

Research Article

Use of Environmental DNA to Determine Fantail Darter (*Etheostoma flabellare*) Density in a Laboratory Setting: Effects of Biomass and Filtration Method

Ramon A. Guivas and Ben F. Brammell 

Department of Science and Health, Asbury University, Wilmore, KY 40390, USA

Correspondence should be addressed to Ben F. Brammell; ben.brammell@asbury.edu

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Estimating fish abundance/biomass holds great importance for freshwater ecology and fisheries management, but current techniques can be expensive, time-consuming, and potentially harmful to target organisms. Environmental DNA (eDNA) has proven an effective and efficient technique for presence/absence detection of freshwater vertebrates. Additionally, recent studies report correlations between target organism density/biomass and eDNA levels, although widespread application of this technique is limited by the number of studies examining this relationship in various species and settings. Additionally, filter clogging is a commonly encountered issue in eDNA studies in environments with significant sediment and/or phytoplankton algae. Frequently, a sample must be split into multiple aliquots and filtered separately in order to process the entire sample. The present study examines both the relationship between biomass and eDNA and the effects of single versus multiple filter sampling on eDNA concentrations of fantail darters (*Etheostoma flabellare*) in a laboratory setting. Tank tests were performed in quadruplicate at four environmentally relevant fantail biomass levels. eDNA samples were collected and processed in parallel (one as a whole through a single filter and one in parts through multiple filters). Species-specific primers and a probe were developed for *E. flabellare* from cytochrome b sequences obtained from locally collected specimens, and real-time quantitative PCR was used to analyze eDNA levels at each biomass. Significant correlations were observed with increasing biomass for both methods, although this relationship was stronger for samples processed by the multiple filter method. These data should be useful in eDNA studies in which turbidity necessitates the use of multiple filters per sample as well as in the use of eDNA to estimate darter populations.

1. Introduction

Since its inception in macroinvertebrate studies in 2008 [1], environmental DNA (eDNA) has become firmly established as an effective method [2–4] and holds great promise for increasing the ease and scope of ecological studies. In aquatic systems, single species eDNA detection has primarily been applied to invasive species monitoring and conservation of threatened or endangered species [5]. Invasive species monitored using eDNA include Asian carp (*Hypophthalmichthys* sp.) [6] and multiple species of molluscs [7–9], crustaceans [10–12], amphibians [1, 13, 14], and reptiles [15, 16]. Rare and endangered taxa eDNA monitoring has included species such as Chinook salmon

(*Oncorhynchus tshawytscha*) [17], eastern hellbenders (*Cryptobranchus alleganiensis*) [18, 19], great crested newts (*Triturus cristatus*) [20], the Japanese crayfish (*Cambaroides japonicas*) [21], and the Trinidad golden tree frog (*Phytotriades auratus*) [22], among others. Single species presence/absence eDNA monitoring is a well-established and widely used technique, in some cases enabling detection of cryptic species where traditional methods were unsuccessful [23, 24].

Recent studies also reveal correlations between organismal abundance and eDNA levels [25–28], indicating the possibility of quantitative eDNA analysis for population assessment. Laboratory studies with jack mackerels (*Trachurus japonicus*) [29], adult sea lampreys (*Petromyzon*

marinus) [30], round gobies (*Neogobius melanostomus*) [31], and common carp (*Cyprinus carpio*) [25] all report positive correlations between eDNA levels and fish density or biomass. However, larval lamprey densities showed no correlation with eDNA in tank experiments [30]. Field studies examining the use of eDNA to quantify tadpole populations of the stream dwelling frog *Odorrana splendida* report a significant, but not strong, correlation between biomass and eDNA levels [27]. A study examining spawning salmon abundance and eDNA found eDNA levels varied with salmon density as well as numerous other factors [28]. Likewise, Lacoursière-Roussel et al. [32] reported a positive but weak correlation between catch per unit effort and lake trout (*Salvelinus namaycush*) eDNA levels. Additionally, numerous studies have observed seasonal fluctuations in eDNA, which are often believed to be associated with reproductive behavior [18, 28, 33]. While an emerging understanding supports the quantitative use of eDNA in population assessment, many factors appear to influence eDNA release [34] and persistence [35] in the environment, and additional studies are needed to validate eDNA as a density detection tool across various habitats and species [28, 36, 37].

Although quality assurance guidelines concerning eDNA collection and extraction have been established [38], numerous sample collection and preservation techniques exist, and the technique has been demonstrated to impact detection probability [39–43]. Although both precipitation and filtration have been used to capture DNA, filtration has been demonstrated to capture more eDNA from water samples [39, 41, 42] and as such is currently overwhelmingly the method of choice in eDNA studies. The clogging of filters by phytoplankton or suspended sediment is a frequently encountered issue in eDNA collection [25, 38, 44], particularly in turbid, lentic waters [45]. Filter clogging is typically countered by prefiltering [25], using larger filter pore sizes [25], or by dividing the sample and utilizing several filters to minimize clogging [19, 38]. Both prefiltering [46] and increasing filter pore size [42, 47] have been shown to reduce eDNA recovery. Although dividing a sample among several filters is a frequently employed technique [19], we are unaware of any studies examining its impact on eDNA efficacy or detection probability. The present study examines the hypothesis that multiple, as opposed to single filter, processing of water samples maintains or improves eDNA efficacy.

Darters are classified as Etheostominae, a subclade of Percidae that contains approximately 250 species endemic to eastern North America, comprising more than 20% of North America ichthyofauna [48]. Significant anatomical and physiological differences exist between darters and other members of Percidae [49, 50]. Darters are frequently in need of study as threatened or endangered species; currently, 27 darter species are listed as federally threatened or endangered on the U.S. Endangered Species List. Fantail darters (*Etheostoma flabellare*), a small, stream dwelling darter, common throughout much of their range in eastern North America [51, 52], were selected for this study as easily accessible members of this wide ranging subclade. We are

aware of only one other darter eDNA study, in which eDNA was used to detect the presence of the federally endangered slackwater darter (*Etheostoma boschungii*) [53]. The present study provides data useful to approaching darter eDNA density studies.

eDNA offers great promise as a compliment [54] and perhaps in some cases a substitute for traditional field studies where such studies are difficult or impractical. This study provides novel data concerning two parameters of great significance to the application of eDNA in field ecology studies:

- (1) The effect of increasing target organism density on eDNA levels
- (2) The effect of single versus multiple filters to quantitatively assess eDNA concentrations

2. Methods

2.1. Fish Collection and Housing. Fantail darters (*Etheostoma flabellare*) were collected from Jessamine Creek (Jessamine County, Kentucky) via electrofishing (KYDFW Permit#1811153) and acclimated to lab conditions in aged tap water over seven days prior to transfer to experimental tanks. Initial temperature of holding water in the lab matched stream temperature (6°C) at the time of collection. Water temperature was increased 2°C a day for seven days until it reached 20°C, and water was maintained at 20°C throughout the remainder of the holding period and the experiment. Fish were housed in a 208-liter tank prior to the experiment and fed commercially purchased blood worms ad libitum. All applicable international, national, and institutional guidelines for the ethical care and use of fish were followed.

2.2. eDNA Trials. Four environmentally relevant fantail darter biomass levels were calculated based on published studies of observed fantail darter densities [55, 56]. Tank tests for each of the four biomass levels were performed in quadruplicate; a negative control tank was included for each level to assess potential cross contamination between tanks. Experimental tanks (38 L) were held in an environmental chamber under regulated conditions (12 h day/12 h night, 20°C, aeration) for 96 hours, and fish were fed blood worms ad libitum during the course of the experiment.

2.3. eDNA Collection. Takahara et al. [25] and Maruyama et al. [57] both reported that eDNA initially spiked and then reached equilibrium near day four in fish tank tests, so a 96-hour end point was utilized for the present study. At 96 hours, all darters were removed, and each tank was homogenized by stirring for 1 minute to homogeneously distribute DNA. Two one liter water samples were collected from each tank using 3.8-liter high-density polyethylene containers not previously exposed to animal DNA and thoroughly washed with bleach and rinsed with distilled water. Water samples were processed through a 47 mm diameter glass microfiber filter (VWR, 0.42 mm thickness

and 0.7 μm pore size) in a manner similar to previous studies [6, 42, 58] with vacuum filtration immediately using one of the two methods: one sample from each tank was filtered through a single filter and the other was divided into three 333 ml aliquots, each of which was filtered separately. Filters were extracted immediately.

2.4. DNA Extraction. eDNA extraction was performed using a DNeasy blood and tissue kit (Qiagen), demonstrated to provide superior yields relative to other extraction methods [59], and a modified version of a published protocol [54]. Briefly, whole filters were cut into 30–40 pieces and incubated at 56°C overnight in 720 μL ATL buffer and 80 μL Proteinase K. Final elutions were performed twice into 400 μL of AE buffer, and the extracted DNA was stored at –20°C until analysis.

Tissue DNA extraction was performed using a DNeasy blood and tissue kit (Qiagen) according to the provided protocol. Tissue was lysed overnight at 56°C in proteinase K and eluted twice to increase DNA yield.

2.5. Primer Design. Cytochrome b was sequenced (GenBank: KT880219.1) from locally collected fantail darters (Jessamine Creek, Jessamine County, KY: 37.859380, –84.630755) using published primers [60]. Using this sequence, a species-specific primer probe assay was designed that amplifies a 118 bp amplicon within *E. flabellare* cytochrome b:

Forward primer: 5'-AAGCGAAGAAGCGAGTTAGG-3'

Reverse primer: 5'-GGTGCTACGGTCATCACTAATC-3'

Probe: 5'-6 FAM/CCCACATAA/ZEN/G GCACTG-CAGAGAGT/3IABkFQ-3'

Three other species of the family Percidae occurring sympatrically with *E. flabellare* in mid-sized central Kentucky streams were considered in the design of the primers and probes. We sequenced cytochrome b from locally collected (Jessamine Creek, Jessamine County, KY: 37.859380, –84.630755) sympatric specimens using the same primer set [60] and designed primers and probes with a minimum of three mismatches in each oligonucleotide (f. primer, r. primer, and probe) based on reported requirements for specificity [61]. Sympatric species considered include *Etheostoma caeruleum* (KT880220.1), *Etheostoma blennioides* (KT880218.1), and *Percina caprodes* (KT880217.1). Nonspecific amplification was tested using tissue extracted DNA from each of these species.

2.6. eDNA Analysis. Dilution of tank-extracted samples showed evidence of inhibition based on reduced product DNA yields despite the use of TaqMan Environmental Master Mix 2.0. Nondiluted samples amplified 9.8% of the DNA yield observed at the 1:20 dilution that was used for analysis (data available in Appendix A). A 1:20 dilution was selected based on previous studies indicating optimal yields observed at 1:20 dilutions [16] and the lower biomass to water ratios utilized in the present study relative to that

study. Environmental DNA was quantified using a StepOnePlus™ Real-Time PCR system (Life Technologies, Carlsbad, CA, USA) in optical 96-well PCR plates. Each plate contained no fish control tank samples to assess contamination and fantail tissue samples as a positive control. Each 20 μL reaction contained the following: TaqMan EMM (10 μL), nuclease free water (7 μL), eDNA extract (2 μL), and *E. flabellare* primer/probe mix (1 μL). Thermocycler conditions were as follows: 95°C for 10 min, 55 cycles of 95°C for 15 s, and 60°C for one minute.

DNA extractions from *E. flabellare* fin clips were used to generate a standard as standards for the qPCR analysis. We used a Qubit 4 Fluorometer (Thermo Fisher) and a Qubit 1X dsDNA HS Assay Kit (Cat. No. Q32850) to quantify the DNA concentrations from the tissue extract. We then diluted a 7.88 ng/ μL fantail darter DNA extract to five levels to use as a standard curve: 0.5 pg/l, 5 pg/l, 50 pg/l, 500 pg/l, and 5,000 pg/l. These dilutions cover the range of DNA concentrations that were observed in tank water-extracted eDNA in this study.

2.7. Statistical Analysis. Type I linear regression was used to examine the relationship of darter biomass and eDNA concentration within a single filter method following the previously published methods [44]. Both eDNA concentration and fish biomass were log transformed following the previously published methods [44, 62]. All tests were performed using IBM SPSS Statistics 25.

3. Results

Data from the single filter method trial indicated a positive correlation between darter biomass and eDNA concentration (Figure 1(a), $p = 0.326$, $r^2 = 0.08$). Interestingly, the multiple filter method demonstrated a significant ($p = 0.034$) and stronger correlation ($r^2 = 0.30$) between these same two variables in parallel analysis (Figure 1(b)).

Amplification was observed in every tank containing darters, but no amplification was observed in any of the no fish control tanks, included in each trial to assess potential transfer of DNA between tanks. PCR efficiency was 97.2% as determined using a standard curve of diluted *E. flabellare* tissue.

4. Discussion

The few laboratory studies that have been completed examining eDNA and biomass utilize biomass ranges up to 200-fold, much greater than the more environmentally realistic 2-, 4-, and 8-fold differences examined in the present study. In tank studies (200 L) with Japanese mackerel (*Trachurus japonicus*) with an average of 6.9 g, 40.2 g, and 319.5 g of total biomass per tank, eDNA shedding rates differed between all groups at 13°C but not at 18, 23, or 28°C [29]. The treatments in this study represent a 6-fold and 46-fold increase in biomass in the upper two treatments compared to the lower. Trials with sea lampreys (*Petromyzon marinus*) reported significant increases in biomass with 0, 2, 20, and 200 adults per 2000 L tank but not with 0, 1, 5, and 25 larva in 28 L

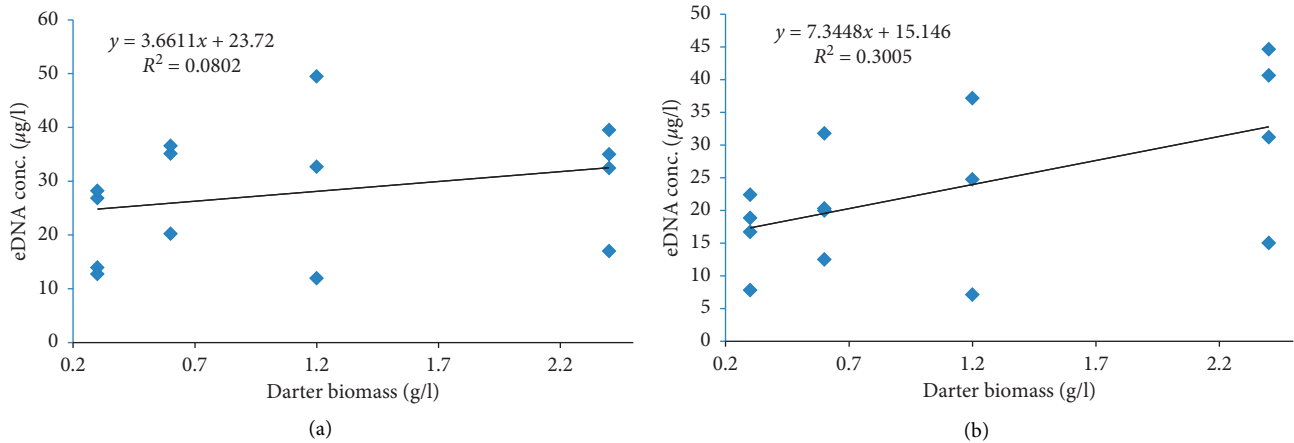


FIGURE 1: eDNA concentrations at various biomass levels for single filter method ($p = 0.326$) (a) and multiple filter method ($p = 0.034$) (b).

aquaria [30]. Assuming homogenous weight of the adult sea lampreys, these treatments exceeded biomass levels in the present study in all but the lowest treatment. Juvenile carp (*Cyprinus carpio*) trials in 9 L tanks found significant correlations between 16 g, 79 g (5-fold), and 158 g (10-fold) biomass and eDNA levels [25], representing similar biomass level treatments to those utilized in the present experiment. The results of the present experiment represent the lowest biomass/eDNA relationships tested to date and are consistent with the results of previous studies, most of which reported positive correlations between biomass and eDNA.

The relationship between biomass and eDNA levels was significant and positive for both filter methods, although the r^2 values were not large (0.29 and 0.49). These values are similar to those observed by Iwai et al. [27]. When comparing tadpole abundance (0.32) and biomass (0.29) to eDNA copy number in lotic samples sites, they observed large variations in the biomass-eDNA relationship within a single stream. Likewise, Baldigo et al. [44] reported r^2 values of 0.44 (density) and 0.25 (biomass) in Adirondack mountain headwater brook trout (*Salvelinus fontinalis*) populations. These data support a growing data set indicating a loose correlation between organism abundance and eDNA concentrations.

The results of the present study support the premise that multiple filters improve the resolving capacity of eDNA as the combined filter method (333 ml per filter) demonstrated a significant relationship between biomass and eDNA, while the same samples processed in a single filter (1 L per filter) did not. Hunter et al. [63] report a 10% increase in DNA yield utilizing multiple as opposed to single filters; although this was not visible in our study, it is possible that a larger sample size would have revealed a similar trend. Filtering larger volumes of water leads to a greater concentration of inhibitors [64], which perhaps could be linked to greater inconsistency in DNA detection in single filter samples if inhibitors are removed more effectively in the multiple filter process as less inhibitor would be present in each filter during the extraction process. Regardless of the mechanism, these results provide evidence that the use of multiple filters to avoid the frequently encountered problem of filter clogging [65–67] is acceptable and perhaps beneficial.

The positive correlation between biomass and eDNA has been previously observed, but not in a small, stream dwelling species at environmentally relevant densities. Doi et al. [26] reported positive correlations between *Plecoglossus altivelis* densities as estimated from snorkeling surveys and eDNA levels. Klymus et al. [68] observed a positive correlation in eDNA and biomass in two species of bighead carp (*Hypophthalmichthys nobilis* and *Hypophthalmichthys molitrix*). Our work builds on these previous efforts by comparing environmentally relevant biomass levels with eDNA concentrations and providing data supporting the validity of utilizing multiple, versus single, filters in field studies.

Data Availability

The data used to support the findings of this study are available upon request to the corresponding author.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Supplementary Materials

Table 1: nondiluted eDNA concentrations for nondiluted samples. These data were not used in the publication because they appear to be inhibited relative to 1 : 20 diluted samples. Table 2: eDNA concentrations for samples diluted 1 : 20 following DNA extraction. Table 3: CT values for samples following a 1 : 20 dilution of extracted DNA. Table 4: CT values for nondiluted samples. Figure 1: eDNA concentrations at various biomass levels for single filter method (A) ($p = 0.031$) and multiple filter method (B) ($p = 0.002$) for nondiluted samples. These data were not used in the paper because they appear to be inhibited relative to 1 : 20 diluted samples. (*Supplementary Materials*)

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