Methods in Ecology and Evolution

British Ecological Society

Methods in Ecology and Evolution 2014, **5,** 676–684 doi: 10.1111/2041-210X.12206

Particle size distribution and optimal capture of aqueous macrobial eDNA

Cameron R. Turner[1]*, Matthew A. Barnes[1,2], Charles C. Y. Xu[1], Stuart E. Jones[1], Christopher L. Jerde[1,2] and David M. Lodge[1,2]

[1]*Department of Biological Sciences, University of Notre Dame, Notre Dame, IN 46556, USA; and* [2]*Environmental Change Initiative, University of Notre Dame, Notre Dame, IN 46556, USA*

Summary

1. Using environmental DNA (eDNA) to detect aquatic macroorganisms is a new survey method with broad applicability. However, the origin, state and fate of aqueous macrobial eDNA – which collectively determine how well eDNA can serve as a proxy for directly observing organisms and how eDNA should be captured, purified and assayed – are poorly understood.

2. The size of aquatic particles provides clues about their origin, state and fate. We used sequential filtration size fractionation to measure the particle size distribution (PSD) of macrobial eDNA, specifically Common Carp (hereafter referred to as Carp) eDNA. We compared it to the PSDs of total eDNA (from all organisms) and suspended particle matter (SPM). We quantified Carp mitochondrial eDNA using a custom qPCR assay, total eDNA with fluorometry and SPM with gravimetric analysis.

3. In a lake and a pond, we found Carp eDNA in particles from >180 to $<0{\cdot}2$ μm, but it was most abundant from 1 to 10 μm. Total eDNA was most abundant below 0·2 μm, and SPM was most abundant above 100 μm. SPM consisted of ≤0·1% total eDNA, and total eDNA consisted of ≤0·0004% Carp eDNA. 0·2 μm filtration maximized Carp eDNA capture (85% ± 6%) while minimizing total (i.e. non-target) eDNA capture (48% ± 3%), but filter clogging limited this pore size to a sample volume <250 mL. To mitigate this limitation, we estimated a continuous PSD model for Carp eDNA and derived an equation for calculating isoclines of pore size and water volume that yield equivalent amounts of Carp eDNA.

4. Our results suggest that aqueous macrobial eDNA predominantly exists inside mitochondria or cells, and that settling may therefore play an important role in its fate. For optimal eDNA capture, we recommend 0·2 μm filtration or a combination of larger pore size and water volume that exceeds the 0·2 μm isocline. *In situ* filtration of large volumes could maximize detection probability when surveying large habitats for rare organisms. Our method for eDNA particle size analysis enables future research to compare the PSDs of eDNA from other organisms and environments, and to easily apply them for ecological monitoring.

Key-words: aquatic ecosystems, ecological monitoring, environmental DNA, genetic monitoring, particle size analysis, rare species, sampling methods

Introduction

Environmental DNA (eDNA) is DNA extracted from bulk environmental samples (e.g. soil, water, air) without isolating target organisms or their parts from the sample. The concept and the term both originate from microbiology (Ogram, Sayler & Barkay 1987) where the target DNA in environmental samples is from abundant live and dead microbes. *Macrobial* eDNA is the DNA of large organisms such as animals or plants that occurs in environmental samples. Although macrobial eDNA has been studied since 1991 in fields such as human forensics (Hochmeister *et al.* 1991), agricultural transgenics (Widmer *et al.* 1997), paleogenetics (Hofreiter *et al.* 2003; Willerslev *et al.* 2003) and faecal pollution source tracking (Martellini, Payment & Villemur 2005), it was only in 2008 that eDNA was first used to detect aquatic macrofauna (Ficetola *et al.* 2008). Aqueous macrobial eDNA has garnered particular interest as a simple and sensitive alternative to directly observing rare aquatic macrofauna. Despite a recent burst of papers demonstrating its use for detection of diverse macrofauna, we have much to learn about the origin (e.g. primary physiological source), state (e.g. intra- or extracellular) and fate (e.g. suspension time) of aqueous macrobial eDNA (Lodge *et al.* 2012). These three domains collectively determine how well eDNA can serve as a proxy for directly observing organisms, and how eDNA should be captured to make robust inferences about organism presence or abundance. In this paper, we determine the size distribution of particles containing eDNA and draw size-based inferences about the state and fate of eDNA.

Martellini, Payment & Villemur (2005) first demonstrated that natural waters contain mitochondrial DNA (mtDNA)

*Correspondence author. E-mail: crt343@gmail.com

from vertebrates inhabiting the watershed. Their research focused on terrestrial mammals for which aqueous eDNA was presumably of allochthonous faecal origin. Autochthonous aqueous eDNA from aquatic macrofauna was subsequently described for amphibians (Ficetola *et al.* 2008), fish (Jerde *et al.* 2011), mammals, crustaceans, insects (Thomsen *et al.* 2012a), birds (Thomsen *et al.* 2012b) and reptiles (Piaggio *et al.* 2014) in lentic, lotic and marine waters. eDNA-based surveys for rare fish or amphibians are more sensitive than traditional methods (Jerde *et al.* 2011; Dejean *et al.* 2012; Thomsen *et al.* 2012b), and eDNA disappears from surface water within 1–25 days of organism absence (Dejean *et al.* 2011; Thomsen *et al.* 2012a,b; Pilliod *et al.* 2014). Aqueous macrobial eDNA concentration is very low (up to 33 mtDNA copies mL^{-1}) and correlates with organism density, at least for amphibians in small ponds and streams (Thomsen *et al.* 2012a; Pilliod *et al.* 2013) and fish in experimental ponds (Takahara *et al.* 2012). Aqueous macrobial eDNA has been captured (i.e. concentrated from water) by precipitation/centrifugation (Martellini, Payment & Villemur 2005; Ficetola *et al.* 2008), chromatography (Douville *et al.* 2007), lyophilization (Poté *et al.* 2009), ultrafiltration (nominal pore size <0·1 μm; Takahara *et al.* 2012) and filtration (nominal pore size >0·1 μm; Martellini, Payment & Villemur 2005; Goldberg *et al.* 2011; Jerde *et al.* 2011; Minamoto *et al.* 2011).

Despite the considerable variation in methods used to capture aqueous macrobial eDNA (Table S1), we are unaware of any systematic comparison between methods or description of the captured particles. The size of aquatic particles helps determine their characteristics and interactions with organisms, other particles and the environment (Burd & Jackson 2009). Thus, the size of aquatic particles containing macrobial eDNA is foundational to our understanding of the origin, state and fate of this material. The PSD of aqueous macrobial eDNA can also inform decisions about sampling effort (e.g. water volume) and particle capture size (e.g. filter nominal pore size). Currently, these decisions seem to have been *ad hoc* or at best based on trial and error or logistical constraints. Finally, increased understanding of the PSD of macrobial eDNA may inform mitigation of one of the most troublesome issues associated with genetic testing for low-level DNA in environmental samples: sample interference (Schrader *et al.* 2012). Non-target material suspended in water, including non-target DNA (Thompson *et al.* 2006), can interfere with eDNA recovery (Lloyd, Macgregor & Teske 2010) and genetic assays (Hedman & Rådström 2013). If the PSD of target macrobial eDNA differs from that of non-target material then size-based, selective capture of target eDNA may be possible.

Here, we describe the PSD of aqueous macrobial eDNA for one fish species. We sequentially filtered water from a lake and pond inhabited by Common Carp (*Cyprinus carpio* Linnaeus, 1758, hereafter Carp) to create a series of size fractions containing the particles retained at each size, from ≥180 μm to <0·2 μm. In aquatic microbiology, 0·2 μm is the filter nominal pore size below which aqueous eDNA is considered to exist as extracellular molecules free in solution (Matsui *et al.* 2004; Maruyama *et al.* 2008), and the vertebrate mitochondrion measures 0·2–8 μm (Flindt 2006 p. 254). Thus, our size fractions spanned from extracellular and extraorganellar DNA free in solution (hereafter free eDNA; <0·2 μm) to particles larger than most vertebrate cells (≥180 μm). For each size fraction, we measured the concentration of Carp eDNA, total eDNA (DNA of any type) and suspended particle matter (SPM; particles of any type). This allowed us to compare PSDs among these three nested types of aquatic matter and evaluate the opportunity for size-based target enrichment.

Materials and methods

TARGET SPECIES

We selected Carp as the target macrobial species because fish species, including Carp, have been the target of many previous studies of aqueous macrobial eDNA (Dejean *et al.* 2011; Jerde *et al.* 2011; Minamoto *et al.* 2011; Takahara *et al.* 2012; Thomsen *et al.* 2012a,b; Collins *et al.* 2013; Takahara, Minamoto & Doi 2013; Wilcox *et al.* 2013). In addition, Carp is listed at number 30 on the IUCN list of the world's worst invasive species (Lowe *et al.* 2004). Because Carp inhabit many continents, our method for particle size analysis can be applied globally to assess the consistency of one species' eDNA PSD across environmental conditions.

WATER BODIES

We collected water from two lentic water bodies, selected to provide a strong contrast in simplicity of the physical environment, in macrofaunal diversity, and in abundance of the target species (Carp). At Potawatomi Zoo in South Bend, Indiana, USA (41·670629, −86·216634), we sampled a small (0·06 ha, maximum depth 2 m) concrete-lined outdoor pond with flow-through treated municipal water that contained approximately 500 large adult Carp. We expected that this extremely high Carp density would provide sufficiently high Carp eDNA concentration to allow quantification in every size fraction following sequential filtration. The more natural, lower Carp density environment was St. Mary's Lake at the University of Notre Dame, Indiana, USA (41·701497, −86·244312), a natural spring-fed mesotrophic lake (10·7 ha, maximum depth of 9·1 m). We lack a quantitative estimate of Carp density for this lake but based on our many visual observations, the density of Carp is far lower in the lake than in the zoo pond.

WATER COLLECTION AND SAMPLING

In December 2011 ('winter'), we conducted our primary sampling event for each water body. In April 2012 ('spring'), we conducted a supplemental sampling event at St. Mary's Lake because Carp eDNA concentrations measured in winter were very low (see Results and Discussion). For each sampling event, we pumped 12 L of water from ≤1 m depth into a carboy using silicone peristaltic tubing with internal diameter of 9·5 mm (Masterflex L/S 36) and a portable, battery-powered peristaltic pump (Alexis, Pegasus Pump Company, Bradenton, FL, USA). At the pond, we pumped water from the east end of the pond where Carp were aggregated by extending the tubing approximately 2 m from shore with a pole. At the lake, we pumped water from a small embayment on the south end as we rowed a boat to and from a point approximately 100 m from shore. Carboys were transported to the laboratory within approximately 20 min, and water was distributed into 300-mL bottles.

We stored the 300-mL bottles on ice without freezing until each was used for particle size fractionation, which began immediately and finished within 10 h.

PARTICLE SIZE FRACTIONATION

We used sequential filtration size fractionation (Fig. 1) to generate the PSDs for SPM, total eDNA and Carp eDNA (see Appendix S1 for details). We used nylon net filters (180, 100, and 60 μm mesh size; EMD Millipore Corporation, Billerica, MA, USA) and polycarbonate track-etched (PCTE) filters (20, 10, 1, 0·2 μm pore size; GE Osmonics, Barney Corporation, Hilliard, OH, USA). From each of the ten (winter) or three (spring) bottles, 250 mL was sequentially filtered through 47-mm diameter filters of decreasing pore size using gentle vacuum pressure (<64 cm Hg). We used 47-mm diameter magnetic filter funnels (Pall Corporation, Port Washington, NY, USA) and 1-L polypropylene vacuum flasks (Nalgene, Thermo Fisher Scientific, Waltham, MA, USA). To avoid filter clogging, we used only 150 mL of the 1-μm filtrate for filtration through the 0·2 μm filter; 15 mL of the 0·2-μm filtrate was transferred to a 50-mL centrifuge tube containing 33·5 mL of 100% ethanol and 1·5 mL of 3 M sodium acetate then frozen at −20°C for subsequent eDNA capture following the precipitation procedure of Ficetola *et al.* (2008) with a centrifuge force of 3220 g. We placed used filters in CTAB buffer (Coyne *et al.* 2005) and stored them at −20°C until subsequent DNA extraction.

For the primary sampling event (winter) only, we conducted size fractionation for SPM analysis at the same time and in the same manner as for DNA analysis except all filters were individually pre-desiccated, pre-weighed and stored in individually labelled Petri dishes. Following filtration, a filter disc was carefully returned to its Petri dish retentate side up and placed in a 60°C desiccation chamber for 24 h prior to weighing. The final size fraction (<0·2 μm) was not included in SPM analysis because we were unable to weigh the tiny pellet produced by precipitation/centrifugation of the 0·2-μm filtrate.

ASSAYING eDNA (qPCR AND FLUOROMETRY)

We extracted eDNA from filters and precipitation pellets following the CTAB extraction of Coyne *et al.* (2005). A complete protocol is provided in Appendix S1. Final eDNA pellets were re-suspended in 100 μL of low TE buffer. We quantified total eDNA using 5 μL of eDNA extract in the Qubit dsDNA High Sensitivity kit and a Qubit fluorometer (Life Technologies, Grand Island, NY, USA). Total eDNA concentrations were scaled to ng L^{-1}. We quantitatively tested eDNA extracts for PCR inhibition using the internal amplification control (IAC) assay of Hartman, Coyne & Norwood (2005) as described in Appendix S1. We quantified Carp eDNA in each eDNA extract using triplicate reactions of the qPCR assay described in Appendix S1. To confirm assay specificity, a subset of qPCR positives from each sampling event were sequenced as described in Appendix S1. Each qPCR plate included a five-point standard curve from 3·1E+04 copies reaction^{-1} down to 3 copies reaction^{-1}. Carp eDNA concentrations were scaled to copies L^{-1}.

ASSAYING SPM (GRAVIMETRIC ANALYSIS)

Desiccated filters containing captured particles were weighed using a microbalance (XP26, Mettler-Toledo, Columbus, OH, USA; readability = 1 μg, repeatability = 1·5 μg, linearity = 6 μg). To eliminate the effect of static charge build-up on microbalance readings, we passed every filter through two 2U500 polonium-210 ionizers held in a Staticmaster positioner (Amstat Industries, Inc., Glenview, IL, USA). SPM concentrations were scaled to mg L^{-1}.

DATA CONVERSION, ANALYSIS AND MODELLING

To compare the PSD shape of Carp eDNA, total eDNA and SPM, we converted concentration in each size fraction to per cent of total (Fig. 2). This assumed that the final fractionation step (precipitation for Carp eDNA and total eDNA, 0·2 μm filtration for SPM) captured all remaining matter for each of the three types, respectively. To evaluate the optimal capture of Carp eDNA, we converted concentration of Carp eDNA, total eDNA and SPM to cumulative per cent captured (capture efficiency, E) at each fractionation step. To allow the most general evaluation possible from our experiment, we pooled the lake and pond capture efficiency data (winter only) without making any assumptions about the similarity or difference between the two water bodies.

To estimate Carp eDNA capture efficiency as a continuous function of filter pore size (i.e. a continuous PSD for Carp eDNA), we modelled our discrete capture efficiency data with the complementary cumulative distribution function (CCDF) of the Weibull distribution, a flexible distribution that was originally developed and is widely used to describe PSDs (Rosin & Rammler 1933; Zobeck, Gill & Popham 1999; see Appendix S1 for details). Finally, we used this continuous model to cal-

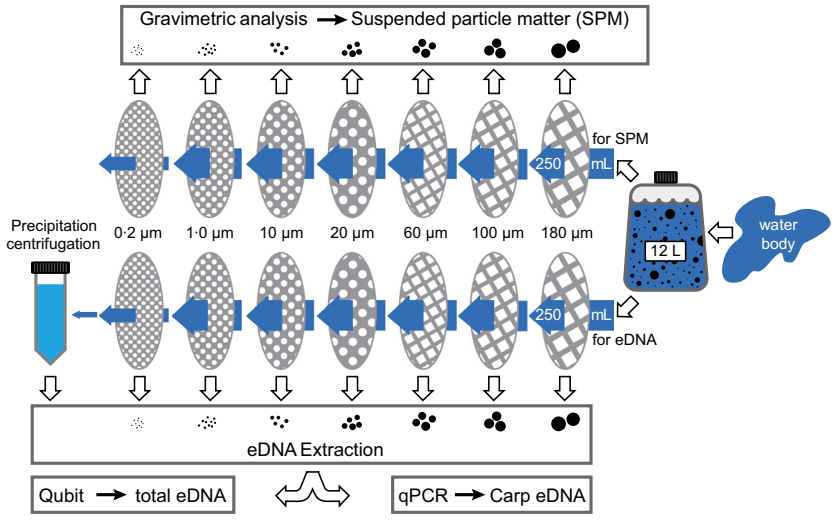

Fig. 1. Diagram of particle size fractionation by sequential filtration. Surface water from throughout the water body was pumped into a 12 L carboy. 250 mL samples from this carboy were size fractionated by sequential filtration through filters of decreasing pore size, 180–0·2 μm. For each water body, ten 250 mL samples provided filter retentate used in gravimetric analysis while ten samples were used for eDNA extraction and eDNA assays. Samples used for eDNA also provided a 15 mL subsample of 0·2-μm filtrate from which eDNA was precipitated and pelleted by centrifugation. Only 150 mL of 1·0-μm filtrate was used for filtration through the 0·2 μm filter to avoid filter clogging.

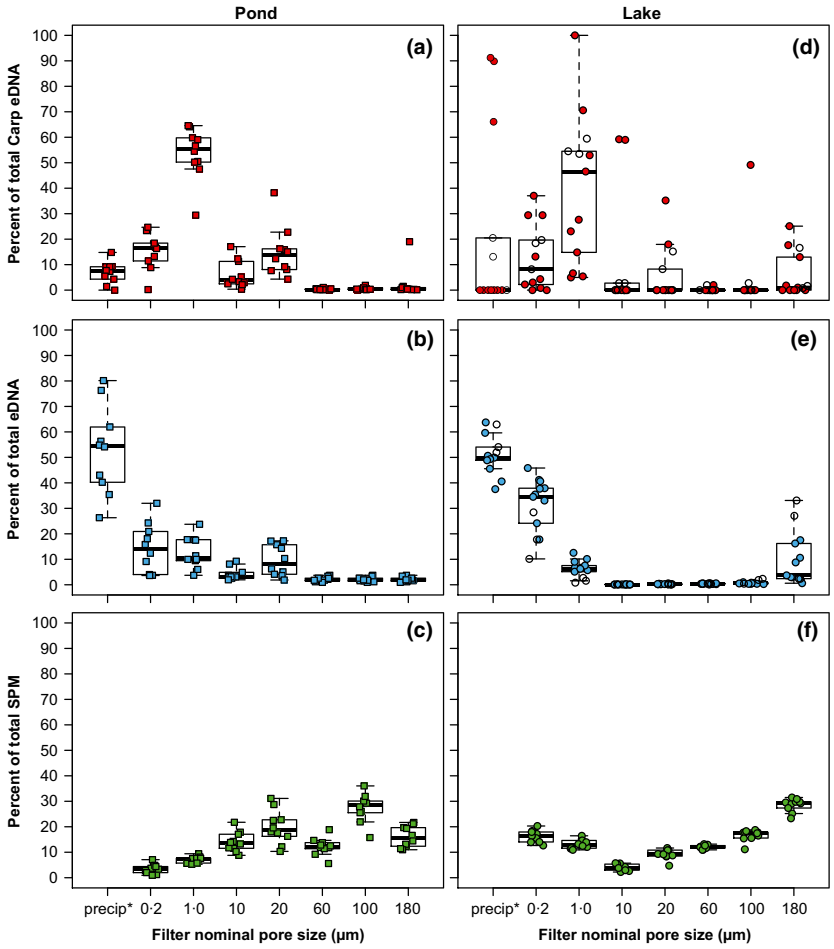

Fig. 2. Per cent of total Carp eDNA, total eDNA and SPM in each particle size fraction per water sample from the pond (a, b, c, respectively) and lake (d, e, f, respectively). *The final fractionation step was DNA precipitation using the 0·2-μm filtrate, but fractionation for SPM measurement did not include this step (see Materials and methods). Filled symbols are winter; open symbols are spring (only the lake was sampled in spring; SPM was not measured in spring). Data points are horizontally 'jittered' within each size fraction to reduce visual overlap. Boxplots include the spring data from the lake in (d) and (e).

culate 'isoclines' of filter pore size and filtered water volume where the Carp eDNA PSD estimates identical captured amounts of Carp eDNA.

To calculate the proportion of total eDNA that was made up by Carp mtDNA, we converted the qPCR-measured Carp eDNA copy number to weight of Carp mitogenomes. This was carried out using the molecular weight of one arbitrarily chosen Carp mitogenome from NCBI GenBank (10244181 g mole^{-1}; accession number: JN105352.1) as calculated by OligoCalc (Kibbe 2007).

Results

All tested samples showed no evidence of PCR inhibition (IAC ΔCq range: −0·15 to 1·15 cycles), and all sequenced Carp qPCR amplicons confirmed assay specificity. All extraction negative controls and qPCR NTCs tested negative for Carp eDNA (no Cq). The standard curve y intercept ranged from 38·7 to 41·0 cycles, slope ranged from −3·39 to −3·88, efficiency ranged from 81% to 97%, and R^2 ranged from 0·97 to 1·00. Based on standard curve amplification, the 95% limit of detection (LOD) was 30 copies reaction^{-1}, and the lowest concentration standard (3 copies reaction^{-1}) amplified 60% of the time. In both water bodies, particles containing Carp eDNA were detected in all of the size fractions, from ≥180 μm to <0·2 μm. Carp eDNA was most abundant in the 1–10 μm particle size fraction (Fig. 2). On average, there was more Carp eDNA in particles larger than 0·2 μm for both water bodies (pond: 10 of 10 replicates; lake: 7 of 10 in winter, 3 of 3 in spring) although this difference was significant only for the pond (chi-square goodness-of-fit test; pond: χ^2 (1, n = 10) = 10·00, P < 0·01; lake-winter: χ^2 (1, n = 10) = 1·60, P = 0·21; lake-all: χ^2 (1, n = 13) = 3·77, P = 0·05). The average per cent of Carp eDNA in particles larger than 0·2 μm was 93% ± 1% (mean ± SEM; pond), 75% ± 13% (lake-winter), and 78% ± 10% (lake-all). These results demonstrate that Carp eDNA can exist in very large particles and suggest that free Carp eDNA (particles <0·2 μm) represents a small portion of the total size distribution of particles containing Carp eDNA. In contrast, total eDNA was most abundant in the <0·2 μm size fraction, and SPM was most abundant in the 100–180 μm size fraction (pond) or the ≥180 μm size fraction (lake) (Fig. 2). Complete data are provided in Tables S2 and S3.

The three types of aquatic matter we measured are nested: Carp eDNA is one component of total eDNA, and total eDNA is one component of SPM. When summed across all size fractions, total eDNA constituted 0·01% ± 0·001% (mean ± SEM) of pond SPM and 0·1% ± 0·01% of lake SPM by weight. Similarly, Carp mitochondrial eDNA constituted 0·0004% ± 0·0001% of pond total eDNA and 0·0000004% ± 0·0000001% of lake total eDNA by weight. These results demonstrate that total eDNA and SPM were nearly 100% non-target material with respect to the target, Carp mtDNA.

Filtration with a 0·2 μm pore size maximized Carp eDNA capture efficiency (85% ± 6%; cumulative data pooled across lake and pond) while minimizing total eDNA capture efficiency (48% ± 3%; Fig. 3). Unfortunately, 0·2-μm-pore-size filters clogged with very small throughput volumes (e.g. we were unable to filter 250 mL of 1·0-μm filtrate through our 0·2 μm filter). Maximum throughput volume could be increased by either increasing filter surface area or increasing filter pore size. Our data cannot provide guidance for increasing filter surface area, but we used the continuous PSD of Carp eDNA to estimate isoclines of pore size and volume that would yield identical amounts of Carp eDNA. The estimated scale and shape parameters (λ and k) from the Weibull CCDF model (i.e. the continuous PSD) of Carp eDNA were 11·85 and 0·351, and the estimated Carp eDNA capture efficiency at 0·2 μm ($E_{0·2}$) was 78·8% (Fig. 3).

Thus, the equation for estimating isoclines relative to 0·2 μm filtration was derived as follows:

$$C * V_{0·2} * E_{0·2} = C * V_x * E_x \qquad \text{eqn 1}$$

where C is the environmental concentration of the target, V is throughput volume, and E is target capture efficiency. Subscripts denote filter pore size for V and E. Solving for V_x yielded:

$$V_x = (V_{0·2} * E_{0·2})/E_x \qquad \text{eqn 2}$$

$V_{0·2}$ is whatever throughput volume the researcher determines is feasible for a 0·2-μm-pore-size filter, $E_{0·2}$ is target capture efficiency at 0·2 μm, and E_x is the continuous PSD of the target, yielding the final equation:

$$V_x = \frac{V_{0·2} * E_{0·2}}{e^{-\left(\frac{x}{\lambda}\right)^k}} \qquad \text{eqn 3}$$

Figure 4 plots isoclines using the continuous PSD for Carp eDNA and several hypothetical values of $V_{0·2}$.

Discussion

METHODOLOGICAL CONSIDERATIONS

As we are not aware of any previous investigations of particle size for aqueous macrobial eDNA, we first consider some methodological points that will be important should other scientists use our approach. Appendix S1 provides extended background on filtration and size fractionation but here we simply emphasize that particle size is an operationally defined property (Buffle and Lepard 1995; Droppo 2006). The filters (PCTE and nylon net) and method (sequential filtration fractionation) applied in this study are the most widely used and recommended for physically separating aquatic particles for chemical-biological analysis of different size classes (Droppo 2006; Brewin *et al.* 2014). However, size data generated by filtration cannot be directly applied to every filter type because the relationship between nominal and effective pore size differs substantially among filter materials (Sheldon 1972; Danielsson 1982; Hickel 1984; Lee, Kang & Fuhrman 1995; Morán *et al.* 1999; Knefelkamp,

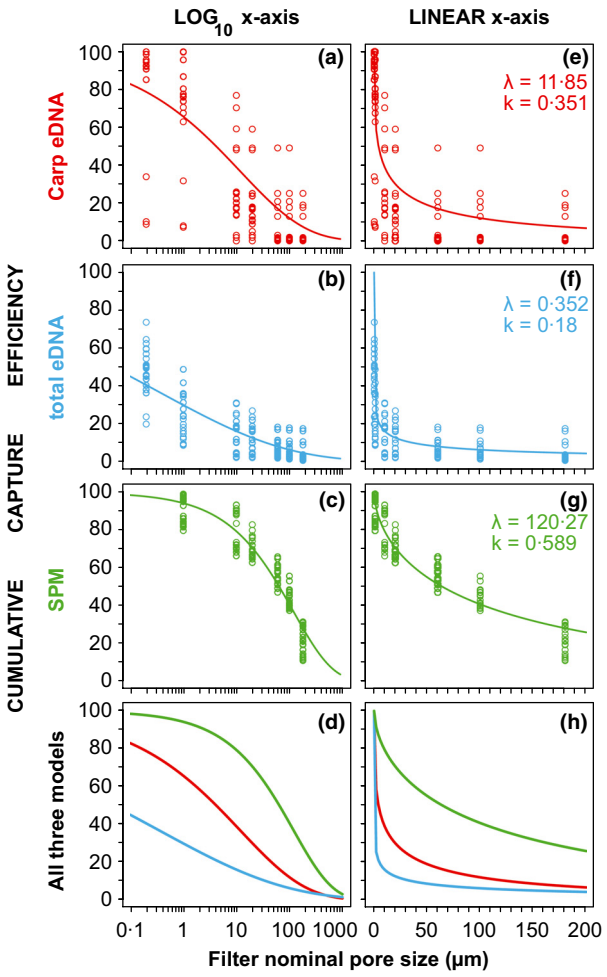

Fig. 3. Cumulative size fractionation data and the PSD models (Weibull CCDF) fitted to these data. Pooled lake and pond data are shown for Carp eDNA (a and e), total eDNA (b and f), and SPM (c and g). Panels (d) and (h) show the PSD models for each type of aquatic matter plotted together, without data points. All plots are shown with a $\log_{10}$ x axis (a, b, c, d) and a linear x axis (e, f, g, h) to allow visualization of both the model fits and the PSD shapes. The estimated scale and shape parameters (λ and k) from the Weibull CCDF model are shown on the plots. Spring data from the lake were not used for PSD modelling because SPM was not measured in spring.

Carstens & Wiltshire 2007; Liang & Keeley 2013). Thus, filters of different materials with identical *nominal* pore sizes actually have significantly different *effective* pore sizes and should not be compared as if their particle retention is equivalent.

The per reaction copy numbers of Carp eDNA measured by qPCR were very low, with a heavily right-skewed distribution and a mode of zero for both water bodies (Fig. S1). This result is consistent with detailed empirical studies of qPCR applied to low-level DNA (i.e. <100 target copies per reaction; Ellison *et al.* 2006). Research using qPCR to quantify eDNA should not discard non-detect reactions (negative reactions from technical replicate sets where at least one reaction was positive). As demonstrated by Ellison *et al.* (2006), non-detect reactions are expected in low-level DNA analysis, and they should be assigned a zero target concentration and included when aver-

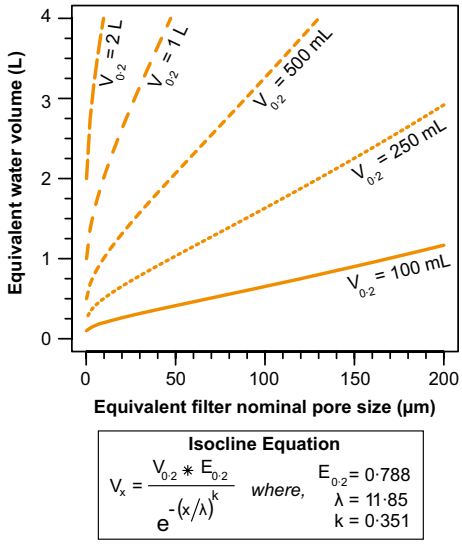

Fig. 4. Isoclines showing combinations of filter pore size (x) and water volume (Vx) where the particle size distribution (PSD) of Carp eDNA predicts identical amounts of Carp eDNA captured. Isoclines are shown for five hypothetical examples of maximum throughput water volume for a 0·2 µm filter pore size ($V_{0·2}$). The equation for calculating isoclines is shown using the 0·2 µm capture efficiency ($E_{0·2}$) we estimated for Carp eDNA by fitting a Weibull CCDF to our cumulative size fractionation data; the scale (λ) and shape (k) parameters from the Weibull CCDF model of Carp eDNA PSD are also used in this equation (see Results and Appendix S1 for details).

aging across technical replicates to provide the best estimate of unknown sample concentration (Appendix S1).

Even with this low-level DNA protocol applied, the frequency of negative reactions (79%) in the lake-winter sampling event was high (Fig. S1). The large variation between samples (Fig. 2d) caused by these extremely low Carp eDNA levels led us to conduct the supplemental spring sampling at the lake, because we expected higher fish activity in the spring would increase Carp eDNA concentration. Indeed, the frequency of negative reactions dropped to 49% in the lake-spring sampling event (Fig. S1). The spring PSD from the lake supported our conclusions that Carp eDNA exists predominantly in particles larger than 0·2 µm and is most abundant between 1 and 10 µm (Fig. 2). The supplemental spring sampling event also confirmed that total Carp eDNA concentration in the lake (summed across all size fractions) had increased fourfold since winter (from 1726 ± 602 to 7144 ± 1165 copies L^{-1}). These data represent a preliminary description of seasonal change in eDNA concentration for aquatic macrofauna and underline the importance of reporting eDNA sampling dates, especially when comparing eDNA sampling with organism sampling.

STATE AND FATE OF AQUEOUS MACROBIAL eDNA

The majority of Carp eDNA was found in particles larger than 0·2 µm in both the pond (93%) and the lake (75–78%), and Carp eDNA was most abundant in the 1–10 µm particle size fraction (Fig. 2). In contrast, total eDNA was most abundant in the <0·2 µm size fraction, and SPM was most abundant in the 100–180 µm (pond) or the <180 µm size fraction (lake) (Fig. 2). Future research from other seasons, environments and species will determine the generality of these results, but even at this early stage it seems appropriate to consider how the PSD can shed light on the state and fate of aqueous macrobial eDNA.

Aqueous eDNA from living macrofauna most likely originates as urine and faeces, epidermal tissues and secretions, and reproductive cells. Much of this source material (e.g. faeces) will enter the water column as large particles (>1000 µm), yet we generally found little Carp eDNA in particles larger than 60 µm. Therefore, we conclude that these sources of eDNA must rapidly settle or break apart. Indeed, particle size is a major determinant of settling velocity, even for complex biological and biomineral aggregates (Maggi 2013). It is also well documented that animal faeces contain viable epithelial cells (10^{-1}–10^{6} cells g^{-1}) and large amounts of the animal's mtDNA (10^{-1}–10^{7} copies g^{-1}) (reviewed in Caldwell, Payment & Villemur 2011) and that faeces from aquatic macrofauna rapidly sink (Robison & Bailey 1981; Wotton & Malmqvist 2001). Thus, at least one obvious source of aquatic macrobial eDNA appears to spend very little time suspended in the water column. We measured PSDs in relatively still water, whereas particle settling should be slower in flowing water, such as streams.

On the small end of the size spectrum, there was generally little free Carp eDNA (<0·2 µm), at least as indicated by the 146 bp mtDNA region our qPCR assay targeted. One might predict an accumulation of smaller particles as discharged materials decompose, ultimately leading to an abundance of free eDNA. However, free eDNA may be exceptionally susceptible to hydrolysis given its full exposure to microbial extracellular enzymes that are abundant in aquatic systems (Matsui, Honjo & Kawabata 2001). Thus, the abundance of Carp eDNA in the 1–10 µm fraction likely reflects a state of eDNA persisting within small cells and/or mitochondria. We cannot exclude the possibility that Carp eDNA in particles larger than 0·2 µm is actually extracellular/extraorganellar DNA aggregated or adsorbed onto larger particles (Burd & Jackson 2009; Suzuki *et al.* 2009), but the fact that animal mitochondria range in diameter from 0·2 to 1·2 µm and in length from 1 to 8 µm (Flindt 2006) suggests mitochondria as a parsimonious explanation for the pattern we observed. Indeed, during the regular apoptotic shedding of epithelial cells, intact mitochondria are released within apoptotic bodies and mtDNA is protected from the endonuclease degradation that rapidly degrades nuclear DNA (Murgia *et al.* 1992; Tepper & Studzinski 1993). For aquatic animals, this process will release whole mitochondria into the water column where their double membrane may resist lysis while the mitochondrial nucleoid further protects mtDNA (Rickwood & Chambers 1981).

The PSD of Carp eDNA indicates that settling, in addition to degradation, plays an important role in the fate of aqueous macrobial eDNA. Particles larger than 1 µm settle in natural waters (Isao *et al.* 1990) and 71% ± 5% of Carp eDNA was in particles exceeding this size (Fig. 3). SPM in the 1–100 µm size range settles at terminal velocities between 0·4 mm h^{-1} and 40 m h^{-1}, depending on size, shape and composition (Maggi 2013). Thus, it appears that most aqueous macrobial eDNA is in a constant state of downward flux, consistent with

observations that microbial extracellular DNA is more concentrated in sediment than the overlying water column (Corinaldesi, Danovaro & Dell'Anno 2005) and that microbial DNA from the water column can progressively accumulate in sediments (Corinaldesi *et al*. 2011). Most research on the fate of aqueous macrobial eDNA has not considered settling (Martellini, Payment & Villemur 2005; Kortbaoui *et al*. 2009; Dejean *et al*. 2011; Thomsen *et al*. 2012a), except one experiment that applied water circulation to eliminate it (Thomsen *et al*. 2012b). Our findings emphasize that degradation of DNA molecules and particle settling combine to reduce aqueous eDNA concentration over time, and future research should try to measure both processes. Past eDNA degradation experiments (except Thomsen *et al*. 2012b) may have underestimated the persistence of aqueous eDNA given that settled particles can return to the water column through bioturbation and water turbulence (Bloesch 1995).

We did detect a small amount of Carp eDNA in particles smaller than 1 μm (Fig. 2). This fraction of aqueous macrobial eDNA is likely to remain in suspension indefinitely until the DNA molecules degrade (Isao *et al*. 1990). Water could advect eDNA in this state over long distances, especially in lotic systems, strong currents, and when extracellular/extraorganellar eDNA is protected via adsorption to other submicron particles (Saeki *et al*. 2011). For example, transgenes from genetically modified corn were detected in river water up to 82 km downstream of a corn cultivation plot (Douville *et al*. 2007), and plant DNA fragments as long as 1000 bp are detectable in groundwater (Poté *et al*. 2009). The stability and advection of extracellular/extraorganellar DNA in aquatic environments is sufficient for use of synthetic DNA as a hydrologic tracer (Sabir *et al*. 2002; Foppen *et al*. 2011). Future work examining the transport and persistence of naturally occurring aqueous macrobial eDNA across long distances will improve our understanding of the spatiotemporal window for inferring organism presence. The size of this window may depend on the particle size fraction that is tested for eDNA.

OPTIMAL CAPTURE OF AQUEOUS MACROBIAL eDNA

Filtration with a 0·2 μm pore size was the best strategy for maximizing Carp eDNA and minimizing non-target eDNA (Fig. 3). However, the continuous PSD of Carp eDNA that we estimated provides simple guidance for overcoming the volume limitations of 0·2 μm filtration by increasing pore size and water volume along an isocline (Fig. 4). These isoclines also highlight the potential to exceed the yield of 0·2 μm filtration using large pore sizes that do not clog easily (e.g. >50 μm) and filtering volumes above the isocline. Given the basic processes discussed above regarding the origin, state and fate of macrobial eDNA, we suspect that these eDNA capture recommendations are applicable beyond Carp. However, as researchers generate eDNA PSDs for additional species and environments, they can easily be compared via Weibull CCDF modelling and our simple equation for calculating isoclines.

The heterogeneous distribution of macrobial eDNA among replicate water samples observed in this study (Fig. 2) and others (Jerde *et al*. 2011; Dejean *et al*. 2012; Thomsen *et al*. 2012a; Pilliod *et al*. 2013) suggests an extensive search mode (i.e. large pore size and large water volume) as the default strategy when conducting eDNA-based monitoring of rare species in large habitats. Devices for *in situ* filtration of large volumes, such as inline filters or plankton nets, could maximize water volume and habitat coverage. Our data indicate that capture methods with extremely small size cut-offs (e.g. precipitation, centrifugation, ultrafiltration) are generally unnecessary compared with filtration (Fig. 3). The >50% increase in non-target eDNA capture (Fig. 2b, e), and the severe volume limitations of these methods further reduce their value relative to filtration (nominal pore size >0·1 μm).

Because macrobial eDNA is only a tiny fraction of the total eDNA and SPM in water, the risk of interference from non-target eDNA and non-DNA inhibitors increases with sample volume (McDevitt *et al*. 2007; Hata *et al*. 2011; but see Gibson *et al*. 2012). Filtration reduces the risk from non-target eDNA because 50% or more passes through a capture filter pore size as small as 0·2 μm (Fig. 2b, e), and pre-filtration could reduce the risk from non-DNA inhibitors as SPM was most abundant on 100 or 180 μm filters (Fig. 2c, f). The concentration and composition of SPM varies between seasons and aquatic environments (Wotton 1994; Håkanson 2006) and its total DNA content reflects plankton density (Paul *et al*. 1991). While non-target DNA (i.e. background DNA) is a potent PCR inhibitor (Thompson *et al*. 2006), many other components of SPM also interfere with DNA extraction and PCR (reviewed in Gallup 2011; Hedman & Rådström 2013), thus inhibition testing is essential for all eDNA studies. In contrast to background eDNA, the interference risk from non-DNA components of SPM can be mitigated by effective DNA extraction and purification; thus, we recommend pre-filtration only if it substantially increases the throughput volume of one's capture filter. For example, Cary *et al*. (2007) applied this 'band-stop' filtration approach for eDNA-based surveillance of the invasive diatom *Didymosphenia geminate*. Further research is needed to determine the generality of these principles gleaned from our particle size analysis of two water bodies.

There is broad interest in research on the reliability of eDNA-based ecological monitoring (Schmidt *et al*. 2013; Sutherland *et al*. 2013; Zhan *et al*. 2013). Particle size analysis, such as that presented here, is one of the many important research avenues needed to better understand the origin, state and fate of aqueous macrobial eDNA (Barnes *et al*. 2014). Our description of particle size for aqueous macrobial eDNA provides immediate guidance for practitioners and a tested method for researchers. For example, because large particles sink faster than small particles (Maggi 2013), eDNA-based surveys aimed at determining very recent and local organism presence may need to target larger eDNA-containing particles. We look forward to future findings that will undoubtedly expand on this initial description by investigating the organismal and environmental determinants of the aqueous eDNA particle size.

Acknowledgements

K.J. Coyne and her laboratory at the University of Delaware provided generous consultation on qPCR and eDNA extraction methods. R.K. Turner helped make Fig. 1. M.A. Renshaw assisted with qPCRs. L. Arriaga at the Potawatomi Zoo provided sampling access to the Carp pond. A.M. Deines provided helpful discussion of PSD modelling. W.L. Chadderton and A.R. Mahon provided helpful discussion of a pilot size fractionation experiment. Two anonymous reviewers provided helpful comments on an earlier version of this manuscript. Funding for this project was provided by the Illinois-Indiana Sea Grant (NA10OAR4170068) and the Great Lakes Restoration Initiative (FY10-S-T0240-O169-2). This work was supported by NSF IGERT grant award #0504495 to the GLOBES graduate training programme at the University of Notre Dame. This is a publication of the Notre Dame Environmental Change Initiative.

Data accessibility

All data are available in the Supporting Information.

Author contributions

CRT designed the experiment, developed the qPCR assay and methods for eDNA capture and extraction, collected, processed, and assayed samples, performed data analysis, and wrote the paper. MAB designed the experiment, collected and processed samples, helped perform data analysis, and helped write the paper. CCYX helped develop the qPCR assay and methods for eDNA capture and extraction and helped write the paper. SEJ helped perform data analysis and write the paper. CLJ performed data analysis and helped write the paper. DML helped write the paper.

References

Barnes, M.A., Turner, C.R., Jerde, C.L., Renshaw, M.A., Chadderton, W.L. & Lodge, D.M. (2014) Environmental conditions influence eDNA persistence in aquatic systems. *Environmental Science & Technology*, **48**, 1819–1827.

Bloesch, J. (1995) Mechanisms, measurement and importance of sediment resuspension in lakes. *Marine and Freshwater Research*, **46**, 295–304.

Brewin, R.J.W., Sathyendranath, S., Lange, P.K. & Tilstone, G. (2014) Comparison of two methods to derive the size-structure of natural populations of phytoplankton. *Deep Sea Research Part I: Oceanographic Research Papers*, **85**, 72–79.

Buffle, J. & Lepard, G.G. (1995) Characterization of aquatic colloids and macromolecules. 2. Key role of physical structures on analytical results. *Environmental Science & Technology*, **29**, 2176–2184.

Burd, A.B. & Jackson, G.A. (2009) Particle aggregation. *Annual Review of Marine Science*, **1**, 65–90.

Caldwell, J., Payment, P. & Villemur, R. (2011). Mitochondrial DNA as source tracking markers of fecal contamination. *Microbial Source Tracking: Methods, Applications, and Case Studies* (eds C. Hagedorn, A.R. Blanch & V.J. Harwood), pp. 229–250. Springer, New York, NY.

Cary, S.C., Hicks, B.J., Coyne, K.J., Rueckert, A., Gemmill, C.E.C. & Barnett, C.M.E. (2007). *A sensitive genetic-based detection capability for Didymosphenia geminata (Lyngbye) M. Schmidt: Phases Two and Three*. Hamilton, New Zealand. http://cber.bio.waikato.ac.nz/PDFs/CBER_62_Early_detection_Cary_Hicks_2007_10_19_final_approved_by_MAFBNZ.pdf

Collins, R.A., Armstrong, K.F., Holyoake, A.J. & Keeling, S. (2013) Something in the water: biosecurity monitoring of ornamental fish imports using environmental DNA. *Biological Invasions*, **15**, 1209–1215.

Corinaldesi, C., Danovaro, R. & Dell'Anno, A. (2005) Simultaneous recovery of extracellular and intracellular DNA suitable for molecular studies from marine sediments. *Applied Environmental Microbiology*, **71**, 46–50.

Corinaldesi, C., Barucca, M., Luna, G.M. & Dell'Anno, A. (2011) Preservation, origin and genetic imprint of extracellular DNA in permanently anoxic deep-sea sediments. *Molecular Ecology*, **20**, 642–654.

Coyne, K.J., Handy, S.M., Demir, E., Whereat, E.B., Hutchins, D.A., Portune, K.J., Doblin, M.A. & Cary, S.C. (2005) Improved quantitative real-time PCR assays for enumeration of harmful algal species in field samples using an exogenous DNA reference standard. *Limnology and Oceanography-Methods*, **3**, 381–391.

Danielsson, L.G. (1982) On the use of filters for distinguishing between dissolved and particulate fractions in natural waters. *Water Research*, **16**, 179–182.

Dejean, T., Valentini, A., Duparc, A., Pellier-Cuit, S., Pompanon, F., Taberlet, P. & Miaud, C. (2011) Persistence of environmental DNA in freshwater ecosystems. *PLoS ONE*, **6**, e23398.

Dejean, T., Valentini, A., Miquel, C., Taberlet, P., Bellemain, E. & Miaud, C. (2012) Improved detection of an alien invasive species through environmental DNA barcoding: the example of the American bullfrog Lithobates catesbeianus. *Journal of Applied Ecology*, **49**, 953–959.

Douville, M., Gagné, F., Blaise, C. & André, C. (2007) Occurrence and persistence of Bacillus thuringiensis (Bt) and transgenic Bt corn cry1Ab gene from an aquatic environment. *Ecotoxicology and Environmental Safety*, **66**, 195–203.

Droppo, I.G. (2006). Filtration in particle size analysis. *Encyclopedia of Analytical Chemistry* (ed R.A. Meyers), pp. 1–16. John Wiley & Sons, Ltd, Hoboken, NJ.

Ellison, S.L.R., English, C.A., Burns, M.J. & Keer, J.T. (2006). Routes to improving the reliability of low level DNA analysis using real-time PCR. *BMC Biotechnology*, **6**, 33.

Ficetola, G.F., Miaud, C., Pompanon, F. & Taberlet, P. (2008) Species detection using environmental DNA from water samples. *Biology Letters*, **4**, 423–425.

Flindt, R. (2006). *Amazing Numbers in Biology*, 6th edn. (ed. D. Czeschlik). Springer-Verlag, Berlin, Heidelberg, Germany.

Foppen, J.W., Orup, C., Adell, R., Poulalion, V. & Uhlenbrook, S. (2011) Using multiple artificial DNA tracers in hydrology. *Hydrological Processes*, **25**, 3101–3106.

Gallup, J. (2011). qPCR inhibition and amplification of difficult templates. *PCR Troubleshooting and Optimization: The Essential Guide* (eds S. Kennedy & N. Oswald), pp. 23–65. Caister Academic Press, Norfolk, UK.

Gibson, K.E., Schwab, K.J., Spencer, S.K. & Borchardt, M.A. (2012) Measuring and mitigating inhibition during quantitative real time PCR analysis of viral nucleic acid extracts from large-volume environmental water samples. *Water Research*, **46**, 4281–4291.

Goldberg, C.S., Pilliod, D.S., Arkle, R.S. & Waits, L.P. (2011) Molecular detection of vertebrates in stream water: a demonstration using Rocky Mountain tailed frogs and Idaho giant salamanders. *PLoS ONE*, **6**, e22746.

Håkanson, L. (2006) *Suspended Particulate Matter in Lakes, Rivers, and Marine Systems*. The Blackburn Press, Caldwell, New Jersey, USA.

Hartman, L.J., Coyne, S.R. & Norwood, D.A. (2005) Development of a novel internal positive control for Taqman based assays. *Molecular and Cellular Probes*, **19**, 51–59.

Hata, A., Katayama, H., Kitajima, M., Visvanathan, C., Nol, C. & Furumai, H. (2011) Validation of internal controls for extraction and amplification of nucleic acids from enteric viruses in water samples. *Applied and Environmental Microbiology*, **77**, 4336–4343.

Hedman, J. & Rådström, P. (2013). Overcoming inhibition in real-time diagnostic PCR. *PCR Detection of Microbial Pathogens* (ed M. Wilks), pp. 17–48. Humana Press, Totowa, NJ.

Hickel, W. (1984) Seston retention by Whatman GF/C glass-fiber filters. *Marine Ecology Progress Series*, **16**, 185–191.

Hochmeister, M.N., Budowle, B., Jung, J., Borer, U.V., Comey, C.T. & Dirnhofer, R. (1991) PCR-based typing of DNA extracted from cigarette butts. *International Journal of Legal Medicine*, **104**, 229–233.

Hofreiter, M., Mead, J.I., Martin, P. & Poinar, H.N. (2003) Molecular caving. *Current Biology*, **13**, R693–R695.

Isao, K., Hara, S., Terauchi, K. & Kogure, K. (1990) Role of sub-micrometre particles in the ocean. *Nature*, **345**, 242–244.

Jerde, C.L., Mahon, A.R., Chadderton, W.L. & Lodge, D.M. (2011) "Sight-unseen" detection of rare species using environmental DNA. *Conservation Letters*, **4**, 150–157.

Kibbe, W.A. (2007) OligoCalc: an online oligonucleotide properties calculator. *Nucleic Acids Research*, **35**, W43–W46.

Knefelkamp, B., Carstens, K. & Wiltshire, K.H. (2007) Comparison of different filter types on chlorophyll-a retention and nutrient measurements. *Journal of Experimental Marine Biology and Ecology*, **345**, 61–70.

Kortbaoui, R., Locas, A., Imbeau, M., Payment, P. & Villemur, R. (2009) Universal mitochondrial PCR combined with species-specific dot-blot assay as a source-tracking method of human, bovine, chicken, ovine, and porcine in fecal-contaminated surface water. *Water Research*, **43**, 2002–2010.

Lee, S., Kang, Y. & Fuhrman, J. (1995) Imperfect retention of natural bacterioplankton cells by glass fiber filters. *Marine Ecology Progress Series*, **119**, 285–290.

Liang, Z. & Keeley, A. (2013) Filtration recovery of extracellular DNA from environmental water samples. *Environmental Science & Technology*, **47**, 9324–9331.

Lloyd, K.G., Macgregor, B.J. & Teske, A. (2010). Quantitative PCR methods for RNA and DNA in marine sediments: maximizing yield while overcoming inhibition. *FEMS Microbiology Ecology*, **72**, 143–151.

Lodge, D.M., Turner, C.R., Jerde, C.L., Barnes, M.A., Chadderton, L., Egan, S.P., Feder, J.L., Mahon, A.R. & Pfrender, M.E. (2012) Conservation in a cup of water: estimating biodiversity and population abundance from environmental DNA. *Molecular Ecology*, **21**, 2555–2558.

Lowe, S., Browne, M., Boudjelas, S. & De Poorter, M. (2004). *100 of the World's Worst Invasive Alien Species, a Selection From the Global Invasive Species Database*. World Conservation Union (IUCN), Auckland, New Zealand. http://www.issg.org/pdf/publications/worst_100/english_100_worst.pdf

Maggi, F. (2013) The settling velocity of mineral, biomineral, and biological particles and aggregates in water. *Journal of Geophysical Research: Oceans*, **118**, 2118–2132.

Martellini, A., Payment, P. & Villemur, R. (2005) Use of eukaryotic mitochondrial DNA to differentiate human, bovine, porcine and ovine sources in fecally contaminated surface water. *Water Research*, **39**, 541–548.

Maruyama, F., Tani, K., Kenzaka, T., Yamaguchi, N. & Nasu, M. (2008) Application of real-time long and short polymerase chain reaction for sensitive monitoring of the fate of extracellular plasmid DNA introduced into river waters. *Microbes and Environments*, **23**, 229–236.

Matsui, K., Honjo, M. & Kawabata, Z. (2001) Estimation of the fate of dissolved DNA in thermally stratified lake water from the stability of exogenous plasmid DNA. *Aquatic Microbial Ecology*, **26**, 95–102.

Matsui, K., Ishii, N., Honjo, M. & Kawabata, Z. (2004) Use of the SYBR Green I fluorescent dye and a centrifugal filter device for rapid determination of dissolved DNA concentration in fresh water. *Aquatic Microbial Ecology*, **36**, 99–105.

McDevitt, J.J., Lees, P.S.J., Merz, W.G. & Schwab, K.J. (2007) Inhibition of quantitative PCR analysis of fungal conidia associated with indoor air particulate matter. *Aerobiologia*, **23**, 35–45.

Minamoto, T., Yamanaka, H., Takahara, T., Honjo, M.N. & Kawabata, Z. (2011) Surveillance of fish species composition using environmental DNA. *Limnology*, **13**, 193–197.

Morán, X., Gasol, J., Arin, L. & Estrada, M. (1999) A comparison between glass fiber and membrane filters for the estimation of phytoplankton POC and DOC production. *Marine Ecology Progress Series*, **187**, 31–41.

Murgia, M., Pizzo, P., Sandona, D., Zanovello, P., Ruzzuto, R. & Virgilioll, F.Di. (1992) Mitochondrial DNA is not fragmented during apoptosis. *Journal of Biological Chemistry*, **267**, 10939–10941.

Ogram, A., Sayler, G.S. & Barkay, T. (1987) The extraction and purification of microbial DNA from sediments. *Journal of Microbiological Methods*, **7**, 57–66.

Paul, J.H., Cazares, L.H., David, A.W., DeFlaun, M.F. & Jeffrey, W.H. (1991) The distribution of dissolved DNA in an oligotrophic and a eutrophic river of Southwest Florida. *Hydrobiologia*, **218**, 53–63.

Piaggio, A.J., Engeman, R.M., Hopken, M.W., Humphrey, J.S., Keacher, K.L., Bruce, W.E. & Avery, M.L. (2014). Detecting an elusive invasive species: a diagnostic PCR to detect Burmese python in Florida waters and an assessment of persistence of environmental DNA. *Molecular Ecology Resources*, **14**, 374–380.

Pilliod, D., Goldberg, C., Arkle, R.S. & Waits, L.P. (2013) Estimating occupancy and abundance of stream amphibians using environmental DNA from filtered water samples. *Canadian Journal of Fisheries and Aquatic Sciences*, **70**, 1123–1130.

Pilliod, D.S., Goldberg, C.S., Arkle, R.S. & Waits, L.P. (2014) Factors influencing detection of eDNA from a stream-dwelling amphibian. *Molecular Ecology Resources*, **14**, 109–116.

Poté, J., Mavingui, P., Navarro, E., Rosselli, W., Wildi, W., Simonet, P. & Vogel, T.M. (2009) Extracellular plant DNA in Geneva groundwater and traditional artesian drinking water fountains. *Chemosphere*, **75**, 498–504.

Rickwood, D. & Chambers, J.A. (1981) Evidence for protected regions of DNA in the mitochondrial nucleoid of Saccharomyces cerevisiae. *Fems Microbiology Letters*, **12**, 187–190.

Robison, B.H. & Bailey, T. (1981) Sinking rates and dissolution of midwater fish fecal matter. *Marine Biology*, **65**, 135–142.

Rosin, P. & Rammler, E. (1933) The laws governing the fineness of powdered coal. *Journal of the Institute of Fuel*, **7**, 29–36.

Sabir, I.H., Torgersen, J., Haldorsen, S. & Aleström, P. (2002) DNA tracers with information capacity and high detection sensitivity tested in groundwater studies. *Hydrogeology Journal*, **7**, 264–272.

Saeki, K., Ihyo, Y., Sakai, M. & Kunito, T. (2011) Strong adsorption of DNA molecules on humic acids. *Environmental Chemistry Letters*, **9**, 505–509.

Schmidt, B.R., Kéry, M., Ursenbacher, S., Hyman, O.J. & Collins, J.P. (2013) Site occupancy models in the analysis of environmental DNA presence/absence surveys: a case study of an emerging amphibian pathogen. *Methods in Ecology and Evolution*, **4**, 646–653.

Schrader, C., Schielke, A., Ellerbroek, L. & Johne, R. (2012). PCR inhibitors - occurrence, properties and removal. *Journal of Applied Microbiology*, **113**, 1014–1026.

Sheldon, R.W. (1972) Size separation of marine seston by membrane and glass-fiber filters. *Limnology and Oceanography*, **17**, 494–498.

Sutherland, W.J., Bardsley, S., Clout, M., Depledge, M.H., Dicks, L.V., Fellman, L. *et al*. (2013) A horizon scan of global conservation issues for 2013. *Trends in Ecology & Evolution*, **28**, 16–22.

Suzuki, H., Daimon, M., Awano, T., Umekage, S., Tanaka, T. & Kikuchi, Y. (2009) Characterization of extracellular DNA production and flocculation of the marine photosynthetic bacterium Rhodovulum sulfidophilum. *Applied Microbiology and Biotechnology*, **84**, 349–356.

Takahara, T., Minamoto, T. & Doi, H. (2013) Using environmental DNA to estimate the distribution of an invasive fish species in ponds. *PLoS ONE*, **8**, e56584.

Takahara, T., Minamoto, T., Yamanaka, H., Doi, H. & Kawabata, Z. (2012) Estimation of fish biomass using environmental DNA. *PLoS ONE*, **7**, e35868.

Tepper, C.G. & Studzinski, G.P. (1993) Resistance of mitochondrial DNA to degradation characterizes the apoptotic but not the necrotic mode of human leukemia cell death. *Journal of Cellular Biochemistry*, **52**, 352–361.

Thompson, D.E., Rajal, V.B., De Batz, S. & Wuertz, S. (2006) Detection of Salmonella spp. in water using magnetic capture hybridization combined with PCR or real-time PCR. *Journal of Water and Health*, **4**, 67–75.

Thomsen, P.F., Kielgast, J., Iversen, L.L., Wiuf, C., Rasmussen, M., Gilbert, M.T.P., Orlando, L. & Willerslev, E. (2012a) Monitoring endangered freshwater biodiversity using environmental DNA. *Molecular Ecology*, **21**, 2565–2573.

Thomsen, P.F., Kielgast, J., Iversen, L.L., Møller, P.R., Rasmussen, M. & Willerslev, E. (2012b) Detection of a diverse marine fish fauna using environmental DNA from seawater samples. *PLoS ONE*, **7**, e41732.

Widmer, F., Seidler, R.J., Donegan, K.K. & Reed, G.L. (1997) Quantification of transgenic plant marker gene persistence in the field. *Molecular Ecology*, **6**, 1–7.

Wilcox, T.M., McKelvey, K.S., Young, M.K., Jane, S.F., Lowe, W.H., Whiteley, A.R. & Schwartz, M.K. (2013) Robust detection of rare species using environmental DNA: the importance of primer specificity. *PLoS ONE*, **8**, e59520.

Willerslev, E., Hansen, A.J., Binladen, J., Brand, T.B., Gilbert, M.T.P., Shapiro, B. *et al*. (2003) Diverse plant and animal genetic records from Holocene and Pleistocene sediments. *Science*, **300**, 791–795.

Wotton, R.S. (ed.) (1994) *The Biology of Particles in Aquatic Systems*, 2nd edn. CRC Press, Boca Raton, Florida, USA.

Wotton, R.S. & Malmqvist, B. (2001) Feces in aquatic ecosystems. *BioScience*, **51**, 537–544.

Zhan, A., Hulák, M., Sylvester, F., Huang, X., Adebayo, A.A., Abbott, C.L. *et al*. (2013) High sensitivity of 454 pyrosequencing for detection of rare species in aquatic communities. *Methods in Ecology and Evolution*, **4**, 558–565.

Zobeck, T.M., Gill, T.E. & Popham, T.W. (1999) A two-parameter Weibull function to describe airborne dust particle size distributions. *Earth Surface Processes and Landforms*, **24**, 943–955.

Received 17 December 2013; accepted 2 May 2014
Handling Editor: M. Gilbert

Supporting Information

Additional Supporting Information may be found in the online version of this article.

Fig. S1. Histograms of qPCR-measured Carp eDNA copies per reaction. Note that the axis scales differ between histograms.

Fig. S2. Micrographs of the pore structure of commonly available filter materials illustrating that only polycarbonate (A) and nylon net (B) filters have uniformly size pores

Appendix S1. Extended methodological details

Table S1. Published methods for capturing aqueous eDNA from macrofauna.

Table S2. Pond concentrations of Carp eDNA, total eDNA and SPM in each size fraction.

Table S3. Lake concentrations of Carp eDNA, total eDNA and SPM in each size fraction.