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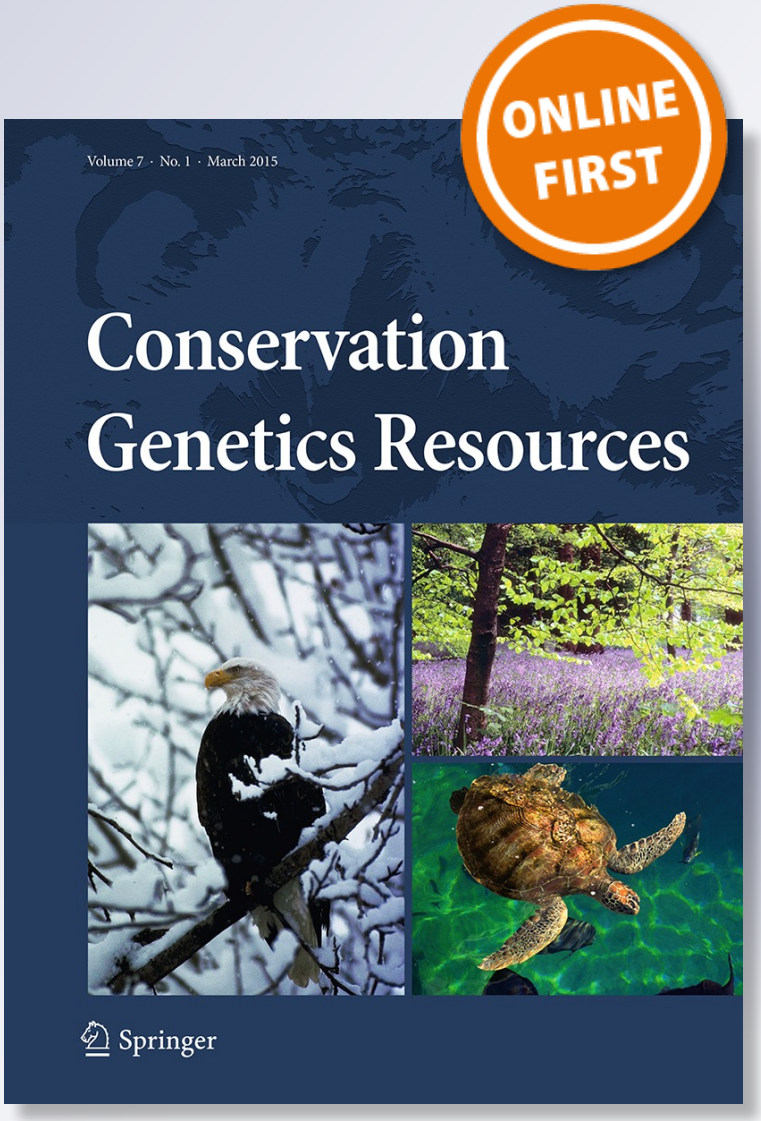

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TECHNICAL NOTE

Environmental DNA particle size distribution from Brook Trout (*Salvelinus fontinalis*)

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Abstract Environmental DNA (eDNA) sampling has become a widespread approach for detecting aquatic animals with high potential for improving conservation biology. However, little research has been done to determine the size of particles targeted by eDNA surveys. In this study, we conduct particle distribution analysis of eDNA from a captive Brook Trout (*Salvelinus fontinalis*) in a flow-through mesocosm. Our data suggest that 1.2–10-µm particles are the most common size of eDNA from Brook Trout, which is consistent with our hypotheses that eDNA in the environment is comprised of loosely aggregated smaller particles, resulting in high inter-sample heterogeneity. These findings are similar to those of a study on Common Carp (*Cyprinus carpio*) in lentic systems.

Keywords Detection · eDNA · Mitochondria · Fish

Sampling water for environmental DNA (eDNA) allows for detection of aquatic organisms without ever seeing a live individual (Lodge et al. 2012). These approaches may be more sensitive and cost-effective than traditional sampling methods, making eDNA a potentially powerful tool for conservation biology. eDNA has recently been applied to detect rare invasive species (e.g., Jerde et al. 2011) and endangered native species (e.g., Biggs et al. 2015) including fishes (Takahara et al. 2013) amphibians (Pilliod et al. 2013), reptiles (Piaggio et al. 2013), mollusks (Goldberg et al. 2013), arthropods (Mächler et al. 2014), and mammals (Foote et al. 2012). However, to fulfill its potential as a conservation tool, we need to learn more about those factors that affect both the production and persistence of eDNA in complex aquatic environments.

A key example is the particle size of eDNA, which has not been determined empirically for most species, but has been attributed in the literature to everything from extracellular DNA to sloughed tissues (e.g., Dejean et al. 2011). DNA of different particle sizes may degrade or be deposited at different rates in the environment, and eDNA sampling approaches (e.g., precipitation vs. filtration) vary in the size of particles that they target. As a result, determining the size of eDNA particles sampled for species detection is a critical step in understanding eDNA dynamics and capture.

Turner et al. (2014) sought to determine the size of eDNA particles from Common Carp (*Cyprinus carpio*) in lentic environments using sequential filtration of environmental samples. Particles 1–10 µm across, corresponding to the size of individual mitochondria or small cells, constituted the most common size class of Common Carp eDNA (Turner et al. 2014). Here, we present the results of a comparable experiment to determine the particle size distribution of eDNA from Brook Trout (*Salvelinus fontinalis*) under different environmental conditions (cold, lotic mesocosms). Brook Trout are a species of special conservation concern within their native range (Hudy et al. 2008) and an important invasive species globally (e.g., Dunham et al. 2002; Bosch et al. 2006). Further, studies of Brook Trout eDNA may be directly transferable to eDNA studies

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of other salmonids of conservation concern (e.g., Laramie et al. 2014).

In August 2014 a single Brook Trout was captured by angling in West Fork Lolo Creek, MT, USA (N 46.687168°, W 114.557371°) and placed in a flow-through mesocosm (0.35 m × 0.5 m × 0.22 m) fed by a fishless, ephemeral tributary. This tributary was confirmed free of Brook Trout DNA by sampling 1 month and again several days prior to the experiment (5-L samples; see protocol below). Twenty-four hours after fish addition, we collected five sequential 0.5-L samples in disposable polyethylene bottles from the tank outflow. The fish was then returned to its capture site.

For each 0.5-L sample, 100 mL was sequentially filtered through four filter pore sizes from largest to smallest. This filter series included three 47-mm-diameter polycarbonate track-etched (PCTE) filters (0.4, 1.2, and 10-µm pores) and one 47-mm-diameter nylon net filter (60-µm pores; no PCTE filter was available in this size; Millipore). We did not assess eDNA particles smaller than 0.4 µm because, in a pilot study, long filtration times made smaller pore sizes impractical for many field applications (≥1 h per filter for 1–5 L sample volumes). As a result, we may not have detected particles of eDNA of smaller sizes, such as extracellular DNA. Filters were held in disposable filter holder funnels (Fisher Scientific) attached to silicone hosing (Masterflex) and were pumped using a peristaltic pump (GeoTech Inc.,) so that the outflow was captured by the next filter in the sequence. New filter holders and hoses were used between each sample. We also filtered 100 mL of distilled water through a sequence of filters as an equipment control. Using new forceps for each sample, filters were folded in half and then rolled into sterile 1.5-mL tubes and stored on ice until arrival at the lab (within 6 h), where they were stored in a −20 °C freezer until extraction. All samples were filtered in the field within 2 h of collection from the mesocosm tank.

We extracted DNA from these samples and equipment controls using the DNeasy Blood and Tissue Kit and QIAShredder columns (Qiagen; protocol adapted from Goldberg et al. 2013; final elution into 100 µL sterile TE; Integrated DNA Technologies; IDT). All extractions were performed in a room reserved for extracting non-invasive genetic samples where no PCR products or other sources of high concentration DNA are handled. Extracted samples were stored in a −20 or −80 °C freezer until qPCR analysis.

We used a species-specific TaqMan-MGB assay (*BRK2*; Wilcox et al. 2013) to estimate Brook Trout mitochondrial DNA (mtDNA) concentrations in all samples and equipment controls. Experiments were performed in 15-µL volumes with 4 µL of template DNA and final concentrations of 1× TaqMan Environmental Mastermix 2.0 (Life Technologies), a 1× assay mix (primers each at 900 nM, probe at 250 nM), and a VIC-labeled exogenous internal amplification control (Life Technologies TaqMan Exogenous Control Kit). We used cycling conditions of 95 °C/10 min (95 °C/15 s, 60 °C/60 s) × 45 cycles on a StepOne Plus Real-time PCR Instrument (Life Technologies). The internal amplification control acted as a test for PCR inhibitors in the samples, as evidence by a >1 cycle threshold (C_t) shift in the amplification curve relative to that in a no template control. There was no evidence of inhibition in this study.

The qPCR plate also included a negative PCR setup control and a series of five known-quantity target standards diluted to 6250, 1250, 250, 50 and 10 copies/reaction. This standard was composed of a linearized, synthetic gene from IDT containing the 139-bp sequence of interest (Wilcox et al. 2013). Samples were quantified by comparison with the standard curve ($r^2 = 0.93$, efficiency = 99.6 %) using the Cy_0 method, which is less sensitive to low levels of PCR inhibitors than the C_t method (Guescini et al. 2008). Cy_0 values were calculated from raw amplification data using the package *qpcR* (Ritz and Spiess 2008) in *R* v. 3.0.1 (R Core Team 2013). All standards, controls, and samples were run in triplicate and copy number is reported as the mean estimate for all triplicate wells.

There was no amplification in any of the equipment or PCR setup controls; there was amplification for all 60 sample reactions (20 samples run in triplicate). For each sequential filtration replicate, mtDNA copy number for all four samples was summed (median = 24 × 10^6, range = 14–41 × 10^6 copies/L), and the copy number estimates from each filter were divided by this total to report the proportion of eDNA in each size class (Fig. 1).

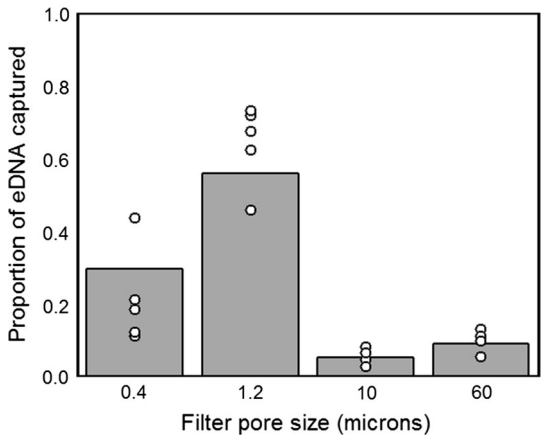

Fig. 1 *Bars* show the mean proportion of the brook trout eDNA that comes from each size class (0.4–1.2, 1.2–10, 10–60, and ≥60 µm) for each of five water samples (individual samples shown with *white points*). As in Turner et al. (2014), particles approximately 1–10 µm across are the most abundant source of the fish eDNA

Our results were generally consistent with the findings of Turner et al. (2014) in that particles 1.2–10 μm were the largest source of Brook Trout eDNA. This suggests that this particle size is likely to be the most useful to target for capture in the field (e.g., via in situ filtration). Also, as in Turner et al. (2014), we observed high inter-sample variability (the maximum DNA concentration was almost 3× the minimum in our five samples), which is surprising because the particle sizes captured by filtration suggest that the eDNA is dominated by individual cells or mitochondria. In Turner et al. (2014) and in this study, a well-mixed solution containing many independent mitochondria or cells should exhibit low intra-sample variability. A parsimonious explanation for this apparent discrepancy is that larger particles were captured, but were broken apart with filtration. This hypothesis—that fish eDNA is transported primarily in weak aggregations that are subject to breakdown—is also consistent with measured eDNA losses in lotic systems (Jane et al. 2015) and high inter-sample heterogeneity observed in other field studies (Pilliod et al. 2013). Jane et al. (2015) observed relatively rapid downstream Brook Trout eDNA losses in streams during low flows, which is consistent with rapid settling expected by larger particle sizes (e.g., Maggi 2013), but also found that small quantities of eDNA were transported distantly downstream, which is consistent with small particle sizes. Previous studies on the physical transport of other organic particles in streams could help us better understand the behavior of fish DNA in the environment, further informing sampling design.

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