Argyrosomus japonicus* detection. The numbers of positive detections at each site (control site marked with ^{C}) from eight water samples are shown, with each sample analysed using six PCR replicates

A negative DNA extraction control was included with the samples from each site and consisted of 100 µl of nuclease-free water and no template DNA. Before qPCR analysis, each sample was checked in triplicate for inhibitors using TaqMan™ Exogenous Internal Positive Control (IPC) reagents (Applied Biosystems). A sample was considered 'inhibited' if the IPC cycle threshold (C_t) was at least two cycles greater than the IPC C_t value in the NTC PCR (Tillotson et al., 2018). Analysis of each sample, as well as the EC, negative DNA extraction control and NTC PCR (using qPCR) used six 20 µl replicates and was performed as described above. Fifty per cent of the positive samples were sequenced at the AGRF (as detailed above) to ensure that the amplified DNA matched the target species.

3 | RESULTS AND DISCUSSION

3.1 | Primer design, probe design and assay specificity

The primers and the hydrolysis probe designed in each species-specific assay successfully amplified 85 and 166 bp fragments from the *M. peelii* and *A. japonicus* 12S rRNA gene, respectively (Table 1). Specificity tests for each assay on tissue-derived genomic DNA with known provenance showed strong amplification of each target species. Subsequent testing, using tissue-derived DNA from co-occurring closely related percichthyids (for *M. peelii*) and sciaenids (for *A. japonicus*), resulted in some unexpected PCR amplification. Follow-up Sanger sequencing of these amplicons confirmed that target-species DNA was present within several non-target samples, suggesting sample contamination during sample collection, DNA extraction or qPCR preparation. Investigation using a suite of negative and positive controls confidently ruled out contamination at the DNA extraction and qPCR preparation stages. Thus, it was concluded that contamination occurred during the tissue sample collection stage. It is likely that this finding relates to a common yet under-reported issue for eDNA assay design and validation. It is prudent therefore that researchers take precautions during fin-clip collection to reduce the risk of contamination (Rodgers, 2017). Follow-up Sanger sequencing confirmed that no amplification occurred in either assay for non-target, co-occurring species in Australia (Table S1). The only non-target amplification observed with the *M. peelii* assay (M.pee_12S_assay) was in two allopatric species: the eastern freshwater cod, *Maccullochella ikei*, and Mary River cod, *Maccullochella mariensis*.

Maccullochella ikei and *M. mariensis* occur in the Clarence River (NSW north coast) and Mary River (south-eastern Queensland coast) catchments, respectively. These closely related allopatric species were included in the specificity test for the *M. peelii* assay because they have an identical 12S rRNA sequence in the region of the gene targeted, and thus the *M. peelii* assay could potentially be applied for their detection. As expected, the *M. peelii* assay amplified genomic DNA samples of these congenerics (Table S1) and is therefore specific to *M. peelii* DNA in the Murray–Darling Basin and south-eastern Australian coastal catchments, except for the Clarence and Mary river systems. Additional field assessments might warrant assessing the performance of the *M. peelii* assay for detecting *M. ikei* and *M. mariensis* in those respective locations. An eDNA approach would probably be useful for surveying both species, given that they are 'Endangered' and rare (International Union for Conservation of Nature, 2021).

There are few relatives of *A. japonicus* co-occurring within its Australian distribution. Specificity testing of the *A. japonicus* assay (A.jap_12S_assay) confirmed no amplification of teraglin, *A. atelodus*, river jewfish, *Johnius borneensis*, or silver jewfish, *Nibea soldado* (Table S1). Distribution records for one closely related sciaenid, the little jewfish, *Johnius australis*, demonstrate the potential for a minor southern distributional overlap with that of *A. japonicus*; however, genomic DNA could not be sourced for the former species. Based on the high number of mismatches (>10 bp) to other *Johnius* species (Table S1), the likelihood of cross-amplification with *J. australis* is low. Nevertheless, using the assay north of the Brisbane River in Queensland, Australia is not recommended without further testing. Therefore, the *A. japonicus* assay is specific to *A. japonicus* DNA within estuaries and coastal waters of southern Australia, from the Brisbane River on the east coast to the Exmouth Gulf (Western Australia) on the west coast.

3.2 | Assay sensitivity and field validation

Each species-specific assay demonstrated high sensitivity, with efficiency and R^2 values greater than 95% and 0.998, respectively (Table 2). The assays were able to detect reliably (with 95% confidence) concentrations of their target DNA down to at least 10 copies per reaction and could accurately determine DNA quantity, to 32 and 70 copies per reaction, for the *M. peelii* and *A. japonicus* assay, respectively. The modelled LOD of each assay (< 10 copies per reaction) was consistent with the modelled LOD values reported for almost 90% of the eDNA assays analysed by Klymus et al. (2020), who proposed standardized LOD and LOQ calculation and reporting. This result highlights the sensitivity of the assays described here and supports an assertion that the assays are capable of detecting DNA at the often low and stochastic concentrations found in the environment.

The field assessments for each assay successfully detected *M. peelii* and *A. japonicus* in all samples at sites where they are known to occur, whereas no detections occurred at control sites (Figure 1). No amplification occurred in the EC, negative DNA extraction control and NTC PCR, confirming no contamination throughout the sample filtration, extraction and amplification procedures. At the two Murrumbidgee River sites where *M. peelii* was detected, DNA was present in all PCR replicates tested, whereas at the Hawksbury River test sites where *A. japonicus* was detected, DNA was present in 89.5% of PCR replicates tested. The PCR inhibition testing found that minor inhibition occurred when analysing samples from the Hawkesbury River sites where *A. japonicus* was known to occur, but the low level of PCR inhibitors did not prevent detections. Moreover, Sanger

sequencing of 50% of the positive samples at all sites produced sequence matches with the respective target species, confirming the specificity of the assays in the field.

3.3 | Conclusions

The two species-specific assays presented here successfully detected *M. peelii* and *A. japonicus* DNA *in silico*, *in vitro* and *in situ*. This rigorous approach to species-specific assay development, supported in guidelines published by Thalinger et al. (2021), Langlois et al. (2021) and Klymus et al. (2020), confirms the specificity, sensitivity and field-based effectiveness of each assay. This outcome will facilitate eDNA-based approaches that will, at a minimum, confirm species presence across both apparent and cryptic habitats. Such methods will become increasingly important for population monitoring if abundances decline to levels where conventional survey methods are ineffective or unethical.

Further validation of each assay might include occupancy modelling to determine detection probability and account for false negatives (Burian et al., 2021; Thalinger et al., 2021). Overall, we suggest that applying comprehensive methods to the development of species-specific assays, as has been done here, will provide researchers and managers with confidence when interpreting and using eDNA detection results (Sepulveda et al., 2020).

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

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Poor utility of environmental DNA for estimating the biomass of a threatened freshwater teleost; but clear direction for future candidate assessments

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ABSTRACT

Environmental DNA (eDNA) can potentially facilitate estimating the abundance of teleosts and might therefore be used for stock assessments. Nevertheless, relationships are species-specific—reflecting several abiotic and biotic factors that require understanding prior to ongoing research investment. Here, we investigated potential relationships between eDNA concentrations and the weights of an endemic and threatened Australian teleost, Murray cod, *Maccullochella peelii* to determine if eDNA could be used to estimate biomass. Three controlled aquaria experiments using juvenile fish were conducted to investigate the time to eDNA stabilisation in tanks following stocking (aquaria experiment 1), and then if water temperature (aquaria experiment 2) and/or fish weight (aquaria experiment 3) explained variability among eDNA concentrations in tanks. In aquaria experiment 1, eDNA concentrations stabilised nine days after stocking, but there were no significant effects of water temperature or fish weight in aquaria experiments 2 and 3. The latter result was verified during a mesocosm experiment using brood stock held in semi-natural outdoor ponds. During a fourth aquaria experiment (experiment 4), ten similar-sized juveniles were held under identical conditions in individual replicate tanks, with significant variability in eDNA concentrations that was not explained by individual fish weights. When all aquaria data were collectively assessed, there was a significant (but not useful) quadratic relationship between eDNA concentrations and fish weight. We conclude eDNA is not suitable for estimating the weight/biomass of Murray cod. Assessing the suitability of future candidate species might benefit from similar initial aquaria experiments under controlled conditions to test the independence of individual fish weight on variability among eDNA concentrations in tanks.

1. Introduction

During the past decade, environmental DNA (eDNA) surveys have been increasingly applied to detect the presence of various organisms in aquatic environments, including many teleosts and cartilaginous fishes (for reviews see Mathieu et al., 2020; Rourke et al., 2022; Thomsen and Willerslev, 2015). More recently, several studies have demonstrated the potential utility of eDNA for estimating the biomass and/or abundance of some aquatic species, precluding the need for lethal sampling and providing a benign option for assessing threatened populations (Rourke et al., 2022).

However, while the growing body of literature implies eDNA

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approaches for estimating abundance are broadly applicable, it is also clear not all species are suitable candidates (Rourke et al., 2022). There are many factors influencing the amount of DNA shed by aquatic species, or the amount that can be detected. Among teleosts, examples of influencing intrinsic factors affecting eDNA release and/or its detection include the species examined as well as their body size (Klymus et al., 2015; Wilcox et al., 2016), distribution (Eichmiller et al., 2014; Itakura et al., 2019), feeding behaviour (Ghosal et al., 2018; Klymus et al., 2015), and reproduction and migration patterns (Bracken et al., 2019). Extrinsic factors often involve hydrological processes affecting the dispersal and persistence of eDNA, especially water flow (Harrison et al., 2019), temperature (Lacoursière-Roussel et al., 2016), and to a lesser extent, depth (Hänfling et al., 2016). Validating the relationship between eDNA and abundance (or biomass) is therefore required for individual species, encompassing the key biotic and abiotic conditions that characterise the assessment environment.

Previous studies assessing correlations between eDNA concentrations and the abundance or biomass of fish have employed various approaches involving aquaria (Klymus et al., 2015), mesocosms (Doi et al., 2015; Takahara et al., 2012), wild environments (Erickson et al., 2017; Hänfling et al., 2016), or combinations thereof (Wilcox et al., 2016). While it is essential causal relationships are investigated in the field to encompass natural variability, it is important that potential trends are first validated under optimal controlled conditions. If relationships between eDNA concentrations and fish abundance or biomass are not detected under such conditions, then they are unlikely to present in field-based studies with stochastic environmental influences (Rourke et al., 2022). This assessment sequence can reduce eventual costs and provide some indication of the likely applicability for the target species.

We followed the above approach to explore the relationship between eDNA and weight (as a proxy for biomass, given known sample sizes) of a threatened Australian freshwater species, Murray cod, *Maccullochella peelii* (Mitchell 1838). This species is an endemic, large-bodied, apex predator (up to 1.8 m total length; TL and 100 kg) with a key ecosystem role in south-eastern Australian inland rivers (the Murray-Darling Basin; MDB), where it inhabits restricted habitat and is subjected to variable water quality, including temperatures of ~10–25 °C. Owing to habitat loss and historical commercial fishing (prohibited since 2001) during the last century, populations of Murray cod declined to the point where it was listed as Vulnerable under the Environment Protection and Biodiversity Conservation Act 1999 (EPBC Act). Populations are currently supported by recovery plans, but the species remains the focus of important inland recreational fisheries, and mostly catch and release (Murphy et al., 2020).

The status of Murray cod populations presents challenges for their monitoring and management. The small number of individuals distributed across a broad spatial range within the MDB render population monitoring via conventional methods inefficient (Lyon et al., 2014) and expensive. The ban on commercial fishing eliminated regular fishery-dependent catch and effort data, which represented a cost-effective input for quantitative population assessments. Fishery-independent electrofishing surveys are now routinely used to monitor Murray cod populations, although these are restricted to small sections of the species' distribution, generating uncertainty regarding overall population status and progress toward recovery.

Environmental DNA surveys have been proposed as one approach to estimate Murray cod population sizes, but there are no available data supporting utility. The main aim of this study was to use a series of controlled experiments to investigate the suitability of eDNA concentrations for estimating the amount of Murray cod. A second aim was to propose an efficient testing strategy for other candidate species.

2. Methods

This study was conducted between July and December, 2020, and involved a series of four indoor aquaria experiments using juvenile Murray cod, along with one outdoor mesocosm experiment using adult Murray cod brood stock at the Narrandera Fisheries Centre (NFC; 34.68° S, 146.61° E), in NSW, Australia. All work was done following established husbandry protocols and animal care and ethics approvals (NSW ref: 20/01).

2.1. Aquaria experimental setup

For the aquaria experiments, three 700-L fibreglass outdoor 'holding' tanks were configured with aeration (via stone diffusers) and supplied with flow-through water (at 30 L min^{-1} and ambient temperature), while 12 smaller polyvinyl chloride 35-L 'experimental' tanks (each containing 32 L of freshwater) were distributed throughout an enclosed room and maintained under an artificial photoperiod (light:dark ratio 12:12 h) (Fig. 1). To eliminate any confounding effects of inflow on eDNA concentrations within (owing to variable circulation) and between (owing to the original water source) experimental tanks, these were maintained without water exchange. A closed system for Murray cod was also justified based on their known water-quality tolerances. Each experimental tank was positioned inside an 81-L 'outer' tank filled with 45 L of flowing temperature-regulated water (via an external heater-chiller unit) that maintained all experimental-tank water temperatures at 18.5–19.5 °C (except during experiment 2; see below).

Prior to each experiment, the 12 experimental tanks were sterilised with a 10% bleach solution, filled with UV-treated bore water, salted (5 g L^{-1}) and pH neutralised (7.0). All experimental tanks were fitted with polycarbonate lids to prevent aerosols from transferring DNA between tanks (Fig. 1). Each lid was fitted with an access hole and plug to allow water to be filtered *in situ*.

The Murray cod used in the aquaria experiments were sourced from an aquaculture facility in Griffith, NSW (34.28° S, 146.03° E) and were the offspring of brood stock originally collected from the MBD. Prior to collection, all fish were anaesthetised in oxygenated tanks containing 50 mg^{-1} benzocaine (ethyl-p-aminobenzoate) (Sigma Aldrich, Shanghai); approximately 600 juveniles (<150 mm TL) were removed using knotless-mesh scoop nets and transferred to a purpose-built transporter (400-L; stocking density < 5 kg fish m^{-3}) containing water at ambient temperature and supplied with pure oxygen. Fish were then transported to the aquaria facility at NFC where they were anesthetised as above prior to transfer to the three holding tanks (~200 individuals $tank^{-1}$). Fish were held in the holding tanks for up to six months, during which time they were monitored following established protocols and fed commercial pellets (Ridley, Australia) at ~2% biomass day^{-1}.

The fish were acclimated for five days in the 700-L tanks to the required experimental temperatures, and not fed for 72 h before starting each experiment (to limit waste impacts on water quality). In addition, fish were not fed during the experiments. Prior to each experiment, all fish in one randomly selected 700-L holding tank were anesthetised (as

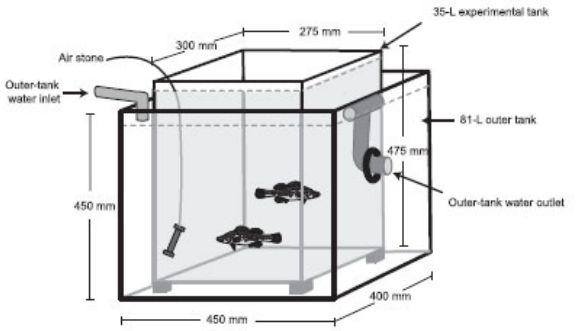

Fig. 1. Diagram of the 35-L experimental tank configurations containing Murray cod, *Maccullochella peelii* used in the four aquaria experiments.

above), and the required number removed, visually assessed (for dermal damage), measured (nearest 1 mm TL) and weighed (nearest 0.1 g). Only fish in good condition and 98–142 mm TL or 12.4–30.9 g (n = 220; see Results) were transferred to the experimental tanks (at the required experimental sample sizes; Fig. 2), with the others returned to the holding tank.

Four aquaria experiments were designed to investigate variability in the Murray cod eDNA concentration $tank^{-1}$ due to biotic and abiotic factors, with the specific hypotheses tested in each experiment outlined below. Common to all experiments was designating two experimental tanks as controls (no fish) and daily monitoring for fish mortality. Water filtering for eDNA assessment also remained consistent and was done after nine days in all experiments (following the results for experiment 1; see Section 3.3) using the Smith-Root eDNA Sampler™ (WA, USA). An equipment control (EC) comprising 100 mL of sterile water was filtered prior to sampling experimental tanks (once day^{-1}) using a 5 µm

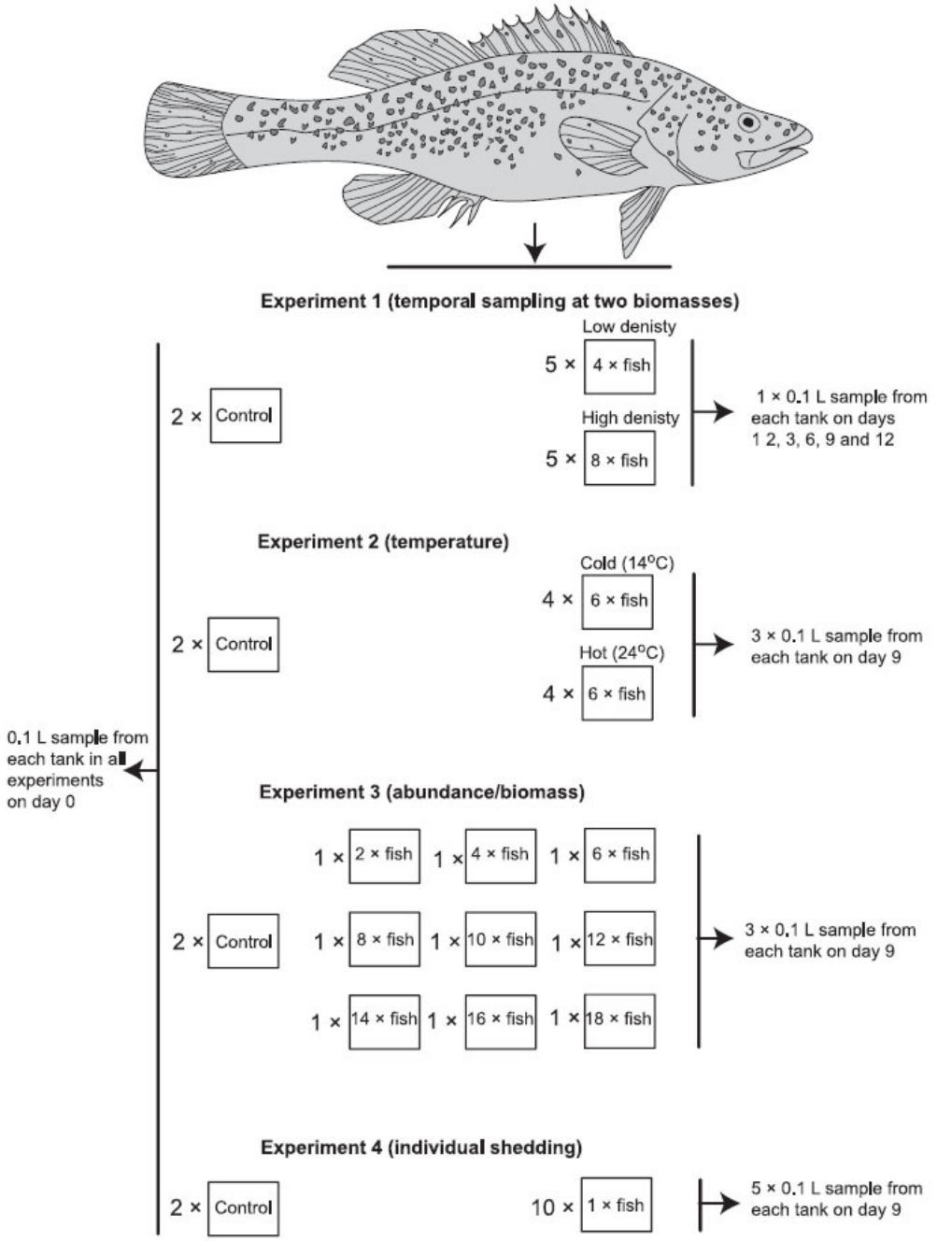

Fig. 2. Schematic representation of the four aquaria experiments, indicating the replicate 35-L experimental tanks used for controls (left) and treatments (right) with stocking densities of Murray cod, *Maccullochella peelii* and tank-specific temporal water sampling for eDNA.

polyethersulfone (PES) filter, enclosed in a sterile self-preserving filter housing (Smith-Root), at 1.0 L min^{-1} and with a max pressure of 69 kPa.

Prior to adding fish to each of the experimental tanks, 100 mL of tank water was filtered to test for existing Murray cod DNA. Water samples (100 mL) were then collected during each sampling event and tested (see Section 2.4) for Murray cod DNA. Each experimental tank was then continuously aerated using stone diffusers (but, as per above, with no water exchanges, except between experimental setups).

2.2. Aquaria experimental workflow

Experiment 1 was designed to assess for any temporal variation in eDNA concentrations $tank^{-1}$ (across two stocking rates) to determine the optimal time to sample (when eDNA concentration in the tanks reach a steady state; Sassoubre et al., 2016). Murray cod were held at two abundances, low ($n = 4$ individuals $tank^{-1}$; each weighing 15.2–22.5 g) and high ($n = 8$ individuals $tank^{-1}$; 15.8–22.8 g each), each replicated across five experimental tanks (Fig. 2). One water sample was collected from each tank at one, two, three, six, nine and 12 days post stocking and analysed for Murray cod DNA as described in Section 2.4.

In experiment 2, the effects of water temperature were investigated (within the range typically experienced by wild fish; ~10–25 °C). Six Murray cod were held in each of four tanks at low (14 ± 1 °C, with fish weighing 17.0–24.4 g each) and high (24 ± 1 °C, fish weighing 14.2–25.8 g) temperatures for nine days, at which point three replicate 100 mL water samples were collected per tank and analysed for DNA (Fig. 2).

During experiment 3, Murray cod were held at nine stocking densities: two (13.7 and 16.3 g); four (19.2–21.2 g each); six (12.5–21.0 g); eight: (13.3–27.5 g); 10 (13.0–24.2 g); 12 (13.7–20.6 g); 14 (14.4–24.9 g); 16 (13.3–23.1 g); and 18 (12.4–20.7 g) fish, for nine days, before three replicate water samples $tank^{-1}$ were taken to test for a continuous and predictable relationship between eDNA concentration and weight (Fig. 2). We increased the numbers of replicate water samples in experiments 2 and 3 after we completed the analyses for experiment 1 to reduce the effect of heterogeneous eDNA distributions on the estimates of eDNA concentration.

In experiment 4, a single Murray cod (27.0–30.9 g) was held in each of 10 tanks and water samples (five samples $tank^{-1}$; increased from the previous experiments to maximise precision) were collected after nine days to determine if there was any individual variability in DNA shedding that was not explained by fish weight (Fig. 2). At the completion of all aquaria experiments, fish were visually inspected and, if healthy, returned to unique holding tanks (to preclude subsequent reuse in an experiment, where relevant).

2.3. Mesocosm experimental setup and workflow

Based on the outcomes of the aquaria experiments (i.e., mostly accepted null hypotheses; see Results), an experiment was conducted using adult Murray cod in seven earthen ponds ('mesocosms' randomly dispersed across an ~8 ha area, separated by between 5 and 250 m and ranging in volume from 2.7 to 4.3 ML; Table S1) at the NFC to test for any effects of fish weight on eDNA concentrations at a larger scale and under semi-natural conditions. The mesocosms were permanent structures used to hold Murray cod brood stock (for decades) to produce offspring for supplementing wild populations. Each mesocosm was aerated with paddle wheels and contained known numbers and weights of Murray cod ($n = 10$–29 and 1.7–14.7 kg).

Normally, the flow-through water source for each mesocosm is the adjacent Murrumbidgee River (34.771° S, 146.572° E), with a known presence of Murray cod (Forbes et al., 2015). However, water was neither added to the mesocosms for two months prior to the experiment nor during, and we therefore assumed that any detected Murray cod DNA was derived exclusively from fish within the mesocosms (Barnes et al., 2014; Eichmiller et al., 2014).

We also hypothesised that under semi-natural conditions in the mesocosms, there would be a balance between eDNA concentration and biomass. There was no rainfall for 15 days prior to sampling and thus we assumed no disturbance of sediments that may have released legacy DNA into the water. The mesocosms contained other small fish such as carp gudgeons, *Hypseleotris* spp. and were accessible to various birds, marsupials and sheep, *Ovis aries*.

All Murray cod were left undisturbed in the mesocosms for at least 30 days prior to starting the experiment. Sampling was conducted during daylight in September, 2020, just prior to the spawning season. On the first day of the experiment, four replicate water samples (2-L target volume) were collected from the surface of each mesocosm using the Smith-Root eDNA Sampler™ by walking in a transect of 30 m in length at approximately equal distances around the perimeter. Each mesocosm was then sampled once for water temperature (°C), pH, turbidity (NTU), conductivity (mS cm^{-1}) and dissolved oxygen (mg L^{-1}) using an Horiba U52 water quality meter (Japan).

2.4. DNA extractions and quantitative PCR

All sample processing occurred in a purpose-built eDNA facility at NFC, with separate UV-sterile rooms (with PCR hoods – AirClean Systems 600, NC, USA) for DNA extraction, PCR preparation and DNA template loading. The DNA was extracted from each filter paper (within four weeks of collection) using the PureLink™ Genomic DNA Mini Kit (Invitrogen, CA, USA) with a modified protocol (Wilkes Walburn et al., 2022). Filter papers were halved and placed into separate 2.0 mL tubes. Cells were lysed using 500 μL of Genomic Digestion Buffer and 20 μL Proteinase K for one hour at 55 °C. Both filter papers were removed, placed into a single QIAshredder™ tube (Qiagen, Germany) and spun down to recover all lysate. The filter papers were discarded, the lysate returned to the tube and 400 μL of Genomic Binding/Lysis Buffer along with 400 μL of 100% ethanol were added. The lysate was then loaded into spin columns and the protocol followed manufacturer's instructions. Final DNA extracts were eluted in 100 μL of Genomic Elution Buffer. Note that a negative extraction control was included with each batch of 16 extractions, which was composed of 100 μL of RNase/DNase free water.

Quantification of Murray cod DNA in each sample was performed using quantitative PCR with a TaqMan™ assay (Applied Biosystems, CA, USA) designed and validated specifically for Murray cod (Wilkes Walburn et al., 2022). An 85 bp fragment of the mitochondrial 12 S rRNA gene was amplified with the primers M.pee_12S_F (5′-CCCTTGTTCCACCAGCC TA-3′), M.pee_12S_R (5′-GTTCTGGGTTGTACCAATTATGCT-3′) and pro be (FAM dye-labelled) M.pee_12S_probe (5′- CCAGCTTACCCTGTG AAGGACCTA-3′). Each sample, including the EC and negative extraction control, was analysed using six replicate 20 μL reactions, comprising 10 μL Environmental Master Mix 2.0 (Applied Biosystems), 1 μL TaqMan Gene Expression assay 20X (Applied Biosystems), 7 μL DNase/RNase free water and 2 μL of DNA template.

The PCR reactions were run on a QuantStudio3 PCR System (Applied Biosystems) with thermal cycling conditions set at 50 °C (2 min) and 95 °C (1 min), followed by 50 cycles of 95 °C for 15 s and 59 °C (60 s). Standards, with six concentrations between 10^{-3} and 10^{-8} ng μL^{-1}, comprising Murray cod 12 S rRNA gene synthetic oligonucleotides (gBlock™ Gene Fragments; Integrated DNA Technologies) diluted in tRNA buffer (1:250 diluted in DNase/RNase water) (Sigma-Aldrich), were included on each plate in triplicate to generate a standard curve. The synthetic 12 S sequence contained an eight base pair reverse complement to enable identifying potential contamination of qPCR samples via Sangar sequencing. Six no template control (NTC) PCR replicates were included on each plate.

There was no expectation of PCR inhibition in the aquaria experiments, but this was deemed possible for the mesocosm experiment due to the presence of various aquatic biota. We subsequently tested for inhibition in a subset of samples from aquaria experiment 3 (a tank with four fish and a tank with 14 fish) and a sample from each pond using an endogenous internal positive control that amplifies a short fragment of

the 16 S rRNA in various fish species (Furlan and Gleeson, 2016). Each sample was screened for inhibition using neat eDNA and 1:10 diluted eDNA. In the absence of inhibitors, we expect the 1:10 dilution to have delayed amplification of 3.3 cycle threshold (Ct) values. Six replicate 20 μL PCR reactions per dilution (neat and 1:10) were prepared as described above with minor modifications including the addition of 0.75 μL of the endogenous control assay (20X; Applied Biosystems) and reducing the DNase/RNase free water to 6.25 μL. The PCR reactions were run as described above.

2.5. Statistical analyses

Variability among eDNA concentrations (ug L^{-1}; response variable) in all experiments, and for selected data sets pooled across aquaria experiments, was first analysed using mixed effects models. For all models, the underlying distributions of eDNA concentrations were assessed and functions applied where required, which dictated linear (e.g., Gaussian) or generalised (e.g., Poisson or gamma) linear approaches. Where positive continuous data with skewed distributions were identified, a gamma distribution with a log-link function was applied. Where non-linear relationships were detected between the *a priori* fixed effects and eDNA concentrations, additional non-linear terms (e.g., cubic and quadratic) were assessed.

Except for aquaria experiment 1, random effects for all experiment-specific models included the replicate water 'samples' that were processed for eDNA (aquaria experiments 2, 3 and 4 and mesocosm experiment) and 'tanks' (aquaria experiments 2 and 4). Fixed effects varied according to the hypothesis being assessed, but included the 'weight' of the fish in tanks or mesocosms (i.e., aquaria experiments 1 and 3 and the mesocosm experiment), 'water sampling time' for eDNA (aquaria experiment 1), 'water temperature' (experiment 2) and 'tanks' holding a single fish (experiment 4). Fish weight was also included as a covariate in aquaria experiment 4. Because the water volume in all aquaria remained consistent (32-L), fish weights were analysed without scaling, but for the mesocosm experiment (which had different volumes) weights were standardised to kg ML^{-1}.

To investigate a general effect of fish weight in tanks on eDNA concentrations, data collected across the four aquaria experiments at the same water-sampling time (nine days; see Section 3.3) were combined and assessed in a single model. In this analysis, the response variable was the mean eDNA concentration in each tank, with fish weight fixed. Significance for all categorical fixed effects in all experiments was determined using a Wald *F* or likelihood ratio tests ($p \leq 0.05$ was considered significant for all statistical tests). All models were run using the glm, glmmTMB, or AsREML functions in R (v. 4.1.3) (Butler et al., 2017; Team, 2021).

3. Results

3.1. Fish husbandry and water quality

The fish used in aquaria experiments 1–4 were all similar in size with mean (± SDs) TLs of 115.2 (4.7), 121.7 (2.3), 114.8 (7.9), and 137.6 (3.3) cm and weights of 18.4 (2.1), 20.4 (2.3), 17.9 (3.2) and 29.6 (1.1) g, respectively. Among 220 fish used in the aquaria experiments, six died and were immediately replaced. Specifically, one fish died during experiment 1 in a high-density treatment tank on day six, one fish died during experiment 2 in a cold-treatment tank on day four, and four fish died during experiment 3 (one in the 18-fish treatment on day four, one in the 16-fish treatment on day six, one in the 12-fish treatment on day eight, and one in the 14-fish treatment on day nine). There were no deaths in any of the seven mesocosms during two months before sampling. Replicate water quality readings in the seven mesocosms after eDNA sampling revealed considerable variability in temperatures (15.81–17.47 °C), pH (7.94–9.01), turbidity (2.3–23.8 NTU), conductivity (0.162–0.264 mS cm^{-1}) and dissolved oxygen (9.72–12.06 mg L^{-1}). However, all parameters were within defined departmental standards for optimal fish welfare.

3.2. Amplification efficiency and qPCR inhibition

The amplification efficiencies from all plates were 94.0–100.7%, with R^2 values of 0.99–1.00. While there was minor qPCR inhibition in the two aquaria samples from experiment 3 (Ct shift of 3.07 and 2.97 for the tanks with four and 14 fish, respectively) and four of the seven mesocosms (Ct shift ranging from 2.25 to 3.16 for inhibited samples; Table S2), this neither biased the study outcomes nor interpretation of results. Specifically, the most inhibited mesocosm (no. 6) had the highest eDNA concentration and three mesocosms with no inhibition had very similar eDNA concentrations despite containing biomasses from 20.7 to 47.3 kg ML^{-1} (Tables S1 and S2).

3.3. Aquaria and mesocosm experiments

The target volume of water was successfully filtered in all experimental tanks (aquaria), but only 0.46–1.99-L could be filtered in each mesocosm due to high turbidity and/or algal loads. No Murray cod DNA was detected in any water samples taken from the control experimental tanks at the starts and ends of the aquaria experiments. In addition, no Murray cod DNA was detected in any treatment experimental tanks prior to adding fish. Murray cod DNA was subsequently detected in all treatment experimental tanks (6.06×10^{-5} to 1.33×10^{-1} ng L^{-1}) and the seven mesocosms (2.99×10^{-7} to 2.21×10^{-5} ng L^{-1}).

In aquaria experiment 1, eDNA concentrations were not significantly different between the two stocking rates (generalised linear model, GLM, $p > 0.05$), although, sampling time was a significant predictor of eDNA variability with support for both quadratic and cubic terms (GLM, $p < 0.01$; Tables 1; S3). The concentrations of eDNA increased during the first three days of the experiment, then decreased to their lowest level at day nine, which supported eDNA stabilisation, and thus we sampled on day nine for the remaining aquaria experiments (Fig. 3). There was no significant interaction between the weight of the fish and sampling time in aquaria experiment 1 (GLM, $p > 0.05$; Table 1). Similarly, eDNA concentrations were neither significantly influenced by

Table 1
Summary of experiments and the significance of relevant effects in mixed effects models explaining variability in Murray cod, *Maccullochella peelii* eDNA concentrations (ng L^{-1}) in water samples from up to twelve 35-L tanks holding juveniles (98–142 mm TL and 12.4–30.9 g) during four experiments in indoor aquaria and seven mesocosms holding adults (1.7–14.9 kg) in one outdoor experiment. Except for aquaria experiment 1, random effects for all experiment-specific models included the replicate water 'samples' (aquaria experiments 2, 3 and 4 and mesocosm experiment) and 'tanks' (aquaria experiments 2 and 4).

Experiment	Weight (wt) of fish	Water sampling (ws) time	Wt × Ws	Water temperature	Tanks (individual fish)
Aquaria no. 1 (temporal sampling at two stocking wts)	–	**	–	Na	Na
Aquaria no. 2 (water temperature)	Na	Na	Na	–	Na
Aquaria no. 3 (wt of fish)	–	Na	Na	Na	Na
Aquaria no. 4 (individual shedding)	Na	Na	Na	Na	***
All aquaria (wt of fish)	***	Na	Na	Na	Na
Mesocosm (wt of fish)	–	Na	Na	Na	Na

Na, not applicable for model; –$p > 0.05$; **$p < 0.01$; ***$p < 0.001$.

temperature (experiment 2; Fig. S1), nor was a relationship detected between eDNA concentrations and fish weight (experiment 3) (generalised and linear mixed models, GLMM and LMM, $p > 0.05$; Table 1).

In aquaria experiment 4, eDNA concentrations were significantly different among the 10 experimental tanks containing similar-sized (132.0–142.0 mm TL and 27.0 −30.9 g; LMM, $p < 0.001$) individuals and were independent of fish weight (Table 1; Fig. 4). For example, individual D shed ten times more DNA than individual A, despite a minimal weight difference (30.9 and 28.0 g, respectively) (Fig. 4). Similar to aquaria experiment 3, the variability of standardised eDNA concentrations in mesocosms was not significantly related to fish weight (GLMM; $p > 0.05$; Tables 1; S3; Fig. S1).

In contrast to the result for experiment 3, a GLM applied to the average overall eDNA concentration tank^{-1} across all aquaria experiments detected a significant quadratic relationship between fish weight and eDNA concentration ($p < 0.001$; Table 1; Fig. 5). Specifically, eDNA concentrations increased in tanks containing up to 180 g of fish and then decreased again in tanks containing > 200 g of fish.

4. Discussion

This study contributes to the growing body of international literature assessing the utility of eDNA to estimate the abundance or biomass of teleosts, and represents one of the few Australian assessments (Hinlo et al., 2018). However, contrary to the general consensus supporting sustained positive correlations between eDNA concentrations and abundance or biomass among many fish species (Rourke et al., 2022), we did not detect a linear relationship. Rather, while we identified a significant relationship between eDNA concentration and fish weight, this has no applied utility simply because eDNA concentrations tank^{-1} increased with fish weight to ~180 g, but then subsequently declined as weights increased to a maximum of 311 g. Thus, our data imply Murray cod is not a suitable candidate for using eDNA to quantify their abundance under controlled, laboratory conditions and so, following the logic in previous studies (e.g., Danziger et al., 2022), we propose the data preclude using eDNA to assess the biomass of wild populations. Nevertheless, by considering key influencing abiotic and biotic factors previously identified to affect other positive-candidate species under controlled conditions, we retrospectively outline a coherent approach for designing future assessments. The lack of a useful relationship between eDNA and the abundance of Murray cod, but the utility of this information for future studies are discussed separately below, although first some comments on our experimental designs are warranted.

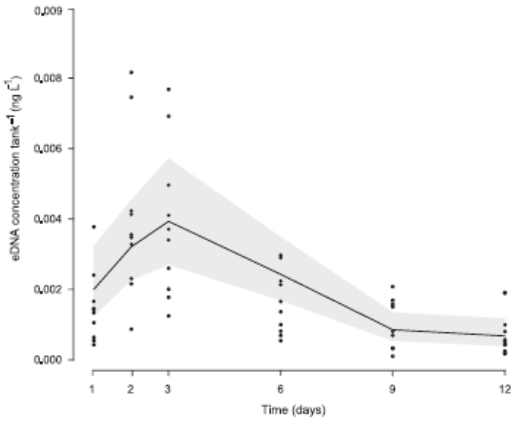

Fig. 3. Relationship between eDNA concentrations and time of sampling assessed during aquaria experiment 1. Data points indicate raw data, the solid line indicates the generalised linear model fit, and the shaded region indicates the 95% confidence intervals.

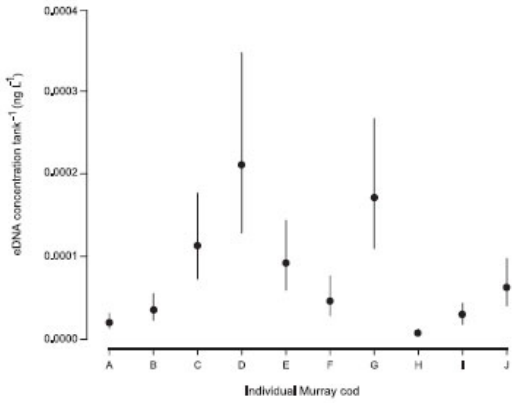

Fig. 4. Mean (with 95% CI) eDNA concentrations sampled from 10 experimental tanks containing individual Murray cod, *Maccullochella peelii* (between 27.0 and 31.9 g).

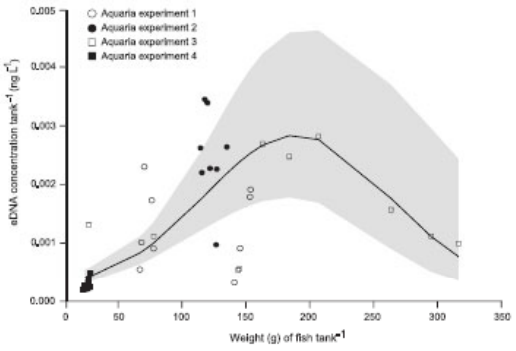

Fig. 5. Relationship between mean eDNA concentrations and the weights of Murray cod, *Maccullochella peelii* using data combined across aquaria experiments 1 (days nine and 12 samples only), 2, 3 and 4.

During the aquaria experiments, the various hypotheses were tested under controlled conditions designed to reduce known confounding biotic and abiotic factors affecting eDNA concentrations, including feeding, water flow and temperature (Jane et al., 2015; Klymus et al., 2015; Lacoursière-Roussel et al., 2016). Nevertheless, a closed system may have stressed some fish and created an imbalance between eDNA accumulation and decay—although aquaria experiment 1 demonstrated eDNA concentrations plateaued around days 9–12, suggesting a balance between eDNA accumulation and decay. There were some mortalities, but these were comparable to natural rates (as observed in the holding tanks), and not attributed to poor conditions. Moreover, previous studies using similar closed systems have detected positive relationships between eDNA concentration and abundance for other teleosts (Doi et al., 2015; Karlsson et al., 2022; Takahara et al., 2012).

For the mesocosm experiment, it is unlikely the established, adult Murray cod were stressed, although beyond the mild qPCR inhibition identified (and deemed not to impact results, discussed below) possible experimental limitations included insufficient replication to account for any influences of natural variability across mesocosms (such as water chemistry, other biota present or poor mixing of water resulting in heterogeneous DNA distribution), which might affect eDNA concentrations (Eichmiller et al., 2014). Nevertheless, such variability would also be present in the field (see Capo et al., 2019). Also, we only sampled water at the surface (precluding any effects of depth), although samples were taken along transects around the perimeter, which should have

encompassed sufficient system variability (Takahara et al., 2012). Vertical mixing and homogeneity of eDNA was also likely assisted via aeration paddle wheels. While we cannot exclude the possibility of external eDNA contamination from the adjacent Murrumbidgee River, previous work has shown fish eDNA in mesocosms degrades within approximately two weeks, and well before the eight-week settlement period we employed here (Barnes et al., 2014; Dejean et al., 2012).

Beyond any fish-husbandry practices affecting empirical conclusions are eDNA processing issues that need to be considered. As with most previous studies, we used water filtration rather than a centrifugation or precipitation methods to capture eDNA (Rourke et al., 2022). The polyethersulfone filter used has been demonstrated to not only preserve eDNA effectively, but yield more than filters preserved in ethanol (Thomas et al., 2019). One concern with qPCR processing is the presence of inhibitors in the sample that persist through DNA extraction. Inhibition was tested in a subset of high and low biomass tanks and although present, it was minor and similar in the low and high biomass tanks. We conclude it was unlikely to influence the results of the aquaria experiments because the water was derived from the same source for all tanks (i.e., UV irradiated bore water). We also detected low or no inhibition in the mesocosm samples, suggesting that inhibition was not responsible for the inconsistent results.

Considering the above, we maintain there is convincing evidence for mostly null effects and a lack of a linear relationship between eDNA concentrations and the weight of Murray cod—a result that remains fairly unique among similar studies of teleosts in controlled environments (Doi et al., 2015; Horiuchi et al., 2019; Klymus et al., 2015; Mizumoto et al., 2018; Takahara et al., 2012). Rather, the few previous studies reporting the lack of coherent positive relationships were conducted under much more variable field conditions in lakes (Capo et al., 2019; Perez et al., 2017), rivers (Deutschmann et al., 2019; Hinlo et al., 2018) and oceans (Fraija-Fernandez et al., 2020; Knudsen et al., 2019). And none of these studies produced a similar quadratic relationship to that identified here, which might warrant further exploration in aquaria studies to include more treatments at low, mid and high densities/-weights, although this would not alter the lack of utility.

Any explanation why Murray cod is not a suitable candidate for quantitative eDNA assessments remains speculative. Possibly, their aggressive and territorial behaviour evokes variable activity among individuals, potentially due to behavioural profiles (Rogers et al., 2020), which may subsequently impact DNA shedding (as demonstrated in aquaria experiment 4). Juvenile fish in hatcheries display aggressive and cannibalistic behaviour, particularly when there is size heterogeneity and at lower fish densities that allow individuals to establish territories (Ingram et al., 2005; Rogers et al., 2020; Ryan et al., 2007). Consequently, it is generally recognised that confined Murray cod should be housed at high densities across similar sizes to avoid excessive aggression (Ingram et al., 2005).

Potential density-specific aggressiveness may have caused variable DNA shedding rates in the aquaria tanks containing more than one fish and might explain why the combined data across those experiments supported a significant quadratic effect of fish weight on eDNA concentration. This model implies a positive relationship between eDNA concentrations and weight to a certain threshold (180 g $tank^{-1}$), after which eDNA concentrations decreased with increasing fish weights. At lower weights (low-density tanks), Murray cod are likely to be more stressed given some will have formed territories and the less aggressive fish may be actively avoiding the aggressive fish. At greater weights (higher stocking densities), fish may have been less stressed and active, avoiding each other to minimise interactions, and consequently shedding less eDNA. Murray cod are known to be less aggressive at higher densities due to their reduced ability to establish a territory, which could explain a concomitant reduced shedding of DNA.

Another factor that can influence stress levels among fish and increase density-dependant aggressive behaviour is starvation (Ingram et al., 2005). We chose not to feed experimental fish because this can potentially affect DNA shedding rates (Ghosal et al., 2018; Klymus et al., 2015), and confound dependant relationships with subsequently measured eDNA concentrations. Juvenile Murray cod > 100 mm TL (i.e. the size of fish used in our aquaria) are unlikely to die after several weeks without food, but any associated stress may have been exacerbated in the lower-density treatments given these fish were more likely to have established territories versus in the greater-density treatments (Ingram et al., 2005). Notwithstanding the possibility for such effects, in the absence of further data, hypotheses concerning intra-specific interactions remain speculative, considering other similarly aggressive species have demonstrated strong positive relationships under comparable experimental conditions (e.g. northern pike, *Esox lucius*; Karlsson et al., 2022).

Regardless of the specific mechanisms contributing towards the lack of a useful relationship between eDNA concentrations and weight for Murray cod, these data can be used to design a coherent strategy for assessing future candidates. Based on published work, there is no consensus on whether aquaria experiments are required before field experiments, and if so, what order they should be conducted in (e.g. individual shedding rate experiments prior to experiments with different biomasses; Rourke et al., 2022). The results here support that for non-schooling species, it would be appropriate first to examine individual variability in eDNA concentrations within controlled aquaria conditions and if this is excessive and/or not related to weight, then additional experiments might not be warranted if the goal is to inform future stock assessments in the wild (Danziger et al., 2022). Certainly, for solitary or territorial species like Murray Cod, excessive individual variability in eDNA concentrations will almost certainly decouple a relationship between eDNA and biomass. However, such logic might require additional testing among schooling species and especially those that form large aggregations. For these species, the effect of individual variability may be overwhelmed by the amount of eDNA produced by the entire aggregation. And larger schools may still produce measurably more eDNA than smaller schools, regardless of individual variability.

Embedded in this approach, it is also imperative that all other factors are controlled or accounted for, including sufficient time for eDNA to stabilise in tanks (i.e., where the rate of shedding approaches that of decay). Here we observed an initial increase, followed by a steady decrease in eDNA concentrations through time consistent with some other studies (Maruyama et al., 2014; Takahara et al., 2012)—but not all (Nevers et al., 2018). Other known abiotic factors warrant absolute standardisation, including tank water volumes, pH, turbidity and temperature. Although not observed for Murray cod, temperature (along with other parameters) could potentially confound relationships between eDNA and fish abundance.

In summary, the results from this study imply that eDNA is not useful for providing weight estimates of Murray cod in the wild, beyond detecting their presence in water samples collected at specific locations. Consequently, electrofishing and recreational catch and effort data should remain the preferred assessment methods. Additional work is required to investigate other regional species that might be more amenable to such quantitative eDNA assays. Based on the results here, we suggest any such work should first quantify the dependence of individual variability in eDNA concentrations on fish weight using closed-system aquaria experiments under controlled conditions, prior to extending these assays to the wild.

CRediT authorship contribution statement

Meaghan L. Rourke: Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. **Jackson Wilkes Walburn:** Data curation, Investigation, Methodology, Visualization, Validation, Writing – original draft. **Matt K. Broadhurst:** Conceptualization, Formal analysis, Funding acquisition, Methodology, Resources, Software, Visualization,

Writing – original draft, Writing – review & editing. **Ashley M. Fowler:** Conceptualization, Formal analysis, Funding acquisition, Methodology, Resources, Software, Visualization, Writing – original draft, Writing – review & editing. **Julian M. Hughes:** Conceptualization, Formal analysis, Funding acquisition, Methodology, Resources, Software, Visualization, Writing – original draft, Writing – review & editing. **D. Stewart Fielder:** Funding acquisition, Methodology, Resources, Writing – review & editing. **Joseph D. DiBattista:** Funding acquisition, Methodology, Resources, Writing – original draft, Writing – review & editing. **Elise M. Furlan:** Funding acquisition, Methodology, Resources, Formal analysis, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.fishres.2022.106545.

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Investigating the Potential Utility of Environmental DNA to Provide a Relative Abundance Index for the Depleted Teleost, Mulloway, *Argyrosomus japonicus*

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Abstract: Non-invasive, low-cost methods for censusing depleted fish populations are being prioritised among many jurisdictions worldwide. Collecting environmental DNA (eDNA) could offer one such option for augmenting fish population assessments. However, candidate species need to be carefully selected because species-specific DNA shedding and decay rates are affected by many biotic and abiotic factors that may influence relative abundance estimates. In this study, we sought to ascertain if the eDNA of a depleted Australian teleost, mulloway, *Argyrosomus japonicus*, reflects its weight under controlled aquaria conditions. With four experiments, we investigated the relationships between mulloway eDNA concentrations and their weight tank^{-1} as a function of: (1) time post-tank establishment; (2) water temperatures (within the species' tolerance range); (3) stocking densities; and (4) among individual, similar-sized fish. The concentrations of eDNA in tanks stabilised after six days, and a positive relationship was found between fish weight and eDNA concentration, despite some variability in shedding rates by similar-sized fish. There was also a positive effect of water temperature on eDNA concentrations, which reinforces the need to control for such abiotic factors. We conclude that there is strong utility in applying eDNA concentrations as an index of relative abundance for mulloway under controlled conditions, which justifies future field-based investigations.

Keywords: fishery-independent sampling; population estimates; qPCR; Sciaenidae; stock assessment; teleost

1. Introduction

Globally, anthropogenic impacts, including habitat destruction, overexploitation, pollution, and introduced alien species, have resulted in many species becoming depleted and fragmented, which represent substantial risks to biodiversity. Of concern are losses to genetic variation and inbreeding, frequently manifesting themselves in populations that have substantially declined [1]. These genetic losses increase the risk of population collapse

and must be carefully managed through reintroducing population connectivity, managing harvests (where appropriate), preserving genetically distinct stocks, or translocating individuals from other populations to improve genetic diversity and reduce inbreeding [2,3].

While the need for effective wildlife management is clear, it requires adequate information on species' ecologies, including population sizes [4]. For harvested aquatic species such as marine teleosts that are not easily censused, it is crucial to have accurate assessments of population sizes to enable appropriate harvest levels to be determined. However, traditional methods for estimating teleost populations remain expensive and are often fishery-dependent (i.e., data are collected during fishing operations), which may create management problems where population issues are identified too late or not at all [5,6]. Relying solely on fishery-dependent data for population assessments is particularly concerning for depleted populations. In some cases, fisheries are eventually limited or closed to fishing, precluding the continued provision of time-series data. Alternative data sources and population assessment methods should therefore be considered in cases where there is the potential for loss of fishery-dependent data series.

An alternative approach that may support traditional population assessments is by sampling environmental DNA (eDNA), which comprises the traces of DNA left behind by an organism in its environment [7]. Beyond simply confirming the presence or absence of a particular species, in some cases, eDNA can also facilitate estimating relative population sizes under controlled and natural conditions [8,9]. However, the utility of eDNA to provide relative population sizes is influenced by several factors including: rates of species-specific DNA shedding and decay; body size, distribution, reproduction, and migrations; hydrological variables affecting DNA dispersal and persistence (especially water temperature); and methodologies for processing samples [8–11]. Therefore, the utility of eDNA for monitoring the abundance or biomass of a species must be carefully verified prior to its application. More specifically, it is evident from the increasing literature focused on eDNA that to fully assess its potential application among individual species, it is important to assess the impacts of various factors such as water temperature under controlled conditions prior to applications under natural conditions [8–11]. Further, such work should be prioritised among those species not only considered of most concern but also inhabiting environments appropriate for sampling and validating eDNA, which often include closed or estuarine systems (e.g., easily accessed on a regular basis and often with known species assemblages) [8].

In eastern Australia, one inshore/estuarine teleost for which there are considerable concerns over long-term sustainability is mulloway, *Argyrosomus japonicus*: an apex euryhaline predator with a key ecosystem role and of considerable economic value [12,13]. Due to historically high fishing mortality, coupled with possible environmental influences (e.g., periods of prolonged drought [14], the regional populations of mulloway in New South Wales (NSW) have been assessed as 'overfished' or 'depleted' (<20% of virgin biomass) for almost 20 years [14–16]. The stock decline has warranted rigorous and representative monitoring and assessment, but approaches currently rely on fishery-dependent data sources.

Mulloway are caught by recreational and commercial fishing sectors, although the primary source of monitoring data comes from the latter. Commercial fishing is increasingly limited owing to management changes, including closures within key estuaries, and more recently, reductions in efforts [15]. Assessing population abundance across the species' range remains a challenge if limited to these traditional fishery-dependent approaches, particularly if further restrictions to fishery operations are implemented in response to continued low biomass. There is therefore a clear requirement to develop fishery-independent population-monitoring approaches that simultaneously encompass sufficient spatial coverage and are cost-effective. Using eDNA as a potential index of relative abundance may be an effective option, but as for all candidate species, there is a need to investigate suitability under controlled conditions as a precursor to rigorous field applications. Here, we sought to address this shortfall by using a series of controlled experiments to quantify the relation-

ships between mulloway eDNA concentrations and their weights across the range of their typical thermal tolerances and schooling densities.

2. Materials and Methods

This study was conducted at the Port Stephens Fisheries Institute (PSFI) aquaria (32.74° S, 152.05° E) in NSW, Australia, from May to August 2021. Four controlled experiments were conducted to investigate the relationships between the mulloway eDNA concentrations and their weight tank^{-1} as a function of: (1) sampling times (i.e., to capture any temporal changes in eDNA concentration); (2) water temperatures; (3) stocking densities; and (4) variability in eDNA shedding among individual fish.

2.1. Aquaria Configuration and Fish Collection

Two enclosed rooms (12:12 h photoperiod) within the PSFI aquaria were configured to hold 18 × 150-L tanks (in groups of four for experiments 1–3) and 10 × 16-L tanks (experiment 4). All 'experimental' tanks were made from polyvinyl chloride and enclosed with lids. Prior to starting the experiments, each tank was sterilised for 24 h with a 2% chlorine (146 g L^{-1}) solution to remove any residual mulloway DNA. All the tanks were supplied with flow-through water at ambient salinity (sourced from Tilligerry Creek, an adjacent tributary) and aerated using ceramic diffusers (at approximately 10 L air min^{-1}) that had been previously sterilised. The 150-L tanks were supplied with ultraviolet (UV)-treated water, pre-heated or cooled to the required temperatures for relevant experiments (1–3) at 2.0 L min^{-1} (±10%), while the 16-L tanks were held in a temperature-controlled room and supplied with water at a rate of 0.1 L min^{-1}. During each experiment, individual tank temperatures were constantly monitored with sterilised probes, and water flows were checked daily and adjusted if beyond a ± 10% range.

Juvenile mulloway were used in the experiments because it was impractical to house adults given they grow to >1 m total length (TL) and >30 kg [16]. The mulloway used in the experiments were hatched at the PSFI aquaria on 5 February 2021 and reared following the methods described by Fielder and Heasman [17]. After 33 days, ~1500 fish (all < ~10 mm TL) were transferred to a 5000-L flow-through (~50% volume replacement hour^{-1}) holding tank, which was situated indoors, supplied with ambient, filtered seawater, and fed daily with a commercial diet at 5% biomass (Nutragard Start Sink, Ridley, Australia). At the start of each experiment, all the fish in the 5000-l holding tank were anesthetised (20 mg L^{-1} AQUI-S®, Fresh by Design, Moss Vale, NSW, Australia), and the required numbers were randomly selected (see below) and weighed (nearest 1.0 g) prior to release into the experimental tanks (Figure 1). Fish were not measured to minimise handling stress. All the experimental fish were fed daily at a reduced rate (1% of their tank biomass) given the lower flow rate in the experimental tanks compared with the holding tank to maintain fish health but not impact water quality. Any uneaten food or waste were siphoned (using sanitised siphons) prior to the following feed. All tanks were monitored daily for mortalities. Prior to most experiments, fish were acclimated for three days to the required experimental temperature (18.0 ± 1.0 °C) in the holding tanks.

To confirm whether experimental tanks were free of mulloway DNA prior to adding the fish, we filtered a 1.0-L water sample from each tank using a Smith Root eDNA sampler™ (EnviroDNA, Brunswick, Victoria, Australia) and a 5-µm polyethersulfone (PES) filter, enclosed in a sterile self-preserving filter housing (Smith–Root, EnviroDNA, Brunswick, Victoria, Australia) at 1.0 L min^{-1} and a maximum pressure of 69 kPa and stored at 4 °C. The 5 µm filter size was selected for consistency with planned field trials that minimise clogging due to the high turbidity typical of many Australian estuaries. All water sampling for the four experiments was conducted as described above. The filters were tested for the presence of mulloway DNA, as described in Section 2.3. An equipment control (EC) consisting of 1.0 L of sterile water was filtered prior to the sampling of tanks at every time point.

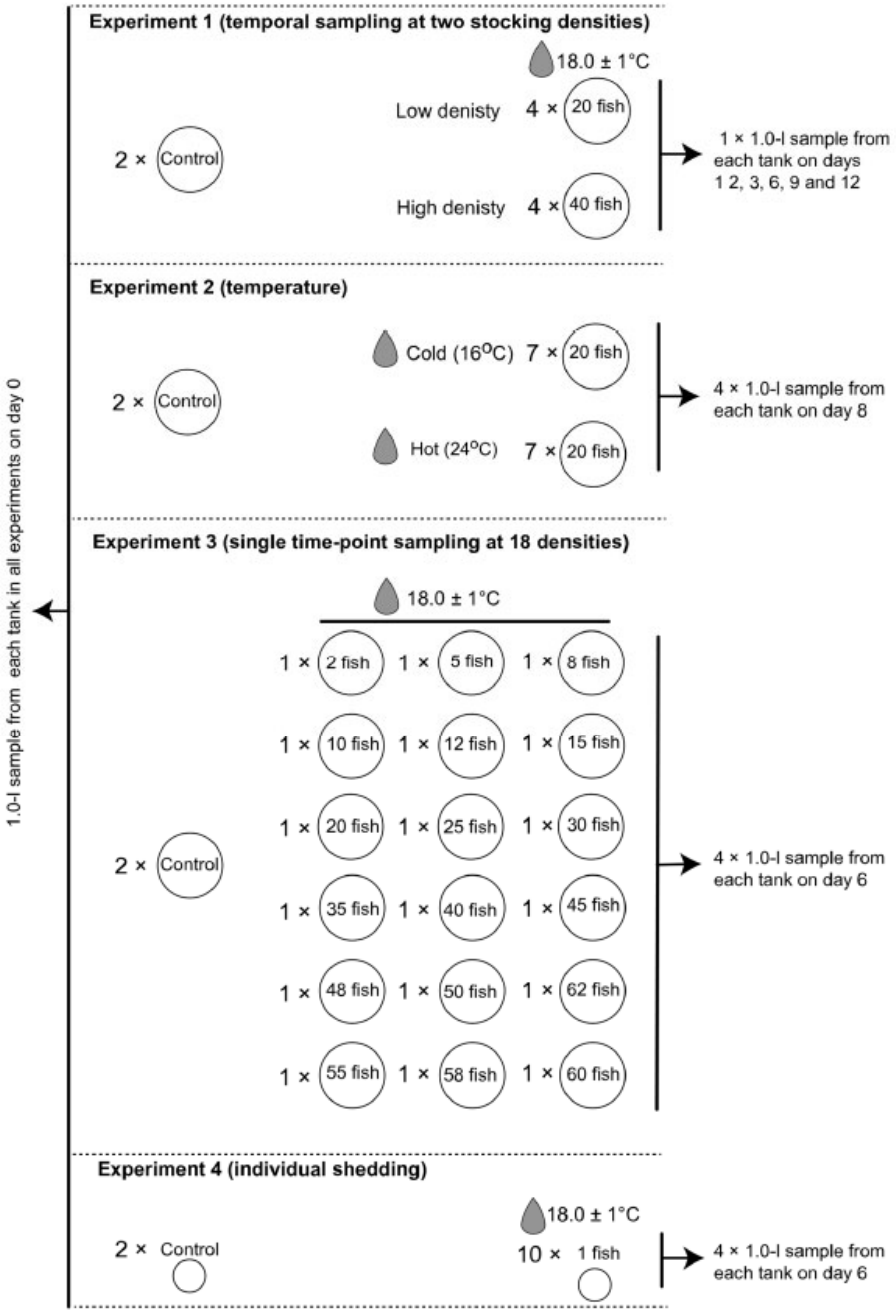

Figure 1. Schematic representation of the four experiments completed at the Port Stephens Fisheries Institute New South Wales, Australia during May to August 2021 to quantify relationships between mulloway, *Argyrosomus japonicus* eDNA concentrations and their weights across the range of their typical thermal tolerances and schooling densities. The replicate experimental tanks used for controls (left) and treatments (right) with stocking densities of mulloway and temporal water sampling for eDNA indicated.

2.2. Experimental Procedure

Common among all the experiments, two tanks were always designated controls (containing no fish) and were sampled to test for mulloway DNA on day zero and at the conclusion of each experiment. For all tanks, the water temperature was maintained at 18.0 ± 1.0 °C, except those used in experiment 2. For experiment 1, we collected one 1-L sample tank^{-1}, while for experiments 2 to 4, we collected four 1-L samples tank^{-1} to account for the unequal dispersion of eDNA in the water [18]. Sampling for experiments was conducted after six (experiments 3 and 4) or eight days (experiment 2, due to a 48 h acclimation period within the experimental tank; see below) (Figure 1; see Results).

In experiment 1, we investigated the temporal changes in eDNA concentrations by sampling from each tank on days 1, 2, 3, 6, 9, and 12 (post-stocking) across two stocking densities (20 vs. 40 fish in each of the four tanks) (Figure 1). In experiment 2, we investigated the effects of temperature on the relationship between eDNA concentration and weight by holding mulloway (20 fish tank^{-1}) at either low (16.0 ± 1.0 °C) or high (24.0 ± 1.0 °C) temperatures, each replicated across seven tanks (Figure 1). Fish were stocked into the experiment at 19.0 ± 1.0 °C and then transitioned to the two treatment temperatures over 48 h. During experiment 3, we investigated the relationship between weight and eDNA concentrations by holding mulloway in tanks at 18 densities ranging from two to 60 fish (with 1 replicate tank density^{-1}) (Figure 1). In experiment 4, we investigated individual variability in eDNA shedding rates by holding 10 mulloway of similar weights (30.5–32.0 g) in individual tanks for six days, after which the tanks were sampled (Figure 1).

2.3. Filters, DNA Extraction, and Quantitative PCR

All the laboratory processing of filters to isolate and amplify DNA took place in a purpose-built eDNA facility at the Narrandera Fisheries Centre (NFC), Australia. This facility includes three separate UV-sterilised rooms each with a PCR hood (AirClean Systems, Ferntree Gully, VIC, Australia) for DNA extraction, PCR preparation, and DNA template loading. The DNA was extracted from the filter papers within four weeks of collection using a PureLink™ Genomic DNA Mini Kit (Invitrogen, Waltham, MA, USA) with a modified protocol [19].

A 166-bp fragment of the mitochondrial 12S rRNA gene was amplified using quantitative PCR (qPCR) with a custom Applied Biosystems TaqMan™ assay (Thermo Fisher Scientific, Scoresby, VIC, Australia)). Mitochondrial 12S rRNA gene fragments were amplified using the primers A.jap_12S_F (5′-CTCACCCTTCTTTGTTTCCC-3′); A.jap_12S_R (5′-CATCATTCGTTTTCTCTGTGTC-3′) and probe (FAM-dye-labelled); and A.jap_12S_probe (5′-CCGTCGTCAGCTTACCCTGTG-3′) [19]. All samples, including the EC and negative extraction control, were analysed using six replicate 20 µL qPCR reactions, comprising 10 µL Environmental Master Mix 2.0, 1 µL TaqMan™ Gene Expression assay 20×, 7 µL DNase/RNase free water, and 2 µL of DNA template. In addition, six no-template control (NTC) PCR replicates were included on each plate to identify the potential contaminants introduced in the molecular workflow. Standards, with six concentrations ranging between 10^{-3} and 10^{-8} ng µL^{-1}, comprising 166 bp of mulloway 12S rRNA synthetic oligonucleotides diluted in tRNA buffer (1:250 diluted in DNase/RNase water; Sigma-Aldrich, Burlington, MA, USA), were included on each plate in triplicate to generate a standard curve for quantifying eDNA concentrations. To facilitate detecting the potential contamination of EC or NTC samples with the synthetic oligonucleotide, we included an eight-base pair reverse complement within the synthetic sequence. The reactions were run on a QuantStudio3 PCR System (Applied Biosystems, Waltham, MA, USA) with thermal cycling conditions set at 50 °C (2 min) and 95 °C (10 min), followed by 50 cycles of 95 °C (15 s) and 56 °C (60 s).

2.4. Statistical Analyses

Variability in eDNA concentrations (ng L^{-1}; response variable) among the replicate tanks in all the experiments was investigated using fixed- or mixed-effect models. For

all models, the underlying distributions of eDNA concentration were first assessed, and functions were applied where required, dictating linear (Gaussian) or generalised linear (gamma) approaches. Except for experiment 1, the blocking effects of 'tanks' were always included as a random factor (i.e., mixed-effect models). Fixed effects varied according to the hypothesis being tested and included the stocking 'density' (two levels) and sampling 'time' (and their interaction) in experiment 1, water 'temperature' (two levels) in experiment 2, and 'weight' of mulloway tank^{-1} for experiments 3 and 4. The significance for all categorical fixed effects in all the experiments was determined using likelihood ratio tests. All models were run using the *glm* or *glmmTMB* functions in R (R Development Core Team 2020).

3. Results

3.1. Experimental Fish

There was minimal intra-experimental variability in the sizes of mulloway selected from the 1500 fish housed in the 5000-L holding tank, but owing to the temporal staggering of the trials, the mean (±SE) fish weights were lower in experiment 1 (9.9 ± 2.4 g) than in experiments 2 (32.2 ± 9.8 g), 3 (33.5 ± 12.6 g), and 4 (31.3 ± 0.8 g). There were no fish mortalities in the 5000-L holding tank or experimental tanks during the study period. The fish used in experiments 1–3 were not visibly disturbed during the water sampling process. In contrast, the mulloway held in individual tanks for experiment 4 were observed to swim rapidly and frequently contacted the tank walls during water sampling.

3.2. Quality Control

One litre of water was successfully filtered across all replicates and in all experiments, indicating no issues related to water turbidity or clogging of the filters. Mulloway eDNA concentrations ranged between 1.94×10^{-6} and 4.16×10^{-4} ng L^{-1} across all the treatments in the four experiments. No mulloway DNA was detected in any of the equipment controls, NTC controls, or the control tanks at the beginnings and ends of experiments 1 and 3. However, for experiment 4, one positive qPCR out of six replicates was recorded in one control tank on day zero and a single positive qPCR replicate was recorded in the other control at the end of the experiment. Two of the experimental tanks also had a single positive qPCR replicate (from six replicates) on day zero. The quantity of DNA in the controls and two experimental tanks at time zero (mean ± SE $7.44 \times 10^{-11} \pm 4.25$ ng L^{-11}) was much lower than the quantity in all treatments at day six ($8.37 \times 10^{-5} \pm 2.54 \times 10^{-5}$ ng L^{-1}) and therefore was not considered to influence the interpretation of the overall results. We attribute these detections (at low relative concentrations) to either residual mulloway eDNA remaining in the tanks prior to adding fish, cross-contamination between the experimental tanks, and/or laboratory contamination.

3.3. Environmental DNA Variability within and among Experiments

In all the experiments, the key fixed factors of interest significantly explained the variability among eDNA concentrations tank^{-1} ($p < 0.05$). For experiment 1, there were significant effects of time (negative coefficient; generalised linear model (GLM); $p < 0.001$), but the interaction term was not significant ($p > 0.05$; Figure 2). The model in experiment 1 was best represented by a cubic polynomial term with the concentrations of eDNA decreasing up to the third day, remaining stable up to day six, peaking at day eight, and then declining gradually until the end of the experiment (Figure 2). Based on these results, we determined six days was the most appropriate time point to collect water samples during the remaining experiments.

In experiment 2, there was a significant main effect of temperature, with predicted eDNA concentrations tank^{-1} ~1.5 times greater at warmer ($1.01 \times 10^{-4} \pm 0.93 \times 10^{-5}$ ng L^{-1}) versus cooler temperatures ($6.86 \times 10^{-5} \pm 0.48 \times 10^{-5}$ ng L^{-1}) (generalised linear mixed model (GLMM); $p < 0.05$). For experiment 3, we detected a significant positive linear relationship between the log eDNA concentration tank^{-1} and the log weight of fish tank^{-1} (linear mixed

model (LMM); $p < 0.001$; Figure 3). In experiment 4, there were significant differences detected among eDNA concentrations in the tanks containing individual fish (LMM; $p < 0.01$), which was not explained by their minimally variable weights (30.5–31.8 g tank^{-1}; $p > 0.05$; Figure 4).

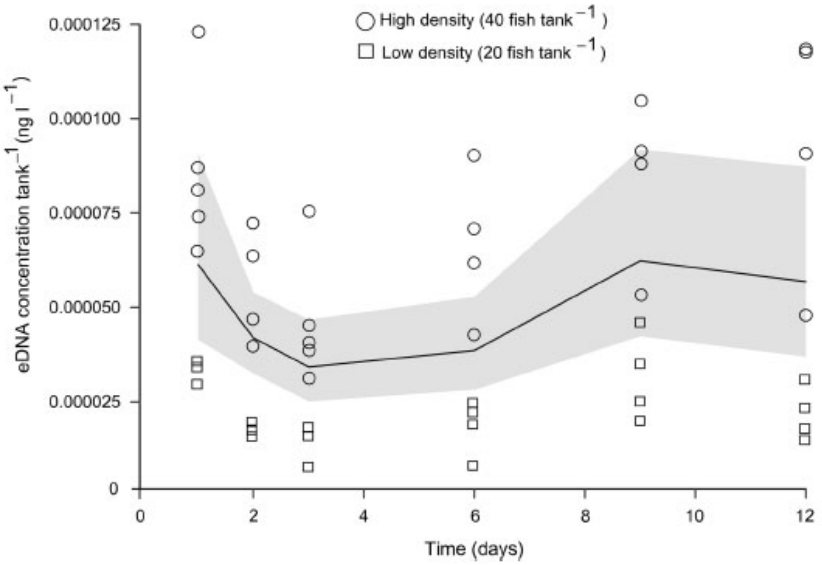

Figure 2. Relationship between eDNA concentration tank^{-1} and time of sampling assessed during experiment 1 completed at the Port Stephens Fisheries Institute, New South Wales, Australia during May, 2021. Data points reflect the raw data from high- and low-density tanks (open circles and squares), whereas solid and shaded lines indicate the generalised linear model fit and 95% confidence intervals, respectively, for the data irrespective of temperature.

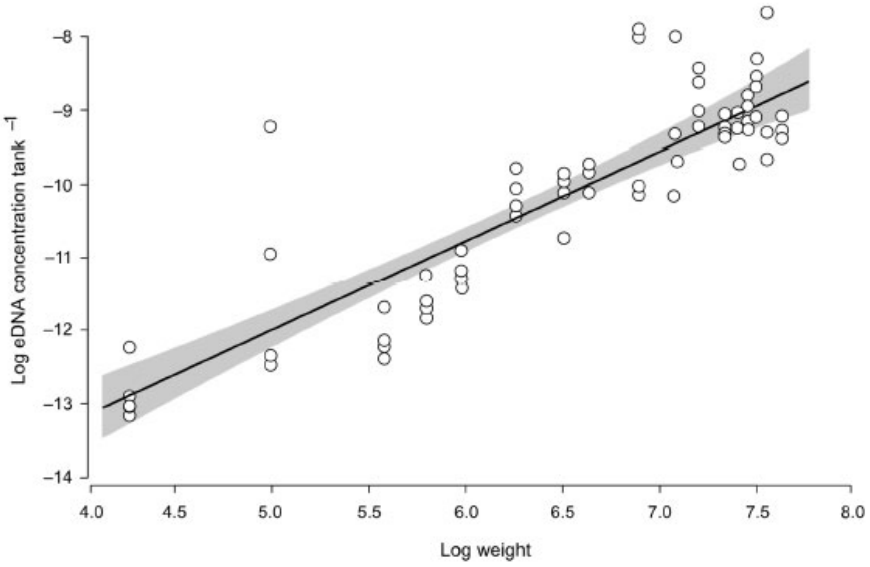

Figure 3. Relationship between log eDNA concentration tank^{-1} and log weight of mulloway, *Argyrosomus japonicus* assessed during experiment 3 completed at the Port Stephens Fisheries Institute, New South Wales, Australia during August, 2021. Open circles are the raw data, whereas the solid and dashed lines indicate the linear model fit and 95% confidence intervals, respectively.

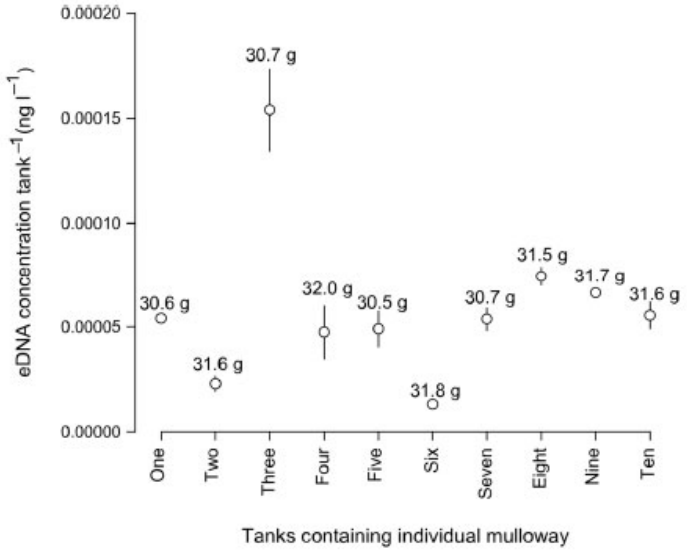

Figure 4. Mean (±SE) eDNA concentrations sampled from 10 identical 16-l tanks containing individual mulloway, *Argyrosomus japonicus* (with their weights displayed above each data point) during experiment 4 completed at the Port Stephens Fisheries Institute, New South Wales, Australia during August, 2021.

4. Discussion

The data from this study imply a strong positive relationship between juvenile mulloway eDNA concentrations and their weights under controlled conditions—an outcome that occurred despite significant variations among individual shedding rates and the influences of temperature. This trend supports the general consensus from the recent literature on the potential for eDNA concentration to serve as a proxy for biomass/abundance [8]. Collectively, the data justify pursuing further research (especially field trials) to test the potential associations between eDNA and the weight/abundance of mulloway in the wild [20]. However, there are some considerations in terms of our chosen experimental design that warrant discussion prior to future regional work with this species, and possibly other species occupying similar habitats and with similar ecologies.

A key consideration is our necessitated use of small fish and any possible confounding effects of body size on the eDNA and weight/abundance relationships. While the positive relationship for juvenile mulloway is consistent with aquaria experiments for numerous other species [8,21,22], it is possible that DNA shedding rates in adult mulloway might not scale linearly with body size. Given mulloway are segregated by size across dynamic estuarine and coastal environments, with juveniles mostly in estuaries and adults moving between estuaries and inshore marine waters, [13,23], eDNA probably will not be useful for predicting absolute biomass or abundances unless size distributions are known [24]. Future research, therefore, warrants assessing the consistency in the relationships between eDNA concentrations and the biomass/abundance of larger fish. The same argument supports re-evaluating the effects of water temperature variability and any size-specific effects. Owing to the limitations of aquaria work, this future field-based research would require concomitant sampling of wild populations (e.g., using fishing gear or hydroacoustic surveys).

A second consideration when interpreting the results presented here is that notwithstanding the significant relationship between eDNA and the abundance of juvenile mulloway overall, there was substantial variability among the tanks containing individual fish (experiment 4) that was not explained by their weight. This result implies that the amount of DNA shed by juvenile mulloway varies between individuals, supporting simi-

lar conclusions in other aquatic studies [21,25,26]. For example, Rourke et al. [26] found individual variation in the shedding rate resulted in no useful relationship between weight and eDNA concentrations for Murray cod, *Maccullochella peelii* under similar controlled conditions. Given Murray cod are typically solitary, such individual variation is likely to be maintained in wild populations where they are more sparsely distributed than schooling species [26]. For species such as mulloway that strongly shoal as juveniles but develop more solitary behaviour as adults, any differences in eDNA shedding rates may confound biomass estimates when different life history stages are present.

Such differences in shedding rates are potentially caused by variations in metabolic rates (either standard or maximum), driving the differences in activity, feeding, and growth [27]. Increased feeding results in increased gastrointestinal transit, potentially increasing DNA shedding from the intestinal wall [21,28]. Boldness may also vary considerably among individual fish, resulting in significant differences in activity levels and therefore shedding from external vs. internal tissue [29].

Alternatively, the observed agitation of solitary juveniles (which would not occur in the wild) during sampling in experiment 4 might have variably affected DNA shedding rates. Specifically, during eDNA sampling, we observed isolated fish to be more agitated, swimming rapidly, and more frequently colliding with the walls of their tanks than those housed with multiple conspecifics. Such stress responses may have been amplified because the fish were not part of an optimally sized school previously shown to reduce predator avoidance behaviour [30]. We did not observe similar agitation among juvenile mulloway held in groups of two or more fish during experiment 3. Hence, under similar conditions, isolated juvenile mulloway may shed DNA more than conspecifics in larger groups.

Irrespective of the causes of significant intra-specific variation in eDNA shedding, caution is required when designing experiments to examine the factors affecting concentrations simply because variable shedding among individuals may overwhelm biologically relevant effect sizes between treatments. It may be beneficial to use small groups of individuals in each replicate tank rather than single individuals to minimise stress and also reduce the effects of any single individual with an unusually high or low shedding rate. For example, when examining the relationship between eDNA concentration and biomass, the same levels of biomass could be established using a large number of small fish rather than a small number of large fish. This approach assumes uniform shedding rates across size classes, which also requires prior validation.

Despite the above caveats of size- and intra-specific variability, our experimental methodology was sufficient to isolate the importance of abiotic factors on eDNA concentration. Specifically, warmer water (24 vs. 16 °C) resulted in ~1.5 times more DNA in the water. Notwithstanding possible size-dependence effects (as discussed above), it is well established that eDNA concentrations can be influenced by water temperature acting via mechanisms of: (1) eDNA degradation and/or, (2) animal physiology and/or behaviour [8,31–34]. Warmer water tends to increase eDNA degradation (but see [35]), although it is rarely due to direct DNA denaturation but rather the indirect activity of hydrolysing microbes and exonucleases [36–38].

Owing to increased activity and metabolic rates in warmer water and greater DNA shedding, species-specific physiology is also likely to affect eDNA concentrations [34,39]. Therefore, warmer temperatures within a species' range could increase the risk of overestimating fish biomass in the field, so temperatures should be recorded and accounted for under natural conditions where possible [39]. This prerequisite is particularly important for juvenile mulloway, given that they inhabit temperate estuaries where the temperature fluctuates widely among seasons and with depth. Conceivably, individuals may seek ideal water temperatures (i.e., depth-dependent) to optimise their routine metabolic rate [40].

Such estuarine environments also present additional challenges for eDNA-based biomass estimates due to: (1) the potential for dilution, deposition, and transport of eDNA away from source individuals; and (2) the increased risk of PCR inhibition due to high turbidity [41–43]. This means that the estimated eDNA concentrations may not reflect the

biomass of mulloway at the sampling location. Consequently, hydrological models are needed to better understand eDNA transport in estuaries [20], which may be unique to each system. Nevertheless, the few studies assessing the tidal influences on eDNA distribution in estuarine environments have shown minimal effects, but accounting for eDNA transport can improve the correlation between concentrations and biomass [44–46]; however, see [47] for a counter-example.

In summary, this study identified a significant relationship between eDNA concentrations and the weights of juvenile mulloway under controlled conditions, which represents a positive first step towards developing a potential fishery-independent, non-lethal, and cost-effective monitoring method. If reproducible under natural conditions, the approach might be developed into a regular monitoring program that supports developing an index of relative biomass which could be used to infer biomass trends through time. However, to move beyond associations between eDNA concentrations and weights and to begin to acquire absolute measures of biomass, considerably more data are required on the abiotic and biotic factors impacting the eDNA concentration in the water [8].

Given the ongoing reductions in costs of processing eDNA samples as technology advances, it may be feasible to collect eDNA samples concurrently with other sampling methods (e.g., trawling or hydroacoustic surveys) to build a reference database to inform future quantitative work. Over sufficient temporal scales, concurrent monitoring using other methods may facilitate the development of field-based eDNA–biomass conversion factors to estimate absolute biomass from eDNA concentrations. However, the feasibility of such an approach depends on identifying and quantifying sources of variance in the eDNA–biomass relationship. Ultimately, the declining cost of eDNA techniques will assist both rigorous field validation and the development of a robust monitoring program for aquatic species, should validation prove successful.

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Appendix 5. Rourke, M.L., Broadhurst, M.K., Fowler, A.M., Hughes, J.M., DiBattista, J.D. and Furlan, E.M. 2024. Correlated eDNA and electrofished-biomass estimates for golden perch, *Macquaria ambigua*. Fron. Fish Sci. 2. Link to article

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Correlated environmental DNA and electrofished estimates of relative biomass for golden perch, *Macquaria ambigua*

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In response to the need to augment electrofishing surveys of golden perch, *Macquaria ambigua* (an important endemic freshwater fish in southeastern Australia), with other non-lethal survey methods, the utility of environmental DNA (eDNA) as an index of relative fish abundance/biomass was investigated. From three adjacent rivers, five sites in each were sampled for eDNA immediately before electrofishing during sequential years. Up to six individuals or 10 kg of electrofished golden perch (200–548 mm total length) were caught or observed site^{-1}. Analyses of concurrent eDNA concentrations revealed no significant relationship with the relative abundance of golden perch, but there was with relative biomass—manifesting as increasing eDNA concentrations between 1.1 and approximately 5.5 kg biomass site^{-1}, after which concentrations stabilized at greater biomasses. Future research warrants assessing the viability of sampling eDNA for spatio-temporally monitoring rivers where low biomasses of golden perch (1–5 kg site^{-1}) are likely to occur.

KEYWORDS

aquatic, biomonitoring, fishery-independent survey, freshwater, Murray–Darling Basin, Percichthyidae

Introduction

Globally, river regulation frequently impedes some teleosts from completing vital life-history movements and so it poses challenges for managing freshwater ecosystems (1, 2). Disrupted river flows fragment populations and/or increase vulnerability to genetic repercussions of diminished population size and can ultimately lead to local extinctions (3). Quantifying population sizes within rivers is therefore essential for prioritizing management strategies but requires careful consideration of methods to minimize impacts while remaining cost-effective, robust, and reliable (4).

Traditional, non-lethal fishery-independent freshwater monitoring often incorporates electrofishing, which involves administering a regulated electrical charge to the water and stunning fish before censusing and releasing them (5). Although useful, electrofishing is expensive and labor-intensive and requires highly trained staff. The method is also

relatively invasive with sub-lethal effects on some species (6). A newer, more benign survey method involves monitoring concentrations of DNA shed into the environment by aquatic organisms (termed 'eDNA') as an index of their relative abundance/biomass (7). Many recent studies have identified useful correlations between abundance/biomass and eDNA concentrations (but not always; e.g., (8, 9)). However, relationships are often stronger in controlled, rather than natural environments—owing to the influence of confounding biotic and abiotic variables among the latter and difficulties with estimating absolute population sizes using conventional methods (10). Such variability supports ongoing species-specific field-based studies to determine if eDNA concentrations may be useful for monitoring spatio-temporal changes in population sizes (11).

One large system of regulated rivers in Australia where native fish populations are censused by electrofishing is the Murray–Darling Basin (MDB). Modified river flows, along with other anthropogenic impacts, have resulted in population declines among various endemic species, including golden perch, *Maquaria ambigua*, a large-bodied (up to 23 kg and 76 cm total length (TL)) (12) recreationally important freshwater species. Golden perch have a complex life history that encompasses large spatial scales (often >1000 km) and is closely linked to river hydrology (13, 14).

As a result, golden perch are highly susceptible to river regulation (12). Nevertheless, owing to both natural recruitment and management, including restocking, the abundance of golden perch in the MDB has increased over recent decades (15). Ongoing population monitoring is required to assess future stocks and prioritize management.

In the absence of commercial fishing or regular angler surveys, assessing golden perch stock status in the MBD has relied on annual electrofishing. There is growing interest from scientists and managers to determine if eDNA could be an alternative or complementary tool for monitoring spatio-temporal changes in populations. Considering the above, the aim of this study was to investigate the extent to which the relative abundance and/or biomass of golden perch estimated using electrofishing in three river systems over two years was correlated with eDNA concentrations in concurrent water samples. This information was then used to propose future sampling strategies.

Methods

A genetic assay was developed to amplify a fragment of the mitochondrial 12S ribosomal RNA (hereafter 12S rRNA) of golden perch (see Supplementary material). Water-sample collection for eDNA was done between 1 March and 14 May 2021 and between 28 February and 11 May 2022 and was followed by boat-based electrofishing at 15 pre-determined sites in the southern MDB. The sites were distributed throughout the Lachlan (n = 5), Murrumbidgee (n = 5), and Wakool rivers (n = 5) and have formed the basis of annual government electrofishing surveys since 2016 (Figures 1A–E). Each site was a 200-m-long stretch of river transect, comprising eight 50-m-long 'cells,' numbered with the first site farthest downstream (Figure 1F). Ninety seconds of electrofishing was carried out in each cell using a 7.5 GPP Smith-Root electrofishing unit. The electrofishing units were adjusted depending on conductivity at each site with a direct current (DC) of 500 or 1,000 V, 120 Hz, a duty cycle of 10–40%, and 4.5–9.8 A applied (see Supplementary material). Key water quality parameters were recorded at each site using an Aqua TROLL 500 sonde.

At each site, the boat was typically launched adjacent to the first cell or allowed to drift downstream to the first cell to avoid liberating DNA that may have accumulated in the sediment. Eight replicate surface-water samples (three from each bank and two moving in a transect upstream from the center of the river (i.e., evenly distributed throughout the 200-m transect; Figure 1F)) were collected with a target volume of 2.0 l using a Smith Root eDNA Sampler (Smith-Root). The filter rate was set at 1.0 l min^{-1} with a maximum pressure of 69 kpa using 5-μm polyethersulfone self-preserving filters (Smith-Root; (16)). An equipment control (EC) comprising 1.0 L of sterile water was filtered at each site before field sampling.

Immediately following water sample collection, electrofishing was conducted across the entire 200-m reach, positioned within each 50-m cell and beginning at cell 1 (Figure 1F). At each cell, any inert fish displaced to the surface were removed (defined as 'caught') and held in an aerated live well for up to 10 min before being measured (to the nearest 1 mm TL), weighed (to the nearest 1 g), and released well downstream of the next upstream cell. Any golden perch displaced to the surface but not retrieved were recorded as 'observed'. The weights of these latter fish were estimated as the averages caught at the site. The total caught and observed weights from cells 1–8 were used as the biomass estimate for each site. The abundance included the observed number of fish.

The DNA was extracted from water samples following conventional procedures, which are described in the Supplementary material. To remove potential inhibitors, all samples were cleaned using a Zymo OneStep PCR Inhibitor Removal Kit (Integrated Sciences) following the manufacturer's instructions. The samples were evaluated for residual polymerase chain reaction (PCR) inhibition (see Supplementary material) and, if present, DNA concentrations were evaluated using 1:10 diluted DNA or (if no apparent inhibition) using neat DNA. Six replicate multiplex quantitative PCR (qPCR) reactions were performed $sample^{-1}$ using a standard curve generated from a dilution series of gBlock™ standards with seven concentrations between 10^{-2} and 10^{-8} ng μl^{-1}, as well as low copy number standards (10, 5, 2, and 1 copies μl^{-1}; see the Supplementary material). The concentrations were then multiplied by 50 (for neat DNA) or 500 (for 1:10 DNA) to calculate the quantity (ng) of DNA $filter^{-1}$. The amount of DNA l^{-1} of water was determined by dividing the quantity of DNA $filter^{-1}$ by the volume of water filtered. The concentration of DNA in copies $reaction^{-1}$ was calculated from the concentration in ng l^{-1} (see Supplementary material).

Data analyses

The analyzed data comprised the concentration of golden perch DNA for each of the eight filters at each site (ng filter l^{-1}) and their

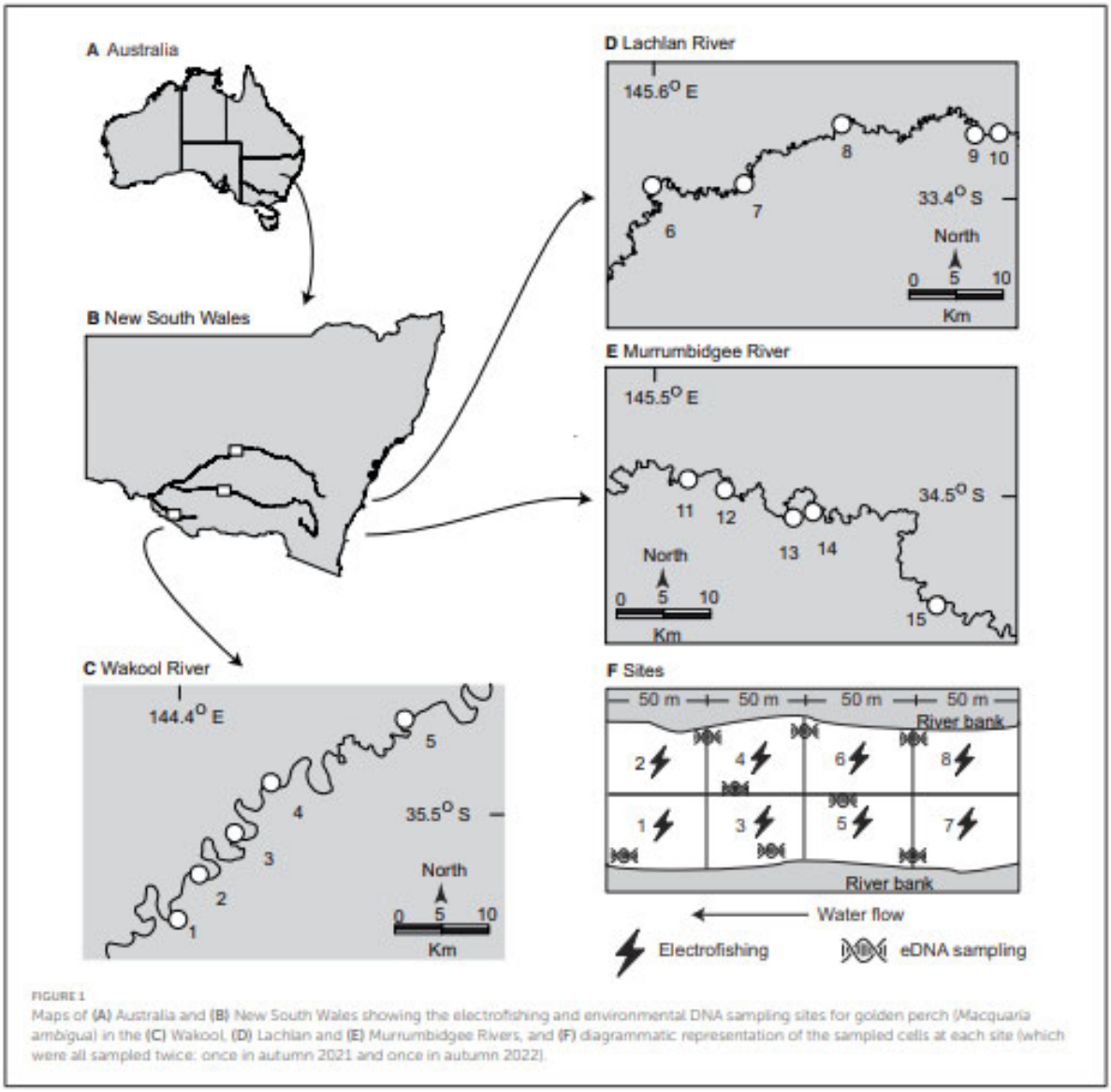

FIGURE 1
Maps of **(A)** Australia and **(B)** New South Wales showing the electrofishing and environmental DNA sampling sites for golden perch (*Macquaria ambigua*) in the **(C)** Wakool, **(D)** Lachlan and **(E)** Murrumbidgee Rivers, and **(F)** diagrammatic representation of the sampled cells at each site (which were all sampled twice: once in autumn 2021 and once in autumn 2022).

observed abundance (no. site^{-1}) and total biomass (kg site^{-1}) at the site level. Any null data for eDNA and fish abundance or biomass were removed but noted. All remaining data were log-transformed to act multiplicatively, and the relationships between eDNA concentrations (ng l^{-1} as the response variable) and abundance and biomass (fixed effects) were initially plotted. Biomasses <1 kg were subsequently removed from analyses because these data (which were very few; see the 'Results' section) created an artificial change in the slope compared to those values ≥1 when log-transformed. The remaining log-transformed data were explored using linear mixed models (LMM). Random effects included 'years,' 'rivers,' and 'sites' with sites nested within rivers. Models were fitted within glmmTMB (v. 1.1.7) in R using penalized quasi-likelihood and with model checking (i.e., over-dispersion) and diagnostics (residual plots; (17, 18)). Because both regressions appeared curvilinear, a quadratic term was included to improve model fit and compared against the linear model via the Akaike information criterion (AIC). Marginal and conditional r^2s were estimated using the function r.squaredGLMM in the MuMIn package (v. 1.47.5) (19).

Results

The water-quality parameters varied considerably between and within rivers, with variability broadly increasing with water flow, which was always greatest in the Murrumbidgee River (Table 1). Furthermore, owing to excessive regional rainfall, the flow was greater across all rivers in 2022 than in 2021 (an average of 4,304 vs. 986 Ml d^{-1}). Water sampling (0.44–1.90 l of filtering) and

TABLE 1 The rivers and sites and their environmental conditions (mean ± *SE* where indicated) were sampled for golden perch, *Macquaria ambigua*, eDNA prior to electrofishing.

Location	Latitude and longitude	Environmental conditions						Mean (± *SE*) eDNA (ng l^{-1})	[b]Mean copies reaction^{-1} (± *SE*)	No. caught	No. observed	Mean (± *SE*) wt (g) caught	Mean (± *SE*) TL (mm)
		Mean (± *SE*) flow (ML d^{-1})	Temp (°C)	pH	DO (mg l^{-1})	Conductivity (mS cm^{-1})	Turbidity (NTU)						
Wakool River		258.1 (22.5)											
Site 1[a]	35.56° S, 144.35° E	NA	19.5 (1.80)	7.5 (0.26)	7.7 (0.39)	0.4 (0.12)	55.1 (10.63)	5.86×10^{-4} (1.94×10^{-4})	0.6 (0.1)	0	0	0	NA
Site 2	35.55° S, 144.35° S	NA	19.6 (2.25)	7.0 (0.23)	8.6 (0.55)	0.4 (0.14)	41.2 (6.20)	1.95×10^{-4} (1.30×10^{-4})	0.6 (0.2)	0	0	0	NA
Site 3	35.54° E, 144.37° S	NA	18.7 (1.91)	7.2 (0.18)	7.6 (0.19)	0.4 (0.12)	46.7 (8.78)	3.05×10^{-4} (8.74×10^{-5})	0.8 (0.1)	1	1	1,923.0	485.0
Site 4	35.53° E, 144.38° S	NA	18.1 (2.05)	7.5 (0.23)	8.3 (0.64)	0.4 (0.13)	51.3 (10.38)	3.40×10^{-4} (7.66×10^{-5})	1.0 (0.2)	6	0	1,741.0 (511.4)	426.7 (63.1)
Site 5	35.51° E, 144.42° S	NA	17.7 (1.52)	7.7 (0.22)	7.9 (0.32)	0.5 (0.13)	46.8 (9.42)	4.67×10^{-4} (9.21×10^{-5})	1.6 (0.3)	1	0	2,124.0	499.0
Lachlan River		2,469.8 (733.5)											
Site 6	33.37° E, 145.59° S	NA	14.6 (0.14)	7.2 (0.27)	8.7 (0.03)	0.3 (0.01)	56.9 (1.81)	1.74×10^{-7} (4.92×10^{-8})	1.3 (0.4)	1	0	1,575.0	450.0
Site 7	33.37° E, 145.66° S	NA	14.3 (0.04)	8.2 (0.01)	9.3 (0.04)	0.3 (0.0002)	67.5 (1.23)	2.64×10^{-7} (3.62×10^{-8})	1.4 (0.3)	4	0	1,413.8 (166.8)	450.0 (20.4)
Site 8	33.34° E, 145.72° S	NA	17.3 (1.34)	8.1 (0.10)	8.4 (0.61)	0.3 (0.02)	92.2 (14.18)	5.63×10^{-7} (9.70×10^{-8})	3.5 (1.1)	4	2	1,589.5 (158.4)	455.3 (14.6)
Site 9	33.34° E, 145.82° S	NA	17.4 (0.05)	8.1 (0.07)	8.7 (0.11)	0.3 (0.004)	90.6 (4.11)	2.50×10^{-7} (8.21×10^{-8})	2.4 (1.1)	5	0	1,386.2 (74.5)	445.2 (9.1)
Site 10	33.34° E, 145.84° S	NA	19.1 (0.64)	8.2 (0.12)	8.7 (0.35)	0.3 (0.01)	118.1 (7.70)	3.75×10^{-7} (5.40×10^{-8})	3.4 (1.0)	2	2	855.8 (533.2)	370.0 (58.0)
Murrumbidgee River		5,207.5 (1037.4)											
Site 11	34.45° E, 145.54° S	NA	14.8 (0.14)	7.7 (0.16)	10.3 (0.40)	0.2 (0.000)	41.2 (0.40)	1.17×10^{-7} (3.55×10^{-8})	1.2 (0.2)	2	0	1,150.5 (134.5)	420.0 (5.0)
Site 12	34.45° E, 145.56° S	NA	19.8 (1.60)	7.5 (0.07)	8.8 (0.81)	0.1 (0.01)	79.5 (7.65)	1.31×10^{-7} (3.15×10^{-8})	0.7 (0.2)	1	0	12.0	97.0
Site 13	34.47° E, 145.61° S	NA	19.8 (1.73)	7.5 0.06)	8.4 (0.63)	0.1 (0.01)	70.8 (5.88)	1.51×10^{-7} (5.66×10^{-8})	1.4 (0.4)	1	0	1,062.0	410.0
Site 14	34.47° E, 145.62° S	NA	16.6 (0.94)	7.0 (0.22)	9.6 (0.39)	0.1 (0.01)	57.6 (11.25)	2.04×10^{-7} (4.96×10^{-8})	1.0 (0.2)	1	2	2,588.0	504.0
Site 15	34.52° E, 145.71° E	NA	19.3 (1.65)	7.2 (0.15)	8.5 (0.44)	0.1 (0.01)	72.4 (2.97)	2.92×10^{-7} (8.32×10^{-8})	1.6 (0.4)	1	0	122.0	200.0

Flow is the mean flow of each sampling day in each river system. The mean (± SE) concentrations of eDNA (ng l^{-1} and copies reaction^{-1}) and the total numbers and mean (± SE) weights (wt) and total length (TL) of 'captured' electrocuted fish and the numbers observed (but not captured) for each site (n = 8 cells site^{-1} combined across two years: 2021 and 2022). NA, not applicable; DO, dissolved oxygen; NTU, Nephelometric Turbidity Units. [a]Includes data from 2022 only, due to evidence of contamination for the 2021 samples. [b]Copies reaction^{-1} were calculated using successfully amplified samples only.

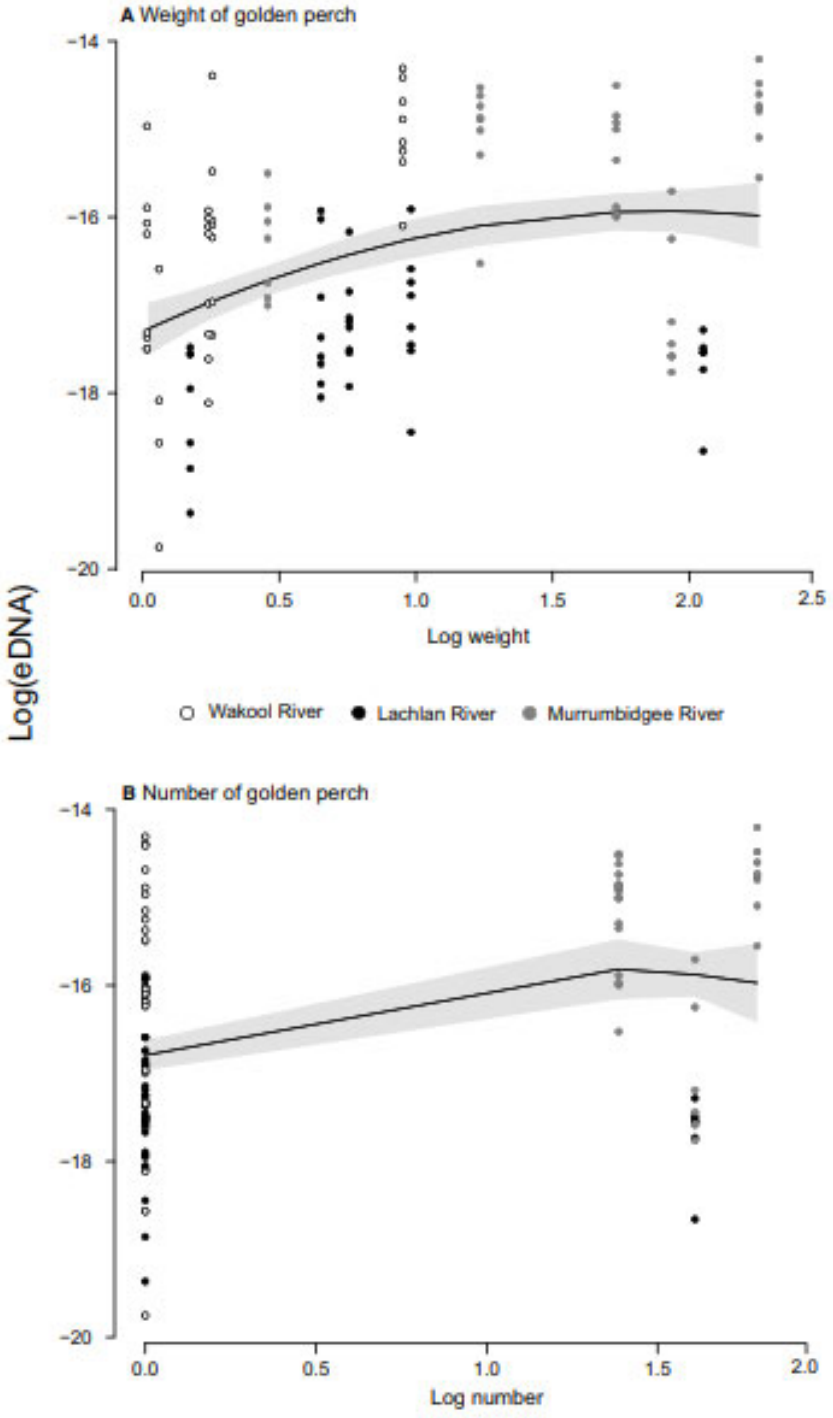

FIGURE 2
Relationships between the log eDNA concentrations (ng l^{-1}) and log **(A)** biomass (kg) and **(B)** numbers of golden perch (*Macquaria ambigua*) at 15 sites across three river systems, during two years in the southern Murray–Darling Basin, Australia. Some weights were estimated (for fish observed but not caught), and abundances included fish caught and observed. Both axes are on the log scale.

electrofishing were successfully completed for all sites during both years, with a total of 30 golden perch (200–504 mm TL) caught and weighed (12–2588 g) and 7 observed (Table 1). At least one golden perch was captured or observed at each site, except for sites 1 and 2 (in the Wakool River), and all but five were >1 kg (Table 1, Figure 2).

TABLE 2 Akaike's information criterion (AIC), marginal r-squared (r^2m), and conditional r-squared (r^2c) values for models with differing random structures (years, rivers, and sites).

Model	AIC	ΔAIC	r^2m	r^2c	Δr^2c
Log (eDNA)~log(biomass) + year + river (site)	281.4	0.0	0.23	0.77	0.64
Log (eDNA)~log (biomass) + year + river	311.8	30.4	0.11	0.60	0.47
Log (eDNA)~log (biomass) + year	356.4	75.0	0.13	0.13	0.00

Sites were nested in the rivers.

Species-specific primers and a hydrolysis probe (TaqMan®) were successfully designed to amplify 200 base pairs (bp) of the 12S rRNA gene (see Supplementary material). A synthetic oligonucleotide (gBlock™) based on the golden perch 12S rRNA gene was designed with an 8-bp reverse complement sequence for use as the positive control on every qPCR plate (see Supplementary material). The 8-bp reverse complement sequence facilitated detecting of false positives due to contaminating gBlock™. Testing of the assay on genomic DNA from co-distributed species in the same family failed to amplify, indicating the assay was specific to golden perch (see Supplementary material). The assay efficiency was 96.7% (R^2 = 0.99, the limit of detection (LoD) (lowest standard with at least 95% positive detection) was 4 copies reaction^{-1} and the limit of quantification (LoQ) (with coefficient of variation (CV) threshold set at 35%) was modeled at 5 copies reaction^{-1} (see Supplementary material).

Of 240 samples (DNA extracts), 171 were partially inhibited following purification (71%). These samples were diluted to 1:10 for subsequent qPCR. A further four samples (all from different sites) exhibited complete inhibition and were not included in subsequent qPCRs (1.7%). Of the remaining 236 samples, golden perch DNA was detected in 220 samples (i.e., in at least 1 of 6 qPCR replicates sample^{-1}) and at every site during both years (Table 1). The eDNA data from site 1 in 2021 were discarded due to endogenous DNA and golden perch DNA in the EC, indicating contamination had occurred during sampling. All other positive and negative controls exhibited strong or null amplification, respectively, indicating no issues with the laboratory methods. There was an average of 0.6–3.5 copies reaction^{-1} across the 15 sites (Table 1). These concentrations were below the LoQ and resulted from diluting the DNA extracts to overcome inhibition, which may affect the precision of concentration estimates. Despite this uncertainty, we are confident that the number of technical replicates increased the accuracy of the concentration estimates.

Overall, the agreement between eDNA and electrofishing, with respect to mutual detections across sites, was 42%. However, the agreement in 2021 was considerably higher (56%) than the agreement in 2022 (28%). Given that golden perch eDNA was detected at all sites in all years, mismatches were driven by zero values obtained from electrofishing, with more non-detections during 2022, particularly in the Lachlan River.

The LMMs investigating the relationships between DNA concentrations and the abundance and biomass of electrofished golden perch across the three rivers were improved when a quadratic term was fitted (AIC abundance: linear = 293.97, quadratic: = 288.03; AIC biomass: linear = 143.30, quadratic: = 134.34). There was a significant relationship between eDNA and estimated biomass, which was positive between approximately 1.1 and 5.5 kg, after which there were consistent eDNA concentrations to 10.3 kg (LMM $p < 0.01$; Figure 2A). Model comparison using AIC supported all random effects in the model, which explained 77% of the variance (Table 2). The fixed effect of biomass in this model explained 23% of the variance in the eDNA concentration. A comparison of marginal and conditional r^2s within and between models indicated that for random effects, rivers explained the greatest variance, followed by sites and then years (Table 2). The relationship between eDNA and abundance was not significant (LMM $p > 0.05$; Figure 2B).

Discussion

The significant relationship detected between the eDNA concentration of golden perch in water samples and their electrofished biomass supports a trend in the literature describing the utility of eDNA for non-lethal aquatic monitoring—although the lack of significance for abundance also reiterates the need for species-specific verification of the approach—which might be best done under controlled conditions, quantifying individual variability in DNA shedding followed by degradation (7, 20). Nevertheless, the data support using eDNA to assess golden perch biomass, albeit with some caveats that warrant consideration.

It is important to reiterate that in this study neither eDNA nor electrofishing provided estimates of absolute abundance. Rather, our focus was a comparative analysis of these two approaches for providing indices of relative quantities and was similar in theme to studies comparing traditional active (trawls and seines) or passive (hooks, traps, and gillnets) fishing gears (e.g., (21, 22)). Ultimately, such comparisons can help ascertain whether alternative methods offer statistically equivalent abundance/biomass indices and might be preferred because of not only lower operational costs but also logistical benefits. Implicit within the latter is an adequate understanding of the limitations of each method.

Both eDNA and electrofishing are affected by various biotic and abiotic factors, including river flow and temperature, which have been discussed in detail elsewhere (e.g., (7, 23, 24)). Here, river flow was not only variable among the three rivers but was also much greater across all rivers in 2022 than in 2021. Golden perch have lower electrofishing capture probabilities when daily flow increases and among individuals approximately <400 mm TL (23). Possibly, electrofishing capture probability was reduced during 2022, especially in the Murrumbidgee River, due to higher flow, deeper water, and increased turbidity following flooding and/or was affected by the relatively large proportion of small fish. Certainly, there was a lower rate of detecting golden perch by electrofishing than via eDNA in 2022. Nevertheless, water flow can also reduce eDNA concentrations and thus detectability (25), which could impact defining relationships with abundance and/or biomass.

Environmental DNA sampling and laboratory processing also have inherent limitations. For example, the number of samples site^{-1} required to accurately estimate mean concentrations in this system was unknown *a priori*. Based on findings from other eDNA studies focused on the species-specific detection of fish in the

MDB and to maximize precision, we collected eight samples site^{-1} (26, 27). Given golden perch DNA was noted in approximately 93% of our filters, it is likely our sampling strategy was adequate to collect sufficient DNA. Furthermore, we assessed every sample for partial inhibition following inhibitor removal and subsequently used either neat or diluted DNA to compensate for the remaining partial inhibition, providing confidence the eDNA concentration estimates were accurate.

Notably, the partial inhibition did not preclude amplification of the endogenous control and its presence would have gone undetected without a comprehensive assessment of all samples. The potential ramifications of partial inhibition on the outcomes of future studies are considerable, potentially leading to a delay in the amplification of the target DNA and uncertain quantification (28). Therefore, it remains crucial for studies assessing eDNA concentrations to address inhibition concerns to ensure the accuracy of results. Additionally, other extraction methods or inhibitor-resistant PCR reagents could be trialed (29, 30), but these are not a panacea. Nonetheless, we suggest checking for low levels of inhibition before commencing qPCR analysis, particularly in embedded riverine ecosystems.

Regardless of the specific influences of any intrinsic and extrinsic factors, collectively, these were encompassed within the observed quadratic relationship between eDNA and electrofishing. This asymptotic relationship deviates from patterns observed in many similar studies comparing eDNA concentration to biomass, where simple linear relationships have been reported, although competing model fits are often not described. Nevertheless, the quadratic relationship reported here is similar to that reported for silver carp, *Hypophthalmichthys molitrix*, in a large river, assessed using eDNA and an acoustic survey (31), and may be influenced by various factors, including, but not limited to, water temperature or flow (discussed earlier), eDNA production, or degradation (7). Deciphering the importance of potentially influencing abiotic or biotic factors warrants future investigation.

Despite the challenges posed by variability within biotic and abiotic factors and processing considerations, and although the data are few, we nevertheless identified a statistically useful relationship between eDNA concentration and biomasses ranging between 1.1 and approximately 5.5 kg per site^{-1}. This result justifies ongoing research to better describe the observed relationship and any important factors affecting variability. Such work might involve additional eDNA sampling concurrent to ongoing electrofishing assessments and would ultimately help inform appropriate future monitoring strategies for golden perch.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was approved by the Fisheries Animal Care and Ethics Committee, Department of Regional New South Wales. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

MR: Writing – original draft, Writing – review & editing. MB: Writing – original draft, Writing – review & editing. AF: Writing – original draft, Writing – review & editing. JH: Writing – review & editing. JD: Writing – review & editing. EF: Writing – review & editing.

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Conflict of interest

This study received funding from the Fisheries Research and Development Corporation. The funder was not involved in the study design, collection, analyses, interpretation of data, the writing of this article or the decision to submit it for publication. All authors declare no other competing interests.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/frish.2024.1358572/full#supplementary-material

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Appendix 6. Rourke, M.L., Broadhurst, M.K., Fowler, A.M., Hughes, J.M., DiBattista, J.D. and Furlan, E.M. 2024. Correlating environmental DNA and hydroacoustic biomass/abundance estimates to monitor the globally Endangered mulloway (*Argyrosomus japonicus*). To be submitted to Fron. Fish Sci

Correlating environmental DNA and hydroacoustic biomass/abundance estimates to monitor the globally Endangered mulloway (*Argyrosomus japonicus*)

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Abstract

Monitoring long-term spatiotemporal changes in the populations of vulnerable species requires non-lethal methods. Here we investigate the utility of environmental DNA (eDNA) for estimating the relative biomass and abundance of the globally Endangered Mulloway (*Argyrosomus japoinicus*) in a southeastern Australian estuary that supports one of the largest coastal fisheries in the country. During minimal tidal movement, ten parallel transects at each of 12 sites were surveyed, involving water samples being collected for determining eDNA concentrations of Mulloway DNA prior to hydroacoustics being used to estimate Mulloway biomass and abundance. The only significant effect on variability among eDNA concentrations was an interaction between season and biomass, with positive and negative regressions observed during autumn and spring, respectively. As non-invasive survey methods, both eDNA and hydroacoustics require further validation, and such efforts should assess the influence of key environmental factors.

Keywords: eDNA, hydroacoustics, Mulloway, fisheries, estuary, qPCR

Introduction

Mulloway (*Argyrosomus japoincus*) occur throughout the Indo-West Pacific and have historically formed the basis of several artisanal, commercial and recreational fisheries off South Africa, India, Pakistan, China, Korea, Japan and Australia (Sasaki, 2001; Silberschneider & Gray, 2008; Silberschneider et al., 2009; Trewavas, 1977; Whitfield & Mann, 2023). Most of the available information describing the life history of Mulloway originates from South Africa and especially Australia where it is regionally distributed across estuaries and nearshore areas south from North West Cape in Western Australia to southern Queensland, with the greatest concentration in the south east off the state of New South Wales (NSW) (Silberschneider & Gray, 2008; Silberschneider et al., 2009).

Mulloway spawn across nearshore areas during late summer, after which larvae move into estuaries, grow to ~50 cm total length (TL) within two years (maximum of ~200 cm TL over 42 years), and attain mean sizes at maturity of 51 and 68 cm TL for males and females, respectively (Silberschneider & Gray, 2008; Silberschneider et al., 2009). Their life cycle makes them very vulnerable to most inshore fishing methods, including trawls, baited hooks, gillnets, seines and even traps. Off NSW, the Mulloway population is considered depleted to ~13% of the unfished biomass, which is reflected in commercial catches (mostly from gillnets and ~50 t p.a.) being ~85% lower than historical highs (Earl et al., 2020). Recreational harvests remain considerably greater (~90 t), but have also historically declined (Earl et al., 2020). Similarly, off South Africa and Vietnam, spawning biomasses have declined to ~1 and 30% of unfished levels, while in other jurisdictions such as China, once-abundant Mulloway are now rarely caught (Fennessy, 2020). Based on the available evidence in 2018, Mulloway were globally listed as Endangered on the ICUN Red list; among only ~5% of ~14000 extant teleosts to receive such a classification (Fennessy, 2020).

Mulloway populations have been prioritised for recovery plans in Australia and South Africa involving legislated size and catch limits (Fennessy, 2020) and, for NSW, restocking (Taylor et al., 2017) along with modifications to problematic gears (mostly penaeid trawlers) to limit unaccounted fishing mortalities (Broadhurst & Kennelly, 1994; Broadhurst & Millar, 2024). Measuring the success of any management strategy to reduce fishing mortality ultimately requires robust spatio-temporal population monitoring (Hill et al., 2023). However, for Mulloway, traditional fishery-dependant monitoring is not ideal considering reductions in fishing effort are required to reduce impacts on the spawning stock. Non-invasive censusing methods are required. One possibility is to utilize visual surveys (either surface or underwater; Palmas et al., 2023), although these require clear water and are not suitable for many inshore or estuarine environments. Two other options are to monitor environmental DNA (eDNA; Rourke et al., 2022) and/or use hydroacoustics (either emitted; Lyons et al., 2021 or received sound; Mouy et al., 2023) to infer relative abundances and/or biomasses over appropriate spatio-temporal scales.

Of these two approaches, eDNA methods are gaining the most recent attention because of mounting evidence that, for many species, concentrations can be positively related to biomass or abundance (Rourke et al., 2022). More specifically, for Mulloway we recently identified positive relationships between eDNA concentrations and their absolute biomass under controlled conditions in aquaria, and recommended subsequent field trials should be done to explore the practicality of any relationships (Rourke et al., 2023). While there are few comparative data supporting hydroacoustic surveys of Mulloway, the technique has been validated for other species as part of fishery-independent/dependant surveys in other jurisdictions and can be validated for Mulloway in the absence of lethal sampling; albeit with some limitations for detecting small sizes of fish (Draštík et al., 2017; Mackinson et al., 2004).

Considering the above, the aim of this study was to investigate any correlation between eDNA and hydroacoustics for estimating the relative biomass and abundance of Mulloway and potential key affecting factors in one estuary (the Hawkesbury River) known to have fish present throughout the year (Broadhurst & Kennelly, 1994; Kennelly, 1993). In doing so, we sought to provide information on the possible utility of each method over the longer term. This investigation builds on the validation

of species-specific qPCR assays developed for Mulloway (Rourke et al., 2022) subsequently applied under aquaria conditions (Rourke et al., 2023).

Methods

Study sites

The work was done in the lower Hawkesbury River (encompassing the known range of Mulloway) within a single year during autumn (16–20 May, 2022) and spring (17–20 October and 1–2 December 2022) (Figure 1). The chosen periods were either side of the peak spawning season (December to March) for Mulloway and so precluded potentially high concentrations of reproductive DNA propagules confounding relationships with biomass or abundance of juvenile and adult fish. The specific sampling dates coincided with optimal salinity profiles in the lower reaches of the catchment (to avoid hydroacoustic biases).

Twelve sites were selected, and each divided into 10 transects (perpendicular to the riverbank and 100 m apart; Figure 1b–d). Prior to starting the biomass/abundance surveys, all sites were bathymetrically modelled (during November 2021). Such modelling is crucial for analysing and interpreting fish biomass data from hydroacoustics (REF). The model created a detailed map of the underwater terrain, which enabled determining tracks for the subsequent biomass survey. Bathymetric data for each site were collected using the Humminbird Helix Mega SI GPS fish finder (Helix) with the boat moving along the transects at each site (Figure 1d, Supplementary material). The data were reviewed using ReefMaster software V2.0 (ReefMaster) and the physical characteristics of each site were generated, including bottom-line (riverbed), zero line (where the water meets the land), area (m^2), depth (m) and volume (m^3). Bathymetric data were also used to determine the optimal angle of the transducer (–3 to –4.5 degrees below horizontal) for the biomass/abundance surveys.

Biomass/abundance survey protocols

To minimise the effect of water flow on eDNA concentrations and maximise the probability of hydroacoustic detections at each site, the biomass/abundance surveys were timed to occur as close to high tide as possible (prioritized from downstream to upstream sites). Before starting sampling, an equipment control (EC – 1 l of sterile water) was filtered to enable potential DNA contamination in the field to be detected. Then, at the start of each transect within each site, the boat was stopped, and a 2-l sample of surface water collected and filtered using a Smith Root eDNA sampler (Smith-Root) and 5-µm polyethersulfone self-preserving filters (Smith-Root) (Thomas et al., 2019). All filters were immediately refrigerated at 4° C.

The vessel then proceeded along the transect for hydroacoustic biomass/abundance surveying (and with concurrent bathymetry surveying to check for any changes in site characteristics). Data detections of individual Mulloway were collected using the BioSonics DT-Z split beam echo sounder and managed with the Visual Acquisition software package (BioSonics), which also concurrently collected bathymetry data. The limits of detection for Mulloway were ≥40 cm TL (to the nearest 10 cm). Total lengths were then converted to weights using the relationship provided by Silberschneider et al. (2009). The Mulloway count (no. m^{-3}) and biomass volume^{-1} (kg m^{-3}) were estimated for each transect and then pooled to provide abundance and biomass estimate site^{-1} (Supplementary material). Two replicate water quality parameter measurements (temperature, salinity and dissolved oxygen) were collected at the first transect prior to commencing sampling.

At the end of each survey period, all filters (for each transect within each site) were transported to a purpose-built eDNA laboratory at the Narrandera Fisheries Centre. The DNA was extracted from each filter within six months of collection and following established protocols and as described in the Supplementary material. We used a Zymo OneStep PCR Inhibitor Removal Kit (Integrated Sciences) to reduce the amount of PCR inhibitors in the samples. We then tested each sample for remaining inhibition following the procedures in Rourke et al., (2024). Briefly, neat (1:1) and diluted (1:10) eDNA for each sample were evaluated for inhibition using six qPCR reactions containing an endogenous control assay (Furlan & Gleeson, 2016). Inhibited samples were those where amplification in the 1:10 dilution was delayed by 3.3 or more cycles threshold (Ct) values. If samples

were inhibited, the 1:10 eDNA was used to evaluate eDNA concentrations. The concentration of eDNA was calculated as described in Rourke (2024) and the supplementary material. Briefly, six qPCR replicates per sample were used to calculate the mean eDNA concentration for each filter in ng/l as well as the copies reaction^{-1}. The qPCR replicates were only included in the calculations if there was successful amplification of the Mulloway or endogenous control assay to ensure that samples that were strongly inhibited or had an error in the laboratory process were excluded. The assay limit of quantification (LoQ) was calculated as described in the supplementary material.

Statistical analyses

The raw data were made up of the concentrations of DNA for each of the filters (ng l^{-1}) at each transect within sites and the hydroacoustically estimated total biomass and abundance for each site standardized to m^{-3} (by volume surveyed). The eDNA data were explored using boxplots, Cleveland plots and scatterplots following the protocol of Zuur et al. (2010) before being log-transformed (with epsilon of the lowest eDNA value for zeros) and analysed using two separate linear mixed models (LMMs) that investigated relationships with the fixed effects of 'seasons' and either 'biomass' or 'abundance' and the interaction term; sites were considered a random factor. The significance of fixed effects was evaluated at the 5% level using Wald tests. All modelling was done in R (ver. 4.3.1, R Foundation for Statistical Computing, Vienna, Austria) using the glmmTMB function from the 'glmmTMB' package (Brooks et al., 2017). Marginal (i.e. proportion of variance explained by fixed factors) and conditional (proportion of variance explained by fixed and random factors) pseudo-r^{2}s were estimated using the r.squaredGLMM function in the MuMIn package (v. 1.47.5; Barton, 2023).

Results

Ten transects were hydroacoustially surveyed at all sites. Although mean depths and survey areas were consistent among sites, water quality was variable (supplementary material). Specifically, water temperatures were typically warmer at downstream sites during autumn versus at upstream sites during spring. In both seasons, salinity steadily declined upstream owing to freshwater inflows (supplementary material) Sites 8–12 were all below 5 practical salinity units (PSU). Dissolved oxygen was consistent throughout the study reach.

Ten eDNA samples were collected for Mulloway at all sites except for two sites where nine eDNA samples were collected across a total of 238 transects. XX% of samples were diluted to 1:10 prior to qPCR for Mulloway DNA concentration estimates due to persistent inhibition. The quantity of DNA reaction^{-1} fell just below the limit of quantification (two copies reaction^{-1}) in 21 out of 24 sites (range 0.01–4.5), most likely because most samples were diluted to 1:10 to overcome persistent inhibition (Table 1). Nevertheless, the number of technical replicates was high, which increased the accuracy of DNA concentration estimates.

Mulloway DNA was detected in 173 out of 238 transects and at 24 out of 24 sites except for the most upstream (no. 12) during spring, while hydroacoustic detections occurred at 16 out of 24 sites; eight during each season (Table 1). Only one site had hydroacoustic detections of Mulloway and no DNA (site 12 during spring) (Table 1). Conversely there were eight sites with eDNA detections but no hydroacoustic detections (Table 1). Equal agreement was observed during each season. Some of the greatest eDNA concentrations were observed at sites where no Mulloway were hydroacoustically detected, including at sites 8 and 9 during spring (Table 1). The mean TLs of Mulloway detected at each site varied, but with smaller and larger fish generally downstream and upstream, respectively (Table 1).

Significance in the LMMs investigating variability among the eDNA concentrations of Mulloway was restricted to the interaction between season and biomass, with positive and negative regressions during autumn and spring; both of which were significantly different from zero (p <0.05; Table 2, Fig. 2a and b). Although not significant, the same trend of slopes was detected for the relationship between eDNA and abundance (p > 0.05; Table 2, Fig. 2c and d).

Discussion

This study contributes towards the few regional data sets determining the viability of eDNA as a tool for monitoring the relative biomass/abundance of Mulloway (Rourke et al., 2023; Wilkes Walburn et al., 2022), and the broader literature for sciaenids (e.g. Wu et al., 2022) and teleosts in general (Rourke et al., 2022). The results provide some support for the positive correlations previously detected for Mulloway under controlled aquaria conditions, and for most assessed species across various scenarios (Rourke et al., 2023; Rourke et al., 2022). But, by including seasonal effects in our analyses, we have also identified considerable negative influences of extraneous factors. These findings can be contextualised within the study limitations and possible influencing environmental factors.

It is important to reiterate that calculating the absolute biomass/abundance of Mulloway in the Hawkesbury River (to reference against eDNA concentrations) is not possible. All traditional methods, including penaeid trawling, electrofishing, gillnetting or trapping either have lethal outcomes or were considered impractical. Hydroacoustic sampling was the only feasible option. This method has been successfully validated and applied to assess fish populations around the world (Draštík et al., 2017; Mackinson et al., 2004), but like all methods, has inherent limitations—notably the inability to identify Mulloway <40 cm TL. We were aware that juvenile Mulloway as small as 5 cm TL inhabit the Hawkesbury River year-round (Broadhurst & Kennelly, 1994), but we assumed that hydroacoustic detections of larger fish would present as relatively greater deviations in eDNA from baseline amounts (i.e. due to smaller fish) given our aquaria trials (using fish <40cm) showed a strong correlation between eDNA concentration and biomass (Rourke et al., 2023). Nevertheless, the absence of data on the absolute biomass/abundance of Mulloway <40 cm TL means the hydroacoustic biomass estimates are incomplete, potentially skewing the observed relationships with eDNA concentrations.

Beyond potential size-specific biases of hydroacoustics are important considerations concerning eDNA assessments. We collected water samples at the surface and at the start of each transect and assumed the extracted DNA represented Mulloway within the entire volume of the site. However, the number of water samples and their appropriate depth required to accurately estimate eDNA concentrations remain unknown (Rourke et al., 2022). While surface sampling may seem counterintuitive for Mulloway, which typically remain associated with the benthos, previous studies have shown surface samples to be suitable for both lentic and lotic water bodies which may reflect mostly shallow depths (Hinlo et al., 2017; Moyer et al., 2014). Nevertheless, it is possible the Mulloway eDNA concentrations here were not an accurate representation at deeper sites (i.e. which ranged from ~6 to 34 m) (Zhang et al., 2023). Further, the surface salinity at the five most upstream sites was quite low across both seasons, indicating potential haloclines. Mulloway prefer at least some salinity, and any reduction in mixing may have resulted in less Mulloway DNA at the surface. The importance of these potential environmental influences is reiterated by the significant interaction detected between season and biomass estimates (and the similar non-significant trend for abundance).

Specifically, while a significant positive relationship between eDNA concentration and biomass was noted in autumn, the opposite occurred in spring. The latter anomaly was primarily driven by high eDNA concentrations when there were very few (or no) hydroacoustic detections of Mulloway. At least two possible explanations for this observation including: (1) relatively larger abundances of undetected Mulloway <40 cm TL; and/or (2) greater influxes of eDNA. Considering the first hypothesis, following spawning in December, and based on penaeid-trawl bycatch, the greatest abundances of juvenile Mulloway (~5–15 cm TL) in the Hawkesbury River typically occur in autumn (when fish are up to ~6 months old), rather than summer (Kennelly, 1993). But, notwithstanding high mortality, many of these 0+ fish would be >20 cm TL by spring (and still undetectable by hydroacoustic scans) when they have lower trawl catchability (Broadhurst & Kennelly, 1994). If present, these larger juvenile fish might be expected to shed relatively more DNA than adult fish (Rourke et al., 2022).

Equally possible are the effects of environmental parameters on DNA inflow from elsewhere. Assuming the hydroacoustic scans remained similarly efficient between seasons, the presence of high concentrations of Mulloway DNA despite few hydroacoustic detections in spring could simply reflect greater rainfall. As evidenced by the lower salinity across most sites, there were greater freshwater flows during spring, which may act to transport DNA to the sampling location from upstream.

Speculation over the causal factors supporting the interaction between season and biomass detection clearly reiterates the need to progress more research with eDNA for use as a relative index of Mulloway biomass/abundance. Such work will remain challenging in estuaries because these present dynamic environments with numerous abiotic factors that can strongly affect relationships (Rourke et al., 2021), despite us controlling for flow and in this study. Because Mulloway are still bycaught and targeted by commercial fisheries in various NSW estuaries and inshore, future surveys would warrant comparing catches against eDNA data across the same space and time. Nevertheless, the challenge remains to compare a method like eDNA which is absent of size-specific biases, against lethal methods that all have catchability constraints (Stoeckle et al., 2020). Future work should aim to compare eDNA signatures between the upper and lower fraction of the estuary, source comparative survey data capable of assessing relative abundances and/or biomasses based on all size fractions of Mulloway, and restrict sampling to low flow conditions or less dynamic estuaries.

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Conflict of interest
This study received funding from the Fisheries Research and Development Corporation. The funder was not involved in the study design, collection, analyses, interpretation of data, the writing of this article or the decision to submit it for publication. All authors declare no other competing interests.

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Table 1. The sites (see Fig. 1) and number of transects assessed during two seasons in the Hawkesbury River, their mean (±SE) Mulloway eDNA concentration, eDNA copies/reaction and hydroacoustically assessed biomass, abundances and total lengths (TL) transect^{-1}.

	eDNA (ng l^{-1})		eDNA (copies/reaction)		Hydro biomass (kg)		Hydro abundance		Mean TL (cm)	
Site number	A	S	A	S	A	S	A	S	A	S
1	1.58×10^{-8} (3.74×10^{-9})	3.29×10^{-9} (1.17×10^{-9})	1.0 (0.24)	0.4 (0.21)	18.3	1.0	10	1	50.0 (2.98)	40.0 (0.00)
2	3.01×10^{-8} (1.17×10^{-8})	1.95×10^{-8} (6.88×10^{-9})	1.7 (0.28)	0.4 (0.15)	7.5	7.1	4	3	50.0 (7.07)	60.0 (5.77)
3	4.43×10^{-8} (1.33×10^{-8})	4.71×10^{-9} (2.13×10^{-9})	1.3 (0.23)	0.3 (0.10)	28.0	5.1	14	2	51.4 (3.76)	60.0 (10.00)
4	7.01×10^{-10} (2.11×10^{-10})	8.51×10^{-9} (2.84×10^{-9})	0.2 (0.05)	0.7 (0.21)	37.9	0	19	0	51.1 (2.41)	0
5	3.24×10^{-9} (3.71×10^{-10})	2.06×10^{-8} (1.04×10^{-8})	0.7 (0.08)	1.3 (0.31)	30.9	3.6	14	3	53.6 (3.25)	43.3 (3.33)
6	1.51×10^{-9} (5.60×10^{-10})	4.75×10^{-8} (1.14×10^{-8})	0.3 (0.11)	1.5 (0.64)	1.6	0	1	0	50.0 (0.00)	0
7	2.11×10^{-10} (1.27×10^{-10})	7.53×10^{-8} (1.94×10^{-8})	0.1 (0.03)	3.5 (0.67)	0	0	0	0	0	0
8	4.02×10^{-8} (1.10×10^{-8})	2.05×10^{-8} (4.86×10^{-9})	3.5 (0.77)	0.8 (0.23)	25.3	23.9	7	7	65.7 (5.71)	65.7 (5.28)
9	4.27×10^{-8} (1.88×10^{-8})	2.31×10^{-8} (6.97×10^{-9})	0.9 (0.24)	0.4 (0.12)	0	0	0	0	0	0
10	1.39×10^{-8} (6.31×10^{-9})	6.30×10^{-9} (2.73×10^{-9})	1.9 (1.27)	0.3 (0.09)	0	64.9	0	26	0	57.3 (2.39)
11	2.29×10^{-8} (1.60×10^{-8})	3.06×10^{-11} (3.06×10^{-11})	0.5 (0.32)	0.01 (0.01)	0	31.7	0	7	0	71.4 (6.70)
12	2.87×10^{-8} (1.91×10^{-8})	0	4.5 (4.26)	0	13.1	9.1	3	3	73.3 (6.67)	66.7 (3.33)

Table 2. Summaries of Wald *F* -values from linear mixed models assessing the effects of seasons and either hydroacoustically estimated biomass or abundance (and the interaction) on variability among the concentrations of eDNA at 12 sites (random factor) in the Hawkesbury River, New South Wales.

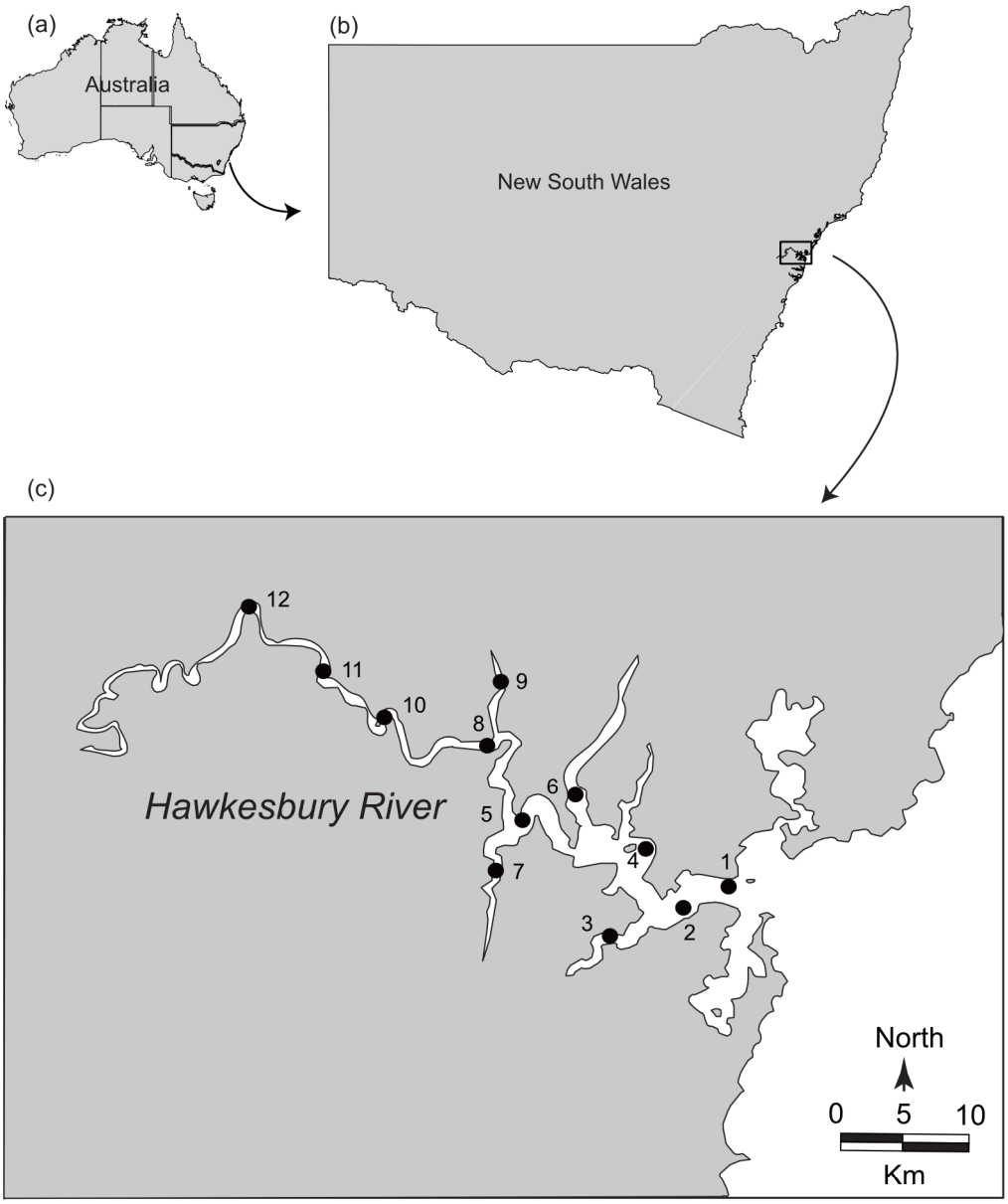
Factor	***F***
Biomass	
Weight m^{-3} (W)	1.06
Season (S)	0.10
W × S	6.36*
Abundance	
Number m^{-3} (N)	NS
Season (S)	NS
N × S	NS

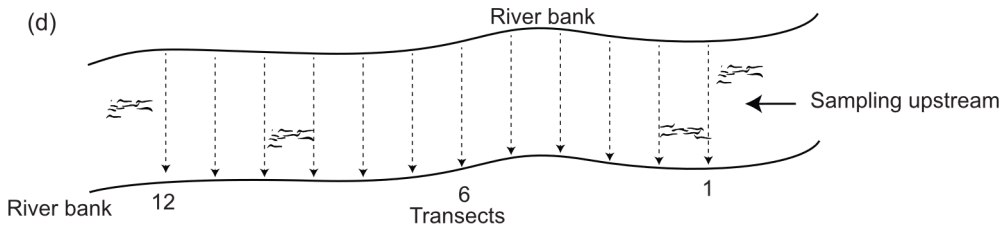
NS, non significant, *$p < 0.05$

Captions to figures

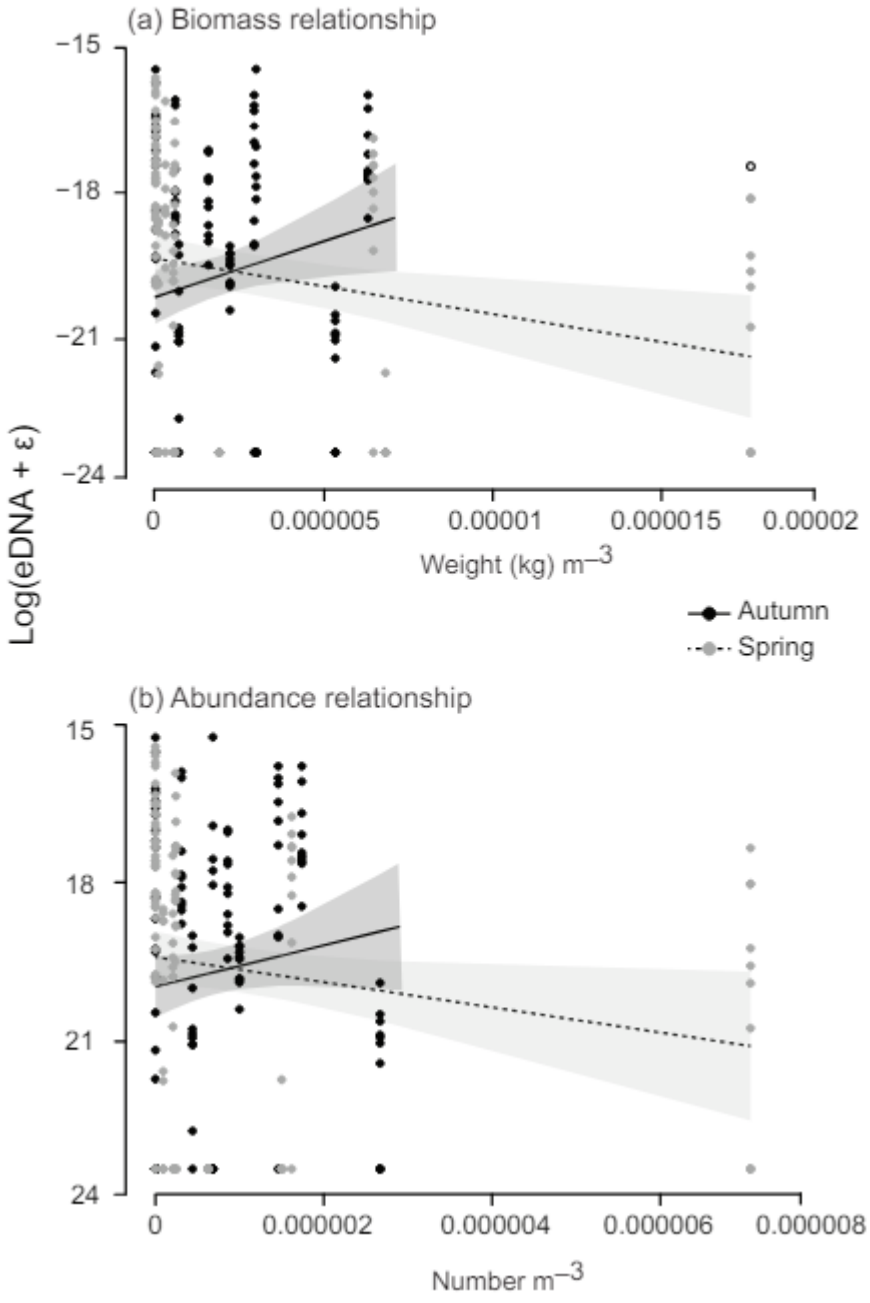
Figure 1. Map of a) Australia and b) New South Wales showing the hydroacoustic and eDNA sampling sites for Mulloway (*Argyrosomus japonicus*) in the Hawkesbury River, and c) diagrammatic representation of hydroacoustic and eDNA transects at one of the sites with the direction of sampling indicated by arrows. Hydroacoustic scans and eDNA sampling were conducted simultaneously along 10 transects beginning at the downstream end of a site and moving upstream. All sites were sampled twice; once in autumn 2022 and once in summer 2022.

Figure 2. Linear regressions between eDNA concentrations (ng l^{-1}) of Mulloway (*Argyrosomus japonicus*) and their hydroacoustically estimated (a) biomass and (b) abundance during autumn and spring. Points indicate raw data, while the lines are the regressions with and 95% confidence intervals either side.


(a)
Australia
(b)
New South Wales
(c)
Hawkesbury River
12
11
10
9
8
7
6
5
4
3
2
1
North
0
5
10
Km


(d)
River bank
Sampling upstream
River bank
12
6
Transects
1

$^{-}$1


(a) Biomass relationship
−15
−18
−21
−24
0
0.000005
0.00001
0.000015
0.00002
Weight (kg) m−3
Log(eDNA + ε)
Autumn
Spring
(b) Abundance relationship
15
18
21
24
0
0.000002
0.000004
0.000006
0.000008
Number m−3

Supplementary material

DNA extraction and inhibitor removal

DNA was extracted using a PureLinkTM Genomic DNA Mini Kit (Invitrogen) modified for use with filter papers (Wilkes Walburn et al., 2019). An extraction control consisting of DNA-free water instead of a filter was included with every batch of 24 filters. Prior to evaluating the concentration of Mulloway eDNA in each sample, inhibitors were first removed using a Zymo OneStep PCR Inhibitor Removal Kit (Integrated Sciences) following the manufacturer's instructions. Each sample was tested for remaining inhibition as described in (Rourke et al., 2024). If no inhibition was detected, Mulloway eDNA concentration was evaluated using neat (1:1) DNA. If there was inhibition detected, the Mulloway eDNA concentration was evaluated for the 1:10 dilution to reduce the effect of the inhibitors.

Calculation of limit of detection (LoD) and limit of quantification (LoQ)

The LoD and LoQ were previously calculated in (Wilkes Walburn et al., 2022). However the LoQ value (70) was reasonably high, potentially because the concentrations tested were not at a fine enough scale. We repeated the LoQ with the standards given in table S1. The curve-fitting model method was used to determine the LoD and LoQ with a 35% CV applied for LoQ (Klymus et al., 2020). The revised LoQ was two copies reaction^{-1} while LoD could not be modelled given no replicates failed. The LoD was determined as the lowest standard concentration where at least 95% of the replicates amplified, which was two copies reaction^{-1}.

qPCR amplification and calculation of DNA concentration

A fragment of the mitochondrial 12S rRNA gene was amplified using the primers A.jap_12S_F and A.jap12S_R and probe (FAM-dye-labelled) (Wilkes Walburn et al., 2019). We included a generic fish assay, which was designed to amplify endogenous DNA present in the filtered sample to ensure the success of the eDNA workflow from extraction to PCR amplification (Furlan and Gleeson 2016). To enable quantification of DNA, a standard curve was generated using six standards ranging in concentration from 10^{-3} and 10^{-8} ng μL^{-1}. Standards comprised of 166 bp of Mulloway 12S rRNA synthetic gBlock™ (Integrated DNA Technologies) oligonucleotides diluted in tRNA buffer (1:250 diluted in DNase/RNase water; Sigma-Aldrich). Six qPCR replicates were conducted for samples (including the EC) in 20 µl reactions comprising 10 µl TaqMan™ Environmental Master Mix 2.0 (Applied Biosystems), 1 µl Golden Perch TaqMan™ Gene Expression assay 20X (Applied Biosystems), 0.75 µl generic fish TaqMan™ Gene Expression assay 20X, 7 µl DNase/RNase-free water and 2 µl of DNA. We included six replicates of no-template controls (NTC) with every qPCR plate, and three replicates of a positive control consisting of synthetic Mulloway 12S rRNA with an eight-base pair reverse complement (gBlock™) to enable false positives from contaminating positive control to be identified. qPCR reactions were run on a QuantStudio3 PCR System (Applied Biosystems) with thermal cycling conditions set at 50 °C (2 min) and 95 °C (10 min), followed by 50 cycles of 95 °C (15 s) and 56°C (60 s). The concentration (ng) of DNA filter^{-1} was calculated by multiplying by 50 (for neat DNA) or 500 (for diluted DNA) given 2 µl of DNA was added to a qPCR reaction out of the 100 µl elution volume). Then the concentration of DNA in the volume filtered (ng/l) to be calculated (quantity of DNA in the filter divided by the volume of water filtered). The copies reaction^{-1} was calculated using the formula:

$$\frac{Concentration\ ng\ ul^{-1} \times (6.0221 \times 10^{23})}{(166 \times 660) \times (1\ \times\ 10^{9})}$$

Where 166 represents the amplicon size and 660 is the average molar mass (g mol^{-1}) per base pair.

Table S1. The data file used to run the LoD-calculator.R. Fluor (fluorescent dye), Sample standard concentrations, SQ (starting quantity), Cq (quantification cycle), and target (ARGJAP; *Argyrosomus japonicus*).

Sample name	Fluor	Samples/Standards	SQ (copies/µl)	SQ (copies/reaction)	Cq	Target
10^-2_1	FAM	10^-2	23041399	46082798	14.4	ARGJAP
10^-2_2	FAM	10^-2	23041399	46082798	11.5	ARGJAP
10^-2_3	FAM	10^-2	23041399	46082798	11.8	ARGJAP
10^-2_4	FAM	10^-2	23041399	46082798	11.4	ARGJAP
10^-2_5	FAM	10^-2	23041399	46082798	11.9	ARGJAP
10^-2_6	FAM	10^-2	23041399	46082798	12.1	ARGJAP
10^-2_7	FAM	10^-2	23041399	46082798	13.5	ARGJAP
10^-2_8	FAM	10^-2	23041399	46082798	13.3	ARGJAP
10^-3_1	FAM	10^-3	2304140	4608280	15.7	ARGJAP
10^-3_2	FAM	10^-3	2304140	4608280	15.0	ARGJAP
10^-3_3	FAM	10^-3	2304140	4608280	15.0	ARGJAP
10^-3_4	FAM	10^-3	2304140	4608280	15.5	ARGJAP
10^-3_5	FAM	10^-3	2304140	4608280	16.0	ARGJAP
10^-3_6	FAM	10^-3	2304140	4608280	15.0	ARGJAP
10^-3_7	FAM	10^-3	2304140	4608280	15.7	ARGJAP
10^-3_8	FAM	10^-3	2304140	4608280	16.7	ARGJAP
10^-4_1	FAM	10^-4	230414	460828	18.7	ARGJAP
10^-4_2	FAM	10^-4	230414	460828	18.1	ARGJAP
10^-4_3	FAM	10^-4	230414	460828	18.2	ARGJAP
10^-4_4	FAM	10^-4	230414	460828	18.3	ARGJAP
10^-4_5	FAM	10^-4	230414	460828	18.6	ARGJAP
10^-4_6	FAM	10^-4	230414	460828	18.6	ARGJAP
10^-4_7	FAM	10^-4	230414	460828	19.9	ARGJAP
10^-4_8	FAM	10^-4	230414	460828	19.7	ARGJAP
10^-5_1	FAM	10^-5	23041	46083	22.5	ARGJAP
10^-5_2	FAM	10^-5	23041	46083	21.9	ARGJAP
10^-5_3	FAM	10^-5	23041	46083	21.9	ARGJAP
10^-5_4	FAM	10^-5	23041	46083	22.3	ARGJAP
10^-5_5	FAM	10^-5	23041	46083	22.8	ARGJAP
10^-5_6	FAM	10^-5	23041	46083	22.2	ARGJAP
10^-5_7	FAM	10^-5	23041	46083	23.7	ARGJAP
10^-5_8	FAM	10^-5	23041	46083	22.9	ARGJAP
10^-6_1	FAM	10^-6	2304	4608	26.2	ARGJAP
10^-6_2	FAM	10^-6	2304	4608	25.8	ARGJAP
10^-6_3	FAM	10^-6	2304	4608	26.8	ARGJAP
10^-6_4	FAM	10^-6	2304	4608	26.1	ARGJAP
10^-6_5	FAM	10^-6	2304	4608	25.4	ARGJAP
10^-6_6	FAM	10^-6	2304	4608	27.4	ARGJAP
10^-6_7	FAM	10^-6	2304	4608	26.9	ARGJAP
10^-6_8	FAM	10^-6	2304	4608	27.8	ARGJAP

10^-7_1	FAM	10^-7	230	461	29.3	ARGJAP
10^-7_2	FAM	10^-7	230	461	28.5	ARGJAP
10^-7_3	FAM	10^-7	230	461	28.9	ARGJAP
10^-7_4	FAM	10^-7	230	461	28.9	ARGJAP
10^-7_5	FAM	10^-7	230	461	29.0	ARGJAP
10^-7_6	FAM	10^-7	230	461	29.0	ARGJAP
10^-7_7	FAM	10^-7	230	461	29.3	ARGJAP
10^-7_8	FAM	10^-7	230	461	30.4	ARGJAP
10^-8_1	FAM	10^-8	23	46	32.8	ARGJAP
10^-8_2	FAM	10^-8	23	46	33.2	ARGJAP
10^-8_3	FAM	10^-8	23	46	32.7	ARGJAP
10^-8_4	FAM	10^-8	23	46	33.4	ARGJAP
10^-8_5	FAM	10^-8	23	46	33.3	ARGJAP
10^-8_6	FAM	10^-8	23	46	33.9	ARGJAP
10^-8_7	FAM	10^-8	23	46	34.9	ARGJAP
10^-8_8	FAM	10^-8	23	46	35.0	ARGJAP
10c_1	FAM	10 copies	10	20	33.4	ARGJAP
10c_2	FAM	10 copies	10	20	33.0	ARGJAP
10c_3	FAM	10 copies	10	20	33.0	ARGJAP
10c_4	FAM	10 copies	10	20	33.5	ARGJAP
10c_5	FAM	10 copies	10	20	33.8	ARGJAP
10c_6	FAM	10 copies	10	20	32.9	ARGJAP
10c_7	FAM	10 copies	10	20	32.9	ARGJAP
10c_8	FAM	10 copies	10	20	34.4	ARGJAP
5c_1	FAM	5 copies	5	10	35.9	ARGJAP
5c_2	FAM	5 copies	5	10	34.5	ARGJAP
5c_3	FAM	5 copies	5	10	34.8	ARGJAP
5c_4	FAM	5 copies	5	10	34.5	ARGJAP
5c_5	FAM	5 copies	5	10	34.4	ARGJAP
5c_6	FAM	5 copies	5	10	34.4	ARGJAP
5c_7	FAM	5 copies	5	10	35.4	ARGJAP
5c_8	FAM	5 copies	5	10	35.3	ARGJAP
2c_1	FAM	2 copies	2	4	36.5	ARGJAP
2c_2	FAM	2 copies	2	4	35.9	ARGJAP
2c_3	FAM	2 copies	2	4	36.3	ARGJAP
2c_4	FAM	2 copies	2	4	35.7	ARGJAP
2c_5	FAM	2 copies	2	4	35.6	ARGJAP
2c_6	FAM	2 copies	2	4	36.0	ARGJAP
2c_7	FAM	2 copies	2	4	37.1	ARGJAP
2c_8	FAM	2 copies	2	4	36.2	ARGJAP
1c_1	FAM	1 copy	1	2	38.4	ARGJAP
1c_2	FAM	1 copy	1	2	38.1	ARGJAP
1c_3	FAM	1 copy	1	2	37.9	ARGJAP

1c_4	FAM	1 copy	1	2	36.6	ARGJAP
1c_5	FAM	1 copy	1	2	38.1	ARGJAP
1c_6	FAM	1 copy	1	2	37.2	ARGJAP
1c_7	FAM	1 copy	1	2	37.9	ARGJAP
1c_8	FAM	1 copy	1	2	39.2	ARGJAP

Table S2. The sites (see Fig. 1) and number of transects assessed during two seasons in the Hawkesbury River, their mean (±SE) depths, volumes and water quality metrics.

	Depth (m)		**Volume (km^3)**		**Temp (^oC)**		**DO ($mg\ l^{-1}$)**		**Salinity (PSU)**	
Site number	A	S	A	S	A	S	A	S	A	S
1	10.3 (0.72)	9.7 (0.71)	1.2×10^6 (0.01×10^6)	1.2×10^6 (0.01×10^6)	21.0 (0.35)	18.3 (0.25)	6.9 (0.08)	8.5 (0.0)	35.0 (0.05)	26.3 (1.60)
2	8.6 (0.11)	8.5 (0.04)	1.3×10^6 (0.03×10^6)	1.1×10^6 (0.01×10^6)	21.1 (0.15)	17.6 (0.10)	6.6 (0.03)	8.5 (0.04)	28.6 (0.70)	11.8 (0.20)
3	10.9 (0.28)	10.7 (0.26)	1.0×10^6 (0.06×10^6)	1.0×10^6 (0.04×10^6)	20.4 (0.20)	19.2 (0.05)	7.2 (0.15)	10.5 (0.34)	28.6 (0.40)	17.3 (0.15)
4	14.0 (0.22)	12.9 (0.27)	0.7×10^6 (0.01×10^6)	0.7×10^6 (0.02×10^6)	19.3 (0.05)	17.7 (0.25)	7.3 (0.04)	8.3 (0.03)	21.3 (0.10)	11.6 (0.05)
5	9.5 (0.3)	8.4 (0.36)	1.4×10^6 (0.04×10^6)	1.4×10^6 (0.03×10^6)	19.2 (0.30)	18.4 (0.65)	7.5 (0.30)	8.3 (0.03)	12.7 (1.65)	8.3 (0.10)
6	3.6 (0.17)	3.2 (0.22)	0.2×10^6 (0.01×10^6)	0.2×10^6 (0.01×10^6)	18.9 (0.0)	20.3 (0.25)	8.2 (0.18)	7.9 (0.02)	20.8 (0.25)	8.3 (0.25)
7	5.1 (0.19)	4.7 (0.18)	0.5×10^6 (0.01×10^6)	0.5×10^6 (0.02×10^6)	19.5 (0.15)	21.5 (0.65)	9.1 (0.02)	9.1 (0.04)	7.0 (0.00)	5.5 (0.40)
8	15.0 (0.42)	12.1 (0.61)	0.4×10^6 (0.02×10^6)	0.4×10^6 (0.02×10^6)	18.5 (0.25)	18.0 (0.10)	8.8 (0.08)	8.4 (0.10)	0.7 (0.41)	3.3 (0.35)
9	3.9 (0.31)	2.3 (0.42)	0.2×10^6 (0.01×10^6)	0.2×10^6 (0.02×10^6)	17.9 (0.25)	22.9 (0.00)	8.7 (0.06)	7.2 (0.04)	1.7 (1.63)	3.3 (0.00)
10	14.8 (0.52)	16.1 (0.98)	0.3×10^6 (0.01×10^6)	0.4×10^6 (0.02×10^6)	17.3 (0.05)	22.5 (0.10)	8.7 (0.02)	8.4 (0.0)	0.2 (0.00)	0.2 (0.01)
11	5.7 (0.22)	13.6 (1.28)	0.5×10^6 (0.02×10^6)	0.5×10^6 (0.04×10^6)	16.9 (0.10)	22.7 (0.15)	9.1 (0.35)	8.3 (0.15)	0.2 (0.01)	0.01 (0.00)
12	11.7 (2.0)	11.5 (1.74)	0.4×10^6 (0.03×10^6)	0.5×10^6 (0.04×10^6)	16.7 (0.05)	22.5 (0.05)	8.6 (0.02)	8.2 (0.05)	0.1 (0.01)	0.01 (0.00)

DO, dissolved oxygen, Temp, temperature

Appendix 7: Rourke, M., Fowler, A., Hughes, J., Broadhurst, M., DiBattista, J., Fielder, S., Wilkes Walburn, J., and Furlan, E. 2022. Estimating the biomass of two iconic Australian fish species using eDNA. 1st Australian and NZ Environmental DNA conference, Hobart, Tasmania, 14–17, February, 2023

Estimating the biomass of one (but not two) iconic Australian fish species using eDNA

Australia's commercial and recreational fisheries are each valued in excess of $2.5 billion per annum. Despite their undeniable collective importance, very few Australian species are supported by appropriate stock assessments. Most species are characterised by severe shortfalls in much of the required basic data, driven by the low value of many individual stocks, the high cost of independent monitoring and the subsequent reliance on low-quality fishery-dependent data, where available. There is a clear requirement to develop fishery-independent population-monitoring approaches for key species that simultaneously encompass sufficient spatial coverage and are cost-effective. A promising genetic approach involves collecting and analysing environmental DNA (eDNA). In the past decade, there have been increasing efforts at estimating the abundance and/or biomass of aquatic organisms from concentrations of eDNA in the water, and with varying levels of success. The objective of this project was to determine if eDNA concentration could accurately estimate the abundance or biomass of two iconic Australian species: Mulloway (*Argyrosomus japonicas*) and Murray Cod (*Maccullochella peelii*). We present the results of a series of aquaria trials to test the effects of time, temperature and fish density on eDNA concentrations. Preliminary results indicate a clear relationship between eDNA concentration and the abundance/biomass of Mulloway but not Murray Cod. These data will be used to inform future field trials testing the relationship between eDNA concentration and biomass as determined from traditional sampling techniques and hydroacoustic surveys.

Duncan M [1,2] Fowler A [3] ,Hughes J [3] , Broadhurst M.K. [4,5] , DiBattista J [6] , Fielder S [7] , Wilkes Walburn J [1] , Furlan E [8]

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[8] Centre for Conservation and Ecology and Genomics, Institute for Applied Ecology, University of Canberra, Bruce ACT, Australia

Estimating the biomass of one (but not two) iconic Australian fish species using eDNA

Meaghan Duncan, Matt Broadhurst, Ashley Fowler, Julian Hughes Jackson Wilkes-Walburn, Stewart Fielder, Joseph DiBattista and Elise Furlan.

1st Australian and New Zealand eDNA Conference 2023

regional.nsw.gov.au

Key challenges for fisheries scientists

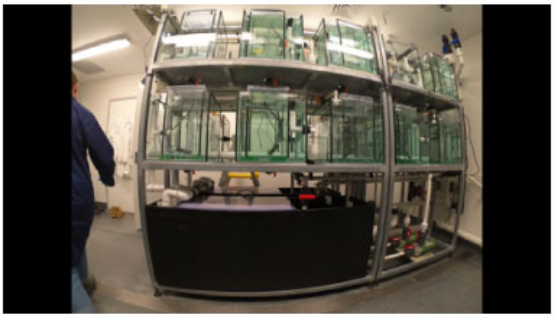
- What species are present?
- What is their distribution?
- How many are there?

Rourke et al. (2022). Environmental DNA (eDNA) as a tool for assessing fish biomass: a review of approaches and future considerations for resource surveys. *Environmental DNA.*

Study species

Murray cod experiments

Narrandera Fisheries Centre 2020.



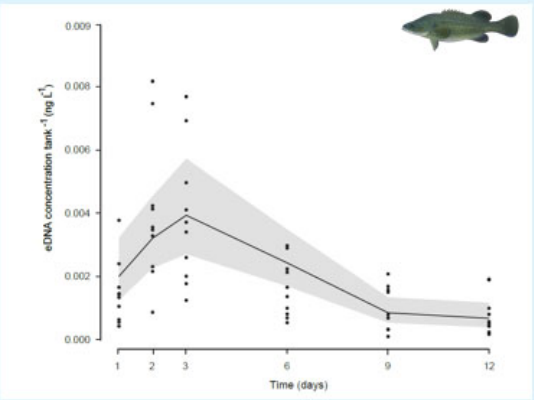
Rourke, M.L.; Walburn, J.W.; Broadhurst, M.K.; Fowler, A.M.; Hughes, J.M.; Fielder, D.S.; DiBattista, J.D.; Furlan, E.M. Poor utility of environmental DNA for estimating the biomass of a threatened freshwater teleost; but clear direction for future candidate assessments. *Fisheries Research* ***2023, 258.***

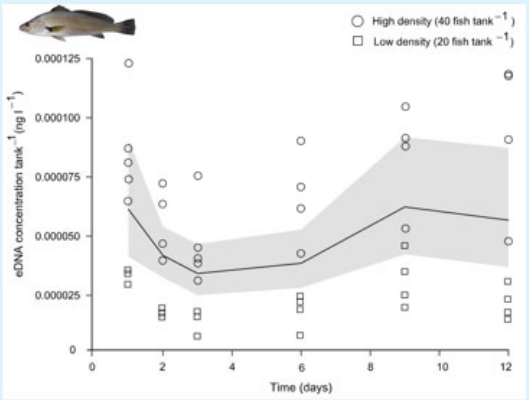
Mulloway experiments

Port Stephens
Agricultural Institute
2021

Rourke, M.L.; Broadhurst, M.K.; Fowler, A.M.; Walburn, J.W.; Hughes, J.M.; Fielder, S.; DiBattista, J.D.; Furlan, E.M. Investigating the potential utility of environmental DNA to provide a relative abundance index for the depleted teleost, mulloway *(Argyrosomus japonicus*). Diversity 2022 (Accepted pending revisions)

Time to eDNA stabilisation



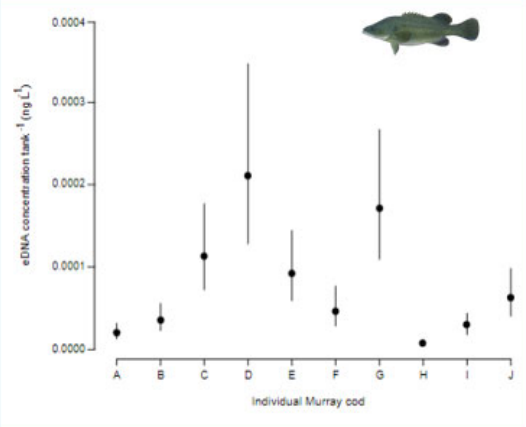


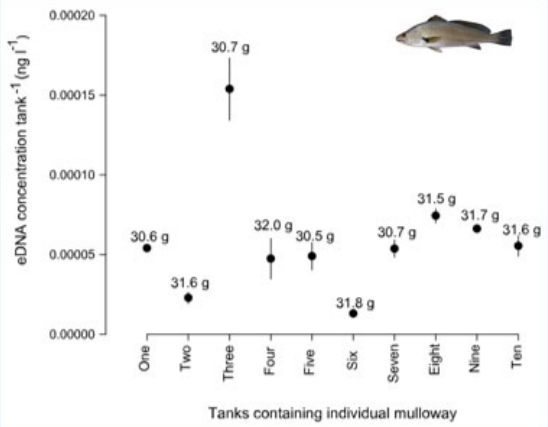
Solid lines indicate the general linear model fit and shaded area the 95% confidence intervals.

Effect of temperature

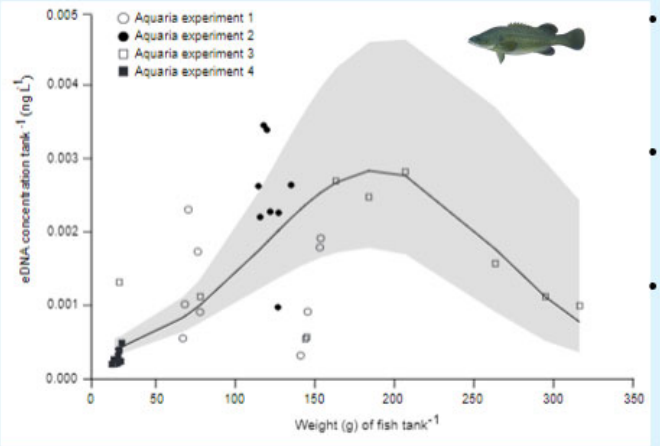
- No effect for Murray cod (put temps in here).
- Significant effect of temperature for mulloway, with predicted eDNA concentrations ~1.5 times greater at warmer versus cooler temperatures (generalized linear mixed model; $p < 0.05$).

Individual variability



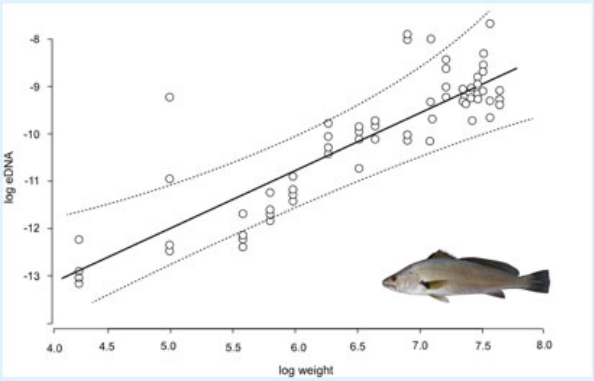


Relationship between eDNA concentration and biomass – Murray cod



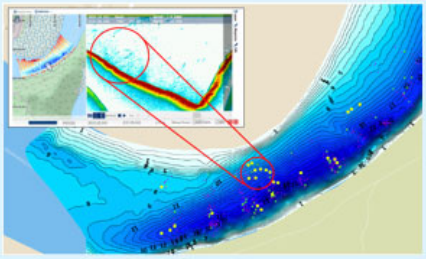
- No relationship between eDNA concentration and fish weight (experiment 3)
- No relationship eDNA concentration and fish weight in mesocosms (adult fish)
- Significant quadratic relationship between fish weight and eDNA concentration for data pooled across experiments (excluding mesocosms).

Relationship between eDNA concentration and biomass – mulloway



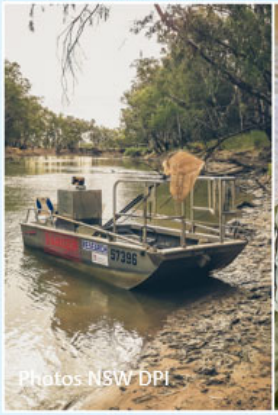
Data points are the raw data, solid and dashed lines indicate the linear model fit and 95% confidence intervals, respectively.

Field validation



Summary

- Click to add text



Acknowledgements

- Funding: FRDC
- Mulloway experiments: Luke Cheviot, Brendan Findlay and Daniel Yee.
- Murray cod experiments: Elka Blackman, Jonathon Doyle, Ian Wooden, Matt McLellan, Lachlan Jess, Stuart Rowland.
- NSW DPI ACEC 20/01

Appendix 8. Initial contact with fisheries managers including the abstract of the literature review

Meaghan Duncan

From: Meaghan Duncan
Sent: Thursday, 27 February 2020 2:16 PM
To: Darren Hale; Bryan van der Walt; Cameron Westaway
Subject: Update on FRDC project 'Estimating the biomass of fish stocks using novel and efficient genetic techniques'

Hi Daren, Brian and Cam,

Last year we (myself, Julian Hughes, Ash Fowler, Matt Broadhurst, Elise Furlan (University of Canberra) and Joey DiBattista (Australian Museum)) put together an application for an FRDC grant for a project to investigate whether eDNA could be used as a fishery-independent method to evaluate the abundance and/or biomass of Murray cod and Mulloway. The original project partner was Doug Ferrell, but he has since moved on. I'm contacting you all as the project is relevant to your roles and may provide useful information to assist with management of these two species once it is complete.

Our application was successful but does have a number of stop-go points that need to be completed prior to commencing the subsequent phase of the project. Our first stop-go point is the completion of a literature review to justify the commencement of the next phase of the project (aquaria trials). We've spent the last four months writing a literature review on the topic with a focus on fish (see abstract below). The results of the review were extremely encouraging with 92% of studies showing a positive relationship between eDNA concentration and abundance or biomass of fish (though with many biotic and abiotic factors influencing results). This justifies the continuation of our project to the next phase – aquaria trials to assess if there is a positive relationship between eDNA concentration and biomass in our two species. We wanted to keep you in the loop as the project progresses and are happy to provide the full review once it has been approved by the FRDC. Please let me know if you have any questions and I will continue to provide updates as the project progresses.

Kind regards,

Meaghan

Abstract

The sustainable harvest of global fisheries resources requires accurate information on spatio-temporal variation in population abundance and/or biomass. For many stocks the required information remains elusive however because of a reliance on, in some case, less-than-accurate fishery-dependent data, or costly fishery-independent data. Among modern quantitative methods, environmental DNA (eDNA) is one approach that is revolutionising our ability to document the biodiversity of aquatic organisms in freshwater and marine environments. In the last decade, increasing efforts have been made to determine if eDNA concentrations are correlated with the abundance or biomass of aquatic species, which ultimately may facilitate the rapid, low-cost quantification of wild fisheries stocks. Here we sought to review the available literature in this emerging field and identify key influencing intrinsic and extrinsic factors to support the future refinement of efforts. In total, 58 papers (2000 to 2020) satisfied the search criteria, of which 92% identified positive and coherent relationships between eDNA and population abundances/biomasses of key species, albeit with some variability in precision compared to conventional fishery-survey methods. The key influencing biotic factors included taxon, life history, diet, metabolism, and behavioural state. Key abiotic factors included mostly hydrological processes affecting source dispersal of eDNA, especially water flow and temperature, although eDNA collection methods were also important for ensuring accurate representation of quantitative data. There is strong evidence to support using eDNA, in some cases, as an immediate, ancillary tool for assessing population biomass and abundance across discrete spatio-temporal scales. Ongoing species-specific characterization will improve the precision of estimates and support boarder application. The development of this approach should be supported considering that it provides a non-lethal means for assessing populations, which is particularly important for exploited stocks.

Appendix 9. Rourke, M.L., Fowler, A.M., Hughes, J.M. Broadhurst, M.K., DiBattista, J.D., Walburn, J.W., Furlan, E.M., Estimating the biomass of two iconic Australian fish species using eDNA. Abstract submitted for consideration at the World Fisheries Congress 2020

Meaghan L. Rourke[1], **Ashley Fowler**[2], **Julian Hughes**[2], **Matt K. Broadhurst**[3], **Joseph D. DiBattista**[4,5], **Stewart Fielder**[6], **Jackson Wilkes Walburn**[1], and **Elise Furlan**[7]

[1]*Department of Primary Industries, Narrandera Fisheries Centre, PO Box 182, Narrandera NSW 2700, Australia.*

[2]*New South Wales Department of Primary Industries, Sydney Institute of Marine Science, Building 19, Chowder Bay Road, Mosman, New South Wales 2088, Australia*

[3]*NSW Department of Primary Industries, Fisheries Conservation Technology Unit, PO Box 4321, National Marine Science Centre, Coffs Harbour, New South Wales 2450, Australia*

[4]*Australian Museum Research Institute, Australian Museum, Sydney, New South Wales 2010, Australia*

[5]*School of Molecular and Life Sciences, Curtin University, Bentley, Western Australia 6102, Australia*

[6]*NSW Department of Primary Industries, Port Stephens Fisheries Institute, Locked Bag 1, Nelson Bay, New South West 2315, Australia*

[7]*Institute for Applied Ecology, University of Canberra, University Drive, Bruce, Australian Capital Territory 2617, Australia*

Correspondence: meaghan.duncan@dpi.nsw.gov.au

Australia's commercial and recreational fisheries are annually valued in excess of $2.5 and $2.6 billion, respectively, yet the biological stock status of many fished species remains uncertain, placing these resources at considerable risk. Most species are characterised by shortfalls in data required to make representative and reliable stock assessments. This is driven by the low economic value of many stocks and the high cost of fishery-independent monitoring, the consequence of which is a reliance on low-quality fishery-dependent data for assessment, where available. There is a clear requirement to develop fishery-independent population-monitoring approaches for such species that encompass sufficient spatial coverage and are cost-effective. A genetic approach showing promise in filling this requirement involves collecting and analysing environmental DNA (eDNA). Like all organisms, fish release mucus, faeces, gametes and skin cells comprising complex mixtures of intra- and extra-cellular DNA into their immediate environment where it remains suspended and detectable for varying periods of time. Simply collecting water samples from aquatic systems can capture the eDNA present at that moment in time and space, enabling the species present to be identified. Additionally, in the past decade, there have been increasing international research efforts towards estimating abundance or biomass of aquatic organisms from the concentration of eDNA in the water, with varying levels of success. The objective of this project was to determine if eDNA concentration could be used to accurately estimate the abundance or biomass of two iconic

Australian fish species, the freshwater Murray Cod (*Maccullochella peelii*) and marine Mulloway (*Argyrosomus japonicus*). Controlled experiments were first used to assess the applicability of this technique to the priority species, to ensure the relationship between eDNA concentration and abundance or biomass are robust. The results of a series of aquaria trials to test the effect of time, temperature and biomass/abundance on eDNA concentration in these two model species are presented. These data will be used to inform future field trials that will test the relationships between eDNA concentration and/or biomass, determined by traditional fishery-independent sampling techniques.

Appendix 10. Non-technical summary distributed to key stakeholders including resource managers

Environmental DNA (eDNA) as a tool for assessing fish biomass: a review of approaches and future considerations for resource surveys

Globally, fisheries resources are under a lot of pressure, simply because of unchecked fishing effort and, in many cases, few data about the biomasses of important stocks. Historically, the required information has been sought by sampling fishing industries and/or scientific cruises, whereby various labour-intensive and expensive methods (including tagging) are applied.

A more recent, alternative approach that might have application for estimating fish populations involves sampling environmental DNA (eDNA). All organisms release DNA into the environment during various excretions. Simply sampling water and assessing the concentrations of DNA may provide information on the numbers of a particular species that recently inhabited that area. A few studies have assessed the utility of eDNA for such a purpose, but until now there has not been any attempt at reviewing this research to determine key benefits/limitations.

We addressed this shortfall by reviewing all studies (55) published between 2000 and 2020 and found 93% identified positive relationships between eDNA concentrations and the abundance and/or biomass of the focus species. Key factors that affected the accuracy of population estimates included the type of species examined as well as their body size, distribution, reproduction and migration, along with water flow and temperature.

Notwithstanding some variability owing to the factors above, there is overwhelming evidence to support using eDNA for assessing fish populations across key areas and times. The main advantages of eDNA monitoring relative to other approaches include reduced costs, increased efficiencies and non-lethal sampling. The research momentum associated with this method implies a strong potential for utility in future fisheries resource assessments throughout the world.

Appendix 11. Letters to recreational fishers and industry to inform of the hydroacoustic surveys to be carried out on the Hawkesbury River (November 2021)

Estimating the biomass of fish stocks using traces of DNA left in the water

A project funded by the FRDC and NSW DPI is underway to determine whether environmental DNA – the DNA that fish shed into the water – can be used to estimate the population size of fish species at a sampling location. This method shows great promise in studies of fish populations around the world and may provide a low-cost and accurate way of assessing fish stocks without the need to physically capture fish.

NSW DPI Fisheries has engaged InfoFish Australia to conduct hydroacoustic surveys of fish populations at several locations on the Hawkesbury River between Wisemans Ferry and the mouth of the estuary for a month starting 8, November,2021. The hydroacoustic surveys will accurately estimate the biomass of fish populations at these sites and then this will be compared to the concentration of environmental DNA collected at the same time. This approach will allow NSW DPI to assess whether environmental DNA shows promise as a technique for fisheries stock assessments.

The project team comprises six researchers from NSW DPI (Meaghan Duncan, Matt Broadhurst, Ashley Fowler, Julian Hughes and Jackson Wilkes Walburn), one from the University of Canberra (Elise Furlan) and one from the Australian Museum (Joey DiBattista). The project will run until 2024 with up to 3 more trips to the Hawkesbury River to collect data.

So far, the work has demonstrated a correlation between eDNA concentration in controlled aquaria conditions. This next field phase of the project will seek to determine if we can demonstrate a similar correlation between eDNA and population size in a natural environment.

For any information on the project, please contact Meaghan Duncan:

meaghan.duncan@dpi.nsw.gov.au

Yours sincerely

Meaghan Duncan
Research Scientist
3 November 2021

To whom it may concern,

The purpose of this letter is to let you know that NSW DPI fisheries researchers are undertaking field work on the Hawkesbury River (between Wisemans Ferry and Broken Bay) during 8–19, November and 1–8, December 2021 (subject to change if the weather is unsuitable). The field work is part of a research project entitled, "Estimating the biomass of fish stocks using novel and efficient genetic techniques", and simply involves our contractors (InfoFish Australia) conducting bathymetry mapping and hydroacoustic surveys. No fishing gears are being deployed. Rather, the vessel will simply complete a zig-zag pattern at several sites. On most days of the surveys, we will also have a DPI scientist on board to filter water samples for environmental DNA. Please see the attached document for a brief background on the project and our experimental design.

InfoFish Australia have been made aware of the need to comply with the NSW DPI Biosecurity - Aquatic Fieldwork Hygiene Procedure to avoid spreading diseases. InfoFish Australia have also provided a list of locations that their vessel has been in the past six months (Attachment A). In addition, the InfoFish Australia vessel (Attachment B) will have a Fisheries Research banner attached to make it clear to the general public and industry that it is conducting research.

Please feel free to contact me if you have any questions.

Yours sincerely,

Meaghan Duncan

Research Scientist
Meaghan.duncan@dpi.nsw.gov.au
3 November 2021

Attachment A. The InfoFish vessel.

Appendix 12. Correspondence with stakeholders (summaries – September 2022)

To whom it may concern,

The purpose of this letter is to let you know that NSW DPI fisheries researchers are undertaking field work on the Hawkesbury River (between Wisemans Ferry and Broken Bay) commencing on Monday the 17th of October and finishing up on Saturday the 23rd of October 2022 (subject to change if the weather is unsuitable). The field work is part of a research project entitled, 'Estimating the biomass of fish stocks using novel and efficient genetic techniques', and involves our contractors (InfoFish Australia) conducting bathymetry mapping and hydroacoustic surveys. No fishing gears are being deployed. Rather, the vessel will simply complete a zig-zag pattern at several sites. On all days of the surveys, we will also have a DPI fisheries technician on board to filter water samples to collect environmental DNA. Please see the attached document for a brief background on the project and our experimental design.

InfoFish Australia have been made aware of the need to comply with the NSW DPI Biosecurity - Aquatic Fieldwork Hygiene Procedure to avoid spreading diseases. In addition, the InfoFish Australia vessel will have a Fisheries Research banner attached to make it clear to the general public and industry that it is conducting research.

Please feel free to contact me if you have any questions.

Yours sincerely,

Meaghan Duncan

Research Scientist
Meaghan.duncan@dpi.nsw.gov.au
27 September 2022

Attachment A. The InfoFish vessel.

NSW DPI research project entitled: Estimating the biomass of fish stocks using novel and efficient genetic techniques

Project background

Australia's commercial and recreational fisheries are annually valued at in excess of $2.5 and 2.6 billion, respectively and comprise a plethora of species. Despite their undeniable importance, very few economically important species are supported by appropriate stock assessments. The recent national Status of Australian Fish Stocks (SAFS) initiative requires biennial updates of population statuses, and with more species added each reiteration. Most species are characterised by severe shortfalls in much of the required basic data, driven by the low value of many stocks, the high cost of independent monitoring and the subsequent reliance on low-quality fishery-dependent data, where available. This project seeks to facilitate a pathway forward for rapidly providing fishery-independent abundance estimates using Mulloway as a case study.

Population declines for both species warrant rigorous and representative monitoring and assessment. Yet both species are challenging to monitor using traditional fishery-dependent approaches. Murray Cod were commercially fished to 2003, but the industry focussed on only a small part of the species' range in the southern Murray-Darling Basin. There is a recreational fishery, which is geographically broad and costly to representatively sample. Mulloway are caught by both fishing sectors, yet the primary source of monitoring data comes from the commercial sector which is increasingly limited owing to variable gear selectivity, the introduction of commercial fishing exclusions within key estuaries, and recent removal of bycatch allowances preventing the retention of individuals smaller than the minimum legal length (MLL) to be retained. There is a clear requirement to develop fishery-independent population-monitoring approaches for both species that simultaneously encompass sufficient spatial coverage and are cost-effective.

One applicable, novel method involves modern genetics to estimate absolute abundances. Such work is in its infancy, but with lowered costs as technology improves, there is a need for cutting-edge approaches to be explored that increase the accuracy and speed of assessments over traditional approaches. More specifically, there is potential for estimating biomass from the concentration of environmental DNA (eDNA) collected from water samples. Put simply, this method does not require labour-intensive sampling—instead relying on capturing DNA shed by the target species (environmental DNA or eDNA) into the water and using those data to estimate abundances.

Experimental design

We have successfully demonstrated that under controlled conditions in aquaria that eDNA concentration is correlated to Mulloway biomass. Therefore, we are now conducting field trials to determine whether eDNA concentration in the Hawkesbury River can be used to estimate the biomass of wild stocks at 12 locations.

Field validation in the Hawkesbury River

Traditional detection approaches have requirements to sample at particular locations or times of the year to enable appropriate comparisons between sites/years. Potentially, eDNA may have a similar requirement. A spatio-temporally stratified design will be used to examine the utility of regular sampling as an indicator of relative abundance at the estuary/system scale. Water samples will be collected at replicate sites within 12 zones of the Hawkesbury River estuary (to target Mulloway), NSW twice per year for two years. The eDNA concentrations will be compared against biomass estimates determined by in-depth hydroacoustic surveys to determine if eDNA concentration is an

accurate predictor of biomass or abundance. We have engaged InfoFish Australia (http://infofishaustralia.com.au/) to conduct the hydroacoustic surveys to accurately estimate the biomass of Mulloway in 12 × ~1-km reaches of the Hawkesbury River while we simultaneously filter water samples for later eDNA extraction. These surveys are made up of two components:

1) Pre-survey; where bathymetric and habitat data are collected to describe the physical shape and depth of a site waterbody and determine the habitats/substrates present at a site/waterbody (to be carried out during November 2021, see Table 1 for dates). Each site will be surveyed at low tide and at high tide to determine which is most suitable for reliably estimating the abundance of Mulloway. eDNA samples from two sites will also be collected at this time to determine whether there is a better correlation with biomass at low or high tide (see eDNA methods below).

2) The fish biomass survey; completed once the pre-survey data have been collected, collated and modelled (to be carried out in December 2021, see Table 1 for dates). eDNA samples will be collected concurrently from each site during the biomass survey.

Pre-survey: bathymetric data

Bathymetric data are required to inform transducer pitch settings and to build a model of each site, which feeds into post-processing of fish biomass data. Bathymetry data will be collected using Humminbird echosounders at each site, collated, and models and maps generated using ReefMaster1. All bathymetry data will be tide adjusted using the latest river height data from the Patonga, Spencer, Gunderman and Wisemans Ferry stations (at least), published by the Bureau of Meteorology. See Images 1 and 2 for examples of boat tracks during bathymetric survey and a generated bathymetric map.

Image 1) boat track during bathymetry survey

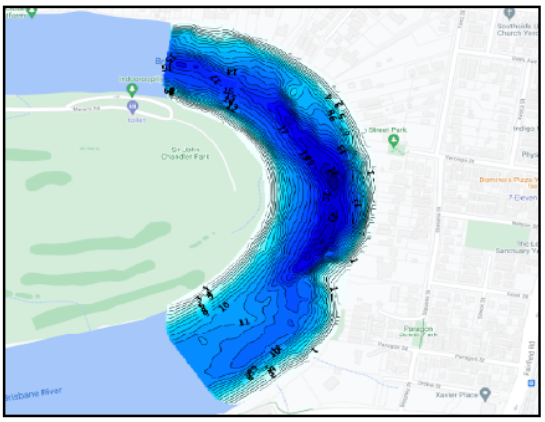


Image 2) generated bathymetric map

Pre survey: habitat data

Habitat data are collected during the bathymetry survey using Humminbird echosounders at each site. The sidescan imagery is recorded to SD cards and habitat mosaics are generated using the ReefMaster software package. Habitat data are used to determine the habitat substrates present at each site (e.g., rock bars, sandy or muddy substrates, etc.).

Fish (Mulloway) biomass survey

Fish biomass data will be collected using the BioSonics DT-X Extreme, split beam scientific echo sounder. For the pilot study, six sites are included. Two sites will be surveyed each day, once each at

the top and bottom of the tide. For the second survey, 12 sites will be surveyed, with frequency and timing as determined by the pilot study.

At each site, a 1-km reach of river will be surveyed with transects spaced at 50m apart (21 per reach). The transducer will be set in a side-scanning configuration with the angle of pitch at each site set depending on the bathymetric profile at each site. Common angles of pitch range from 3o in shallow (<10 m) water to 8o in deeper (>30 m) water.

The DT-X provides quantitative data on fish abundance, target strength (a measure of the area of a sonar target) and, when analysed with bathymetry models, location in the water column. From target strength data, fish length can be calculated, and fish-target size graded.

Raw fish biomass data will be extracted from Visual Acquisition. All required information (object identification, fish counts, size grading, GPS coordinates, volumetric calculations, etc.) are analysed in RStudio. From the analysis, models and maps can be created which include distribution and density. Biomass estimates will be calculated and reported as fish per hectare or fish per megalitre.

Table 1. The timing of the bathymetry and biomass surveys in November and December 2021.

Item	Details	November																				
		8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
Pre-Survey	Bathymetry Mapping					*																
	Shoreline Imagery					*																
	Habitat Imaging / Mapping					*																
	Site Physical Data Prep																					
	Survey Reach Finalisation / Approval																					
Survey	Fish Biomass																					
Post-processing	Biomass Data Prep																					
	Data Analysis																					
	Output Generation and Reporting																					
* Contingency																						

Item	Details	December																				
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Pre-Survey	Bathymetry Mapping																					
	Shoreline Imagery																					
	Habitat Imaging / Mapping																					
	Site Physical Data Prep																					
	Survey Reach Finalisation / Approval																					
Survey	Fish Biomass																					
Post-processing	Biomass Data Prep																					
	Data Analysis																					
	Output Generation and Reporting																					

eDNA collection and analyses

The eDNA will be collected using an eDNA backpack (Smith-Root) to collect and filter water in a single step. This purpose-built eDNA sampling unit uses a new sterile, self-preserving filter housing and filter paper (supplied in individual sealed packages) for each sampling event, thus reducing the risk of contamination compared to traditional water sampling that involves water collection, transport and filtration steps. Samples will be collected from immediately below the surface. To account for uneven dispersion of eDNA in the water, we will take ten, 2-L replicate water samples from each sampling site.

At each sampling location, an equipment control will be taken by filtering 2 L of UV sterilised water. This will be treated as an additional replicate that is processed in the laboratory in the same way as the other replicates in order to check for contamination of equipment. Following collection of the equipment control, the ten replicate sites will be sampled covering a distance of approximately 1km. Sampling will be conducted in evenly spaced transects. A fresh filter housing containing 5-µm filters

will be used at each site with the eDNA backpack set at a flow rate of 1L/min, with a pressure threshold of 10 psi until a minimum of 2 L of water is filtered or until the filter is clogged (flow ≤ 0.1 L/min) (Thomas et al., 2018). If the filter paper does clog, a fresh filter unit will be fitted until the full 2 L of water has been successfully filtered (eDNA will be extracted from both filter papers in separate extractions and then the eDNA will be combined. A maximum of two filters will be used for a single replicate.

If the full 2 L of water cannot be filtered, the volume filtered will be noted to enable this to be accounted for during data analysis. Upon returning to the laboratory, filter will be stored in the refrigerator 4°C for later eDNA extraction. All filter papers will be treated in the same way to preclude any confounding effects. Following completion of water filtration at each sampling site, 3 L of 1% bleach solution will be run through the tubing and pump system and then fully flushed with clean water to avoid any damage to the unit. The body of the unit will also be wiped thoroughly with a 1% bleach solution.

Species-specific primers will be designed to amplify a short mitochondrial 12S rRNA gene region in Mulloway. The 12S rRNA gene is preferred given its utility to differentiate among fish species combined with minimal within-species variation. We will download all available Mulloway sequences from GenBank as well as all native and introduced species. We will then align all the sequences and design primers using Geneious Prime and will ensure primers have a maximum number of mismatches to non-target species. The selected primer pair for each species will be tested on closely related species (at least three individuals per species) that occur anywhere in Australia as well as on species with the lowest number of mismatches (2-4 base pairs) to the primers by performing PCR reactions to identify any amplification of non-target species.

Laboratory work will be undertaken in the Narrandera Fisheries eDNA laboratory. This laboratory has separate rooms for DNA extraction and PCR set-up. It has UV lights to enable overnight decontamination of surfaces and the air and all steps of the DNA extraction and PCR set up will be carried out within UV hoods. In addition, all equipment, where possible, will be sterilised with 10% bleach solution. Each PCR plate will include positive and negative controls, as well an endogenous control designed for eDNA in freshwater systems to ensure PCR reactions were successful (Furlan & Gleeson, 2016).

Data analyses

All data will be analysed using linear models (LMs) or generalised linear models (GLMs) fitted in R to examine the correlation between eDNA concentration and biomass.

Appendix 13. Lay summaries for each publication

Lay summary for literature review.

Globally, fisheries resources are under a lot of pressure, simply because of unchecked fishing effort and, in many cases, few data about the biomasses of important stocks. Historically, the required information has been sought by sampling fishing industries and/or scientific cruises, whereby various labour-intensive and expensive methods (including tagging) are applied.

A more recent, alternative approach that might have application for estimating fish populations involves sampling environmental DNA (eDNA). All organisms release DNA into the environment during various excretions. Simply sampling water and assessing the concentrations of DNA may provide information on the numbers of a particular species that recently inhabited that area. A few studies have assessed the utility of eDNA for such a purpose, but until now there has not been any attempt at reviewing this research to determine key benefits/limitations.

We addressed this shortfall here by reviewing all studies (55) published between 2000 and 2020 and found 93% identified positive relationships between eDNA concentrations and the abundance and/or biomass of the focus species. Key factors that affected the accuracy of population estimates included the type of species examined as well as their body size, distribution, reproduction and migration, along with water flow and temperature.

Notwithstanding some variability owing to the factors above, there is overwhelming evidence to support using eDNA for assessing fish populations across key areas and times. The main advantages of eDNA monitoring relative to other approaches include reduced costs, increased efficiencies and non-lethal sampling. This method will become the mainstay of future fisheries resource assessments.

Lay summary for eDNA assay development paper.

- Analysis of environmental DNA can facilitate a comprehensive understanding of the presence and distribution of aquatic species. However, species-specific environmental DNA detection using quantitative PCR requires validated and standardised assay design for each target species.
- Murray Cod*, Maccullochella peelii* and Mulloway, *Argyrosomus japonicus* are two iconic and ecologically vulnerable species that have been subject to recreational and commercial exploitation in Australia.
- This study presents two environmental DNA assays to detect *M. peelii* and *A. japonicus*, based on small fragments of the mitochondrial 12S ribosomal RNA gene. A comprehensive description of species-specific assay development, from assay design to testing in silico, in vitro and in situ has been included to guide effective assay design and validation in future studies.
- The results of this study indicate that the *M. peelii* and *A. japonicus* assays are highly specific and highly sensitive to as little as 4 and 10 copies of the 12S gene fragment per PCR reaction, respectively. We demonstrate that these assays are effective tools for detecting their target species in situ from environmental DNA samples, which will assist in the conservation and management of two important freshwater and marine fish species.

Lay summary for Mulloway (*Argyrosomus japonicus*) aquaria trial paper.

- Mulloway are an economically important coastal species off southeastern Australia, but stocks have suffered excessive fishing pressure.

- Detailed data describing Mulloway distributions and abundances are required, and one non-invasive way of obtaining such information might involve analysing their so-called 'environmental DNA' (i.e. their individual signatures in an area from their constantly shed DNA).
- Before applying eDNA approaches, confirmation on the suitability of Mulloway as a candidate species is required.
- Recent experiments have shown evidence of a useful positive association between eDNA concentrations and the abundance of Mulloway in aquaria.
- Additional field trials are planned, and it is hoped these trials will support analysing eDNA as a practical solution for learning more about Mulloway populations in NSW.

Lay summary for Murray Cod (*Maccullochella peelii*) aquaria trial paper.

Effective management of fisheries resources requires accurate information of the biomass of important stocks. A new approach to estimate biomass using fragments of DNA that fish leave behind in the water (environmental DNA or eDNA) is increasingly being implemented around the world.

This study used a range of aquaria and pond experiments to determine whether the concentration of Murray Cod eDNA was related to biomass to determine whether this may be an effective tool for estimating biomass in wild populations.

Results showed that there was no useful relationship between eDNA concentrations and biomass and thus eDNA is not a suitable method for monitoring the biomass of Murray Cod in the wild.

Lay summary for Golden Perch (*Macquaria ambigua*) field trial paper.

- Inexpensive, non-lethal methods of estimating fish biomass are required to minimise costs and environmental impacts. Collecting so-called 'environmental DNA' in water samples has been postulated to satisfy the above criteria but requires species-specific validation.
- This study confirmed the utility of eDNA for providing a similar relative index of Golden Perch biomass as electrofishing (at sites with up to ~5 kg of fish).
- Future surveys might benefit from using eDNA to monitor population changes among Golden Perch across areas with known low biomasses.

Appendix 14. Lectures/seminars where the project was disseminated to stakeholders

- Invited lecture: BIOS3081 Ocean to Estuarine Ecosystems Undergraduate Course, University of New South Wales, Australia, April 2023.
- Invited public seminar: Underwater Research Group NSW, Griffith University, Gold Coast, QLD, April 2023, The Oaks Hotel, Neutral Bay, NSW, March 2023.
- Public seminar: Future Science Talks Series, East Village Hotel, Darlinghurst, NSW, March 2023.
- Invited Seminar: Lord Howe Island Museum, Lord Howe Island, December 2022.
- Invited Seminar (virtual presentation): NSW Marine Parks Submissions Working Group, November 2022.
- Invited Seminar (virtual presentation): Applied Research Center for Environment & Marine Studies Research Institute, King Fahd University of Petroleum and Minerals, Dhahran, Saudi Arabia, October 2022.
- Invited lecture: Mulloway harvest strategy working group (comprising various stakeholders), May, 2022.
- Invited lecture: Port meeting in Brooklyn (commercial fishers and fishing managers), May 2022.

Appendix 15. FishNews article Link to article

A proof of concept using environmental DNA paves the way for more efficient and affordable sampling to estimate relative fish biomasses in rivers and estuaries.

By Catherine Norwood

Environmental DNA, or eDNA, holds new promise for identifying not only the presence of different species in any given habitat, but also for estimating their relative biomasses over space and time.

Over the past decade, with improved sequencing technology, eDNA has rapidly gained momentum for use in environmental surveillance on land and in water.

Jackson Wilkes Walburn gathering water samples from a Golden Perch larval pond at the Narrandera Fisheries Centre for eDNA testing. Source: NSW DPIRD

What is eDNA?

Environmental DNA (eDNA) is the genetic materials left by organisms in the environment. Every organism sheds its DNA in both life and death. It can be found in tissue fragments skin cells or eggs, in mucus, faeces and urine. Every sample of river or seawater is effectively a kind of DNA soup.

Fisheries scientists can look for DNA of a specific species in a sample – a process particularly suited to biosecurity surveillance. Alternatively, all the DNA in a sample can be sequenced, to identify the community of organisms in a particular habitat.

This process is made possible by the advent of new technology that makes high throughput sequencing possible and cheap.

Both processes rely on having sample DNA in a reference to match DNA sequenced to that of a known organism. Global DNA libraries collectively already contain DNA sequences for hundreds of thousands of organisms, with entries growing daily.

> **While eDNA is already well recognised for its ability to determine the presence or absence of species, less certain has been its ability to assess the biomass of a species. Biomass is defined as the weight of all individuals in each volume.**

This is a "rapidly advancing area of research" says Dr Meagan Duncan from the NSW Department of Primary Industries and Regional Development's (DPIRD) Narrandera Fisheries Centre.

Meaghan leads the new eDNA laboratory at Narrandera and leads an FRDC-funded research project testing whether eDNA can identify fish biomass in rivers and estuaries (2019-016).

"There are potential cost savings with eDNA and also offer a less invasive way of sampling fish biomass than electrofishing or relying on catch-and-effort data which might not be available for many species," she says.

Tank and field trials

Mulloway (*Argyrosomus japonicus*) was chosen as the marine species for tank trials with Murray Cod (*Maccullochella peelii*) and Golden Perch (*Macquaria ambigua*) as the freshwater species.

A juvenile Mulloway used in eDNA tank trials. Source: NSW DPIRD

> **These trials showed a positive relationship between fish biomass stocked in tanks and eDNA analysed in water samples for both Mulloway and Golden Perch. The results for Murray Cod were less clear.**

Field trials for Mulloway were conducted at 12 sites on the Hawkesbury River. Fisheries monitoring consultancy InfoFish used hydroacoustics to identify Mulloway fish numbers at selected sites. Soundwaves have a distinct acoustic pattern or signature when they encounter Mulloway, making the species easy to identify. Water samples to test for eDNA were collected at the same time.

Meaghan says that while Mulloway live in both estuarine and marine waters, depending on the stage of their life cycle, this project focused on estuarine sampling in spring and autumn. The autumn sampling provided a clear positive relationship between the hydroacoustic populations detected and the eDNA results.

Sampling of Golden Perch was done in conjunction with an electrofishing sampling project funded by the Commonwealth Environmental Water Holder. Samples were taken from five sites on each of the Lachlan, Murrumbidgee and Wakool Rivers.

Jarrod Chapman collecting water samples using the eDNA backpack in the upper Murrumbidgee River. Source: NSW DPIRD

The results showed a positive relationship between the biomass of Golden Perch found in the electrofishing and the eDNA sampling.

"We were also able to compare the electrofishing and eDNA results for Bony Bream (*Nematalosa erebi*) and Murray Cod, and found a positive relationship for both, although the Murray Cod results were the weakest."

eDNA benefits

Meaghan says collecting water samples for eDNA makes it a faster, cheaper and less labour-intensive technique than electrofishing.

> **"The beauty of eDNA sampling is that anyone can collect water samples because it doesn't require a lot of training.**

"Our results essentially provide a 'proof of concept' for using eDNA to assess biomass in freshwater and estuarine environments. But using eDNA to estimate relative biomass is likely going to become feasible in the future," says Meaghan.

FRDC Related Project

2019-016 Estimating the biomass of fish stocks using novel and efficient genetic techniques