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Fish environmental DNA is more concentrated in aquatic sediments than surface water

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ABSTRACT

Genetic identification of aqueous environmental DNA (eDNA) provides site occupancy inferences for rare aquatic macrofauna that are often easier to obtain than direct observations of organisms. This relative ease makes eDNA sampling a valuable tool for conservation biology. Research on the origin, state, transport, and fate of eDNA shed by aquatic macrofauna is needed to describe the spatiotemporal context for eDNA-based occupancy inferences and to guide eDNA sampling design. We tested the hypothesis that eDNA is more concentrated in surficial sediments than in surface water by measuring the concentration of aqueous and sedimentary eDNA from an invasive fish, bigheaded Asian carp (*Hypophthalmichthys* spp.), in experimental ponds and natural rivers. We modified a simple, low-cost DNA extraction method to yield inhibitor-free eDNA from both sediment and water samples. Carp eDNA was 8–1800 times more concentrated per gram of sediment than per milliliter of water and was detected in sediments up to 132 days after carp removal – five times longer than any previous reports of macrobial eDNA persistence in water. These results may be explained by particle settling and/or retarded degradation of sediment-adsorbed DNA molecules. Compared to aqueous eDNA, sedimentary eDNA could provide a more abundant and longer-lasting source of genetic material for inferring current-or-past site occupancy by aquatic macrofauna, particularly benthic species. However, resuspension and transport of sedimentary eDNA could complicate the spatiotemporal inferences from surface water sampling, which is currently the predominant eDNA-based approach. We discuss these implications in the context of conservation-oriented monitoring in aquatic ecosystems.

1. Introduction

Conservation and management of biodiversity relies on effectively monitoring rare or patchily distributed species across large areas. However, directly observing and identifying such species is often difficult and expensive (Bogich et al., 2008). Less direct methods such as camera traps, acoustic surveys, and noninvasive genetic sampling can be easier, cheaper, and less harmful (Beja-Pereira et al., 2009; Jewell, 2013; Stanley and Royle, 2005). For rare aquatic macrofauna, aqueous environmental DNA (eDNA) sampling is a recent extension of noninvasive genetic sampling in which a sample of bulk environmental material (i.e., water or suspended solids) is assayed for the presence of species-specific DNA fragments without isolating target organisms or their parts from the sample (Ficetola et al., 2008). This method provides inferences about occupancy (Dejean et al., 2012) and abundance (Pilliod et al., 2013; Takahara et al., 2012; Thomsen et al., 2012b) that are simple and inexpensive to obtain (Sigsgaard et al., 2015), once robust sample collection and assay protocols are established (Hayes et al., 2005; Wood et al., 2013). Importantly, development of robust sample collection and assay protocols is difficult, expensive, and time-consuming, making eDNA methods less valuable for abundant organisms that are easily observed and identified by direct methods (discussed in Biggs et al., 2015).

Determining how well eDNA can serve as a proxy for directly observing organisms is an area of active research that will influence how eDNA methods should be applied to biological conservation (Foote et al., 2012; Lodge et al., 2012; Pilliod et al.,

Abbreviations: CTAB, cetyl trimethyl ammonium bromide; eDNA, environmental DNA; IPC, internal positive control; KUFS, University of Kansas Field Station; NCBI, National Center for Biotechnology Information; PCR, polymerase chain reaction; qPCR, quantitative real-time PCR.

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2013, 2014). Guidance is available from other fields that make inferences about organisms based solely on indirect genetic evidence from environmental samples. These include microbiology (Liang and Keeley, 2013), forensics (van Oorschot et al., 2010), paleogenetics (Knapp et al., 2012), fecal pollution tracking (Caldwell et al., 2011), and agricultural transgene monitoring (Nielsen et al., 2007). Across these fields, three major features differentiate detection of eDNA from detection of organisms: **contamination**, **time**, and **space**. First, contaminating DNA molecules from the target organism(s) can enter eDNA samples at any point in the sampling process, from preparation of supplies to genetic assay (Kowalchuk et al., 2007). High-concentration DNA such as polymerase chain reaction (PCR) products and fresh tissue produce the greatest risk of contamination that cannot be objectively distinguished from real eDNA detection (Champlot et al., 2010). Second, eDNA can persist for days to thousands of years, depending on starting concentration and degradation conditions (Levy-Booth et al., 2007). Third, organisms can move long distances from where they shed eDNA and physical forces can move eDNA far from its organismal source (Douville et al., 2007). These features of eDNA detection create uncertainty whose characterization and appropriate use requires better understanding of eDNA in four domains: **origin**, **state**, **transport**, and **fate**. The **origin** of eDNA describes its physiological sources, commonly hypothesized to be feces, urine, gametes, skin, and decomposition (Caldwell et al., 2011). The **state** of eDNA describes its mutable physical forms, such as particle-bound or freely dissolved DNA molecules (Turner et al., 2014). The **transport** of eDNA describes its movement after leaving the source organism, including settling and downstream flow in water (Deiner and Altermatt, 2014). The **fate** of eDNA describes its transformation from intact genomic DNA within living cells into extracellular DNA fragments too small for identification (Barnes et al., 2014). Discoveries in these four domains are beginning to establish the spatial and temporal context for eDNA-based inferences and guide eDNA sampling design. eDNA surveys can effectively inform conservation efforts only when this uncertainty information is used.

In the present study we aimed to gain new understanding about the transport of eDNA shed by fish, specifically its relative concentration in two alternative locations: surface water and surficial sediments. Comparing eDNA concentration in water and sediment is valuable because aquatic particles readily move between them (Leff et al., 1992) and because transport and degradation of aquatic particles can differ substantially between them (Pietramellara et al., 2009). Furthermore, surface water sampling is the primary sampling strategy for eDNA surveys of aquatic macrofauna and the spatiotemporal context of its inferences could be linked to sedimentary eDNA by settling and resuspension dynamics. Depending on eDNA concentration and persistence, sediment samples might be more useful than water samples for monitoring rare macrofauna, particularly benthic species. Previous work on the origin and state of macrofaunal eDNA led us to hypothesize that fish eDNA concentration is higher in sediment than water. Feces are a major source of aqueous macrofaunal eDNA, because they are regularly expelled in large quantities and can contain high concentrations of DNA (Caldwell et al., 2011; Corse et al., 2010). Most animal feces rapidly sink (Robison and Bailey, 1981; Saba and Steinberg, 2012; Wotton and Malmqvist, 2001) and many eDNA-bearing particles of different origins likely sink as well. For example, Turner et al. (2014) recently demonstrated that most of the aqueous eDNA-bearing particles for Common Carp (*Cyprinus carpio*) are too large to stay suspended indefinitely (>1 μm; Maggi, 2013). Settling should lead to higher concentrations of fish eDNA in sediment than water.

Previous work on the transport and fate of microbial eDNA also informed our hypothesis. eDNA concepts and terminology originated in microbiology (Ogram et al., 1987) yet an important distinction must be made between microbial and macrobial eDNA. Microbial eDNA includes both intraorganismal and extraorganismal eDNA from microorganisms such as bacteria. Water, sediment, and virtually any environmental material contains abundant living microbes with active, replicating DNA (intraorganismal eDNA), along with some extracellular DNA from dead microbes (extraorganismal eDNA) (Corinaldesi et al., 2005). When this original concept of eDNA is applied to large organisms the collected material is less likely to contain live macrobes (discussed in Thomsen and Willerslev, 2015). Thus macrobial eDNA originates primarily as shed, excreted, or dead matter (extraorganismal eDNA) where the DNA molecules may still be inside tissue, cells, or organelles (Turner et al., 2014). Macrobial eDNA that is free of cellular and organellar membranes is most analogous to microbial extracellular eDNA, and the term extramembranous comprises both. Recent studies have shown that extramembranous DNA is found in higher concentrations in sediment than the overlying water column (Corinaldesi et al., 2005) and that microbial DNA from the water column can progressively accumulate in sediments (Corinaldesi et al., 2011). These findings suggest that settling and/or preservation of extramembranous DNA could cause fish eDNA to be more concentrated in aquatic sediment than in water.

To test our hypothesis, we measured the concentration of eDNA from an invasive fish in surface water and surficial sediment from experimental ponds and natural rivers. We adapted a simple, low-cost DNA extraction method to produce sedimentary and aqueous eDNA that was free of polymerase chain reaction (PCR) inhibition. Comparison of the sedimentary and aqueous reservoirs of fish eDNA provides a more comprehensive understanding of the processes that affect observed eDNA concentrations, potentially providing further insight to inferences made when using eDNA as an indirect detection method. To our knowledge, this comparison represents the first evaluation of sediments as a source material for eDNA-based monitoring of aquatic macrofauna.

2. Material and methods

2.1. Target species

One of the first and largest conservation programs with eDNA-based monitoring as a central instrument is focused on bigheaded Asian carp (*Hypophthalmichthys* spp., hereafter bigheaded carp) (USACE, 2013; USACE et al., 2013; Jerde et al., 2013; USFWS, 2013). Bigheaded carp were imported to North America as two separate species, Bighead Carp (*H. nobilis*) and Silver Carp (*H. molitrix*). However, since establishing in the Mississippi River basin, introgressive hybridization is widespread, including fertile post-F1 hybrids and F1 hybrid frequency estimates as high as 73% for the silver carp morphotype (Lamer et al., 2010; Stuck, 2012). This hybrid swarm may be developing into a new species complex (Lamer et al., 2010) as the genus expands its range northward (Kolar et al., 2007; USGS, 2013). These large planktivorous fish threaten native fishes because of their dietary overlap with native filter feeders (Sampson et al., 2008) and their tendency to reach high abundance and biomass in their invaded range (Chapman and Hoff, 2011). These characteristics have implicated bigheaded carp in the decline of at least two commercially important fish species in the Mississippi basin, Gizzard Shad (*Dorosoma cepedianum*) and Bigmouth Buffalo (*Ictiobus cyprinellus*) (Irons et al., 2007). Recent analyses predict that small introductions of bigheaded carp could become established (Cuddington et al., 2013) and cause significant ecological and economic harm in many coastal embayments, wetlands, and tributaries of the Laurentian Great Lakes (Cooke and Hill, 2010; Cudmore et al., 2012).

2.2. Pilot sampling

The primary sampling design described in this study was informed by pilot sampling conducted earlier. Here we describe the details of pilot sampling that differed from the primary sampling design. Pilot sampling used four ponds located at the United States Geological Survey (USGS) Columbia Environmental Research Center (CERC) in Columbia, Missouri, USA (LatDD/LonDD: 38.911980, −92.276825). The earthen ponds measured 37 m by 21 m with a maximum depth of 1 m. Each pond contained multiple bigheaded carp until July 2011 when they were emptied of fish, drained of water, and renovated with earthmoving equipment. Renovation included scraping and removing soft surface sediments from the clay substratum and lining the banks with gravel. At the time of sampling on October 21, 2011 three ponds had been partially filled with well water to approximately 0.3 m for the first time since renovation and one pond was still empty. We collected and filtered five 2 L water samples from the three partially filled ponds following the protocol of Jerde et al. (2013). We collected five sediment samples from all four ponds by hand using sterile 50-mL tubes. Sampling containers filled with sterile water were included as collection negative controls for water and sediment. Aqueous eDNA was extracted following Jerde et al. (2013) and sedimentary eDNA was extracted from 5 g of each sediment sample using PowerMax Soil DNA Isolation Kits (MO BIO Laboratories, Carlsbad, California, USA). pGEM-3Z plasmid (Promega, Madison, Wisconsin, USA) was added to the first extraction solution at 0.02 ng μL^{-1} as internal positive control (IPC) DNA for PCR inhibition testing (Coyne et al., 2005). We tested all eDNA extracts for PCR inhibition using a pGEM-specific IPC assay (Coyne et al., 2005) with pGEM amplification providing qualitative evidence for a lack of inhibition. Inhibited extracts were diluted until pGEM amplified. All other details, including qPCR assay for bigheaded carp eDNA, were as described below.

2.3. Primary sampling sites

We sampled both experimental ponds and natural rivers containing bigheaded carp. The experimental ponds were located at the University of Kansas Field Station (KUFS) in Lawrence, Kansas, USA (LatDD/LonDD: 39.047452, −95.191526). Ten earthen ponds had been stocked with at least one bigheaded carp on May 30, 2012, and one additional pond (Pond 311) contained no fish and served as a negative control site. Bigheaded carp had never been present at KUFS prior to May 30, 2012 and quantitative real-time PCR (qPCR) testing of the well water and sediment from each pond prior to stocking detected no bigheaded carp eDNA (data not shown). The ten ponds with bigheaded carp also contained at least one Bluegill Sunfish (*Lepomis macrochirus*), Redear Sunfish (*Lepomis microlophus*), White Crappie (*Pomoxis annularis*), Common Carp (*Cyprinus carpio*), and Grass Carp (*Ctenopharyngodon idella*). The number of bigheaded carp stocked ranged from zero to 46 per pond and the number of total fish ranged from zero to 57 per pond. The density of bigheaded carp stocked per pond ranged from zero to 0.016 g L^{-1} and the density of total fish ranged from zero to 0.02 g L^{-1}. Between June 4 and June 5, 2012 the only bigheaded carp stocked in KUFS pond 321 died and was removed within 24 h of death. The ponds measured 21 m^2 with a maximum depth of 3 m and were filled to approximately 450 m^3 with KUFS well water. After filling there was no water flow through the ponds. Water and sediment samples were collected on October 8–15, 2012. The experimental pond study at KUFS was conducted in accordance with a protocol for field research on live vertebrates (protocol number 211-01) approved by the University of Kansas Institutional Animal Care and Use Committee.

The natural sites were located on the Wabash River in West Lafayette, Indiana, USA; the Kansas River near Desoto, Kansas, USA; and the Wakarusa River below Clinton Reservoir near Lawrence, Kansas, USA. Bigheaded carp have been captured at each of these sites (USGS, 2013).

The Wabash River is a large, unchannelized tributary of the Ohio River with a 1924–2012 mean annual flow between 46 and 352 $m^3 s^{-1}$. The Wabash River stretches 810 km and drains approximately 103,500 km^2 of Ohio, Indiana, and Illinois. Our Wabash River sampling site (LatDD/LonDD: 40.430281, −86.897993) was a borrow pit connected to the main river channel and located approximately 600 m upstream of the nearest United States Geological Survey (USGS) gaging station (USGS 03335500), which reported a mean daily flow of 78 $m^3 s^{-1}$ on the day of sampling (November 13, 2013).

The Kansas River is a large, sand-bottom prairie river characterized by a relatively wide, shallow channel (100–500 m wide, 0.5–3 m deep) and flood control levees on both banks with a 1918–2013 mean annual flow between 38 and 866 $m^3 s^{-1}$. The Kansas River stretches 283 km and drains approximately 155,000 km^2 of Kansas, Colorado, and Nebraska. Our Kansas River sampling site (LatDD/LonDD: 38.984901, −94.97385) was approximately 800 m upstream of the nearest USGS gaging station (USGS 06892350), which reported a mean daily flow of 27 $m^3 s^{-1}$ on the day of sampling (November 26, 2013).

The Wakarusa River is a relatively narrow, shallow (15–20 m wide, 0.5–2 m deep) tributary of the Kansas River that spans 130 km and drains approximately 1100 km^2 of eastern Kansas. It had a 1930–2013 mean annual flow between 0.3 and 21 $m^3 s^{-1}$. The lower Wakarusa River is constrained by incision and dominated by outflows from a large reservoir (Clinton Lake, 28 km^2) with peak daily flows ranging from 17 to 227 $m^3 s^{-1}$. Our Wakarusa River sampling site (LatDD/LonDD: 38.928506, −95.321393) was 800 m downstream from the reservoir outfall. The nearest USGS gaging station (USGS 06891500; approximately 8 km downstream of the sampling site) reported a mean daily flow of 0.15 $m^3 s^{-1}$ on the day of sampling (December 2, 2013).

2.4. Sample collection, preservation, and storage

Water samples were collected following the protocol described by Ficetola et al. (2008). We submerged a sterile 50-mL centrifuge tube slightly below the water surface, allowing it to fill with 15 mL of surface water (measured with the tube graduations), added 1.5 mL of 3 M sodium acetate and 33.5 mL absolute ethanol, and then stored the tube on ice for 10–120 min until it could be frozen (−20 °C). At KUFS we collected three water samples from the shore of each pond at positions chosen by randomized selection from the entire shoreline divided into 20 sections. At the natural sites we collected three water samples from a boat along an approximate transect across the river. At each site we included a 'collection negative control' that consisted of a 50-mL tube containing 15 mL of tap water. This negative control tube was transported to the site alongside sample tubes and was treated as a sample from that point on. All samples were driven or shipped overnight on dry ice to the University of Notre Dame, and stored at −80 °C until eDNA extraction. At every site, we collected all water samples prior to sediment sampling in order to avoid collecting eDNA in the water column that may have been suspended from the sediment by our sediment collection. This precaution prevented any within-site pairing of water and sediment samples.

Our sediment collection method was modified from a method originally developed for collecting sediment diatoms (USEPA, 2007). Sediment samples were collected using Wildco (Yulee, Florida, USA) hand corers and Wildco K-B corers with 5 cm internal diameter, 51 cm long stainless steel core tubes. We collected three

samples along an approximate transect across the pond or river. At the Wakarusa River, outflow from the Clinton Reservoir dam had previously scoured away most of the unconsolidated sediment and we were able to collect only two sediment samples. For each sample we inserted a clear plastic liner tube into the corer and vertically dropped the corer from a small boat. The hand corer was fitted with additional weight to increase the sediment penetration depth. After gently pulling the corer to the surface, we took precautions to minimize disturbance of the sediment-water interface, including submerged plugging of the bottom end of the corer tube with a sediment core extruder, slowly extruding the core upward through the liner tube, and carefully pipetting the last few milliliters of water from atop the sediment core without extruding it past the top end of the liner tube (Glew et al., 2001). We collected 5 mL of wet surficial sediment from the top 2 cm of the sediment core using a 5-mL scoop and transferred this to a 50-mL centrifuge tube containing 10 mL of cetyl trimethyl ammonium bromide (CTAB) buffer (Coyne et al., 2006, 2001). The only exception to this protocol was at the Kansas River site, where sand-dominated sediments were not sufficiently cohesive to maintain core integrity. Thus Kansas River sediment samples were collected by hand in shallow water (~0.75 m) from the top 2 cm of the sediment surface with a sterile 50-mL centrifuge tube. The wet weight of each sample was measured by weighing the sample in its tube and subtracting off the previously measured weight of the tube and CTAB. Sediment samples from the Kansas and Wakarusa Rivers were added to empty centrifuge tubes, stored on ice for 10–120 min, frozen (−20 °C), and shipped overnight on dry ice to the University of Notre Dame where they were thawed, weighed, and preserved in CTAB immediately before eDNA extraction. All other samples were immediately preserved in CTAB, stored on ice for 10–120 min, weighed, frozen (−20 °C), driven on dry ice to the University of Notre Dame, and stored at −80 °C until eDNA extraction.

In between collection of each sediment sample, the corers, liner tubes, nosepieces, extruders, scoop, cables, and ropes were treated with a 4-step decontamination process. First, most visible sediments were rinsed and scrubbed into the pond or river water. Second, all equipment was submerged in solution of hot tap water, 10% bleach, and detergent, then scrubbed until all visible sediment traces were removed. Third, all equipment was submerged in solution of tap water and 10% bleach for 10 min. Fourth, all equipment was rinsed in tap water. In between sites we decontaminated field equipment (boat, waders, boots, etc.) by scrubbing away all visible sediment traces and spraying exposed surfaces with a solution of tap water and 10% bleach. We collected two negative control samples at each site to test for contamination during sampling. The 'corer negative control' consisted of 5 mL of water collected below an internal/external rinsing of a decontaminated and reassembled corer (corer, liner tube, nosepiece) and added to 10 mL of CTAB in a 50-mL centrifuge tube. The 'collection negative control' consisted of swirling the decontaminated measuring scoop in 10 mL of CTAB in a 50-mL centrifuge tube. Upon creation in the field these negative controls were treated as samples from that point on.

2.5. eDNA extraction and purification

DNA extraction was performed in a strictly pre-PCR laboratory separate from our post-PCR laboratory. During extraction we added an 'extraction negative control' to every batch of samples. This consisted of an empty 50-mL centrifuge tube containing 5 or 15 mL of autoclaved reverse osmosis water, which was subsequently treated as a sample. Including separate extraction and collection negative controls allowed us to distinguish between collection-derived and extraction-derived contamination, if any was detected.

Dissolved and suspended particulate matter was precipitated and pelleted from the water samples by 35 min centrifugation at 6 °C and 3220 g. eDNA was extracted from the pellet using a CTAB protocol (Coyne et al., 2005, 2006, 2001), and the final aqueous eDNA pellet was re-suspended in 100 μL of 1X TE Low EDTA buffer (USB Corporation, Cleveland, Ohio, USA) and stored at 4 °C until qPCR assay (Jerome et al., 2002). For sediment samples, we modified the CTAB extraction protocol of Coyne et al. (2005, 2006, 2001). A step-by-step description of our protocols is provided in Supplementary Appendix A1. The final sedimentary eDNA pellet was re-suspended in 1 mL of 1X TE Low EDTA buffer. At this stage we measured PCR inhibition on a subset of 27 sediment samples using the internal positive control assay described below. Results of this testing identified PCR inhibition in some samples (see Results) thus 200 μL of eDNA extract from all 27 samples was further purified using a OneStep Inhibitor Removal Kit (Zymo Research, Irvine, California, USA), according to the manufacturer's instructions. Testing of these purified extracts showed no evidence of PCR inhibition (see Section 3) thus all remaining sediment samples were extracted, purified, and tested for inhibition accordingly.

2.6. eDNA quantification

We measured bigheaded carp eDNA concentration in each eDNA extract using the Minor Groove-Binding (MGB) hydrolysis probe qPCR assay described in Turner et al. (in press), which has a 95% limit of detection of 30 copies reaction^{-1}. This assay targets a 100-base pair (bp) section of the mitochondrial control region (D-loop) of bigheaded carp and uses a FAM-labeled hydrolysis probe. We performed all reactions on a Mastercycler ep realplex2 S thermocycler (Eppendorf, Hauppauge, New York, USA) with the following reaction conditions: 50 °C for 2 min, 95 °C for 10 min, and 55 2-step cycles of 95 °C for 15 s and 60 °C for 1 min. Fluorescence data collection using the FAM filter (520 nm) occurred during the 60 °C step. We performed 20-μL sextuplicate reactions using 10 μL of TaqMan Environmental Master Mix 2.0 (Life Technologies, Carlsbad, California, USA), final primer concentrations of 300 nM each, a final probe concentration of 200 nM, and 4 μL of eDNA extract. To minimize variation between qPCR replicates (technical replicates) caused by imperfect pipetting of small eDNA extract volumes (Ellison et al., 2006), we combined eDNA extract and master mix (all other reagents) for six reactions into one tube then dispensed to six plate wells using an electronic repeating pipette (Xplorer 5–100 μL, Eppendorf). All liquid handling for qPCR used low bind tubes and low bind aerosol barrier pipette tips (Ellison et al., 2006) and each qPCR plate included two qPCR negative control reactions (no template controls – NTCs). qPCR setup was performed inside of an AirClean 600 dead air box with ultraviolet (UV) light (AirClean Systems, Creedmoor, North Carolina, USA) that was decontaminated with 15 min of UV irradiation after every use. Sealed qPCR plates were carried from the pre-PCR laboratory to the post-PCR laboratory for thermocycling.

We used a copy number standard curve made of complete D-loop (1022 bp) (Liu and Chen, 2003) PCR amplicon from tissue-derived Silver Carp DNA that was quantified using a Qubit fluorometer and the Qubit dsDNA High Sensitivity kit (Life Technologies). Standard curve DNA weight was converted to DNA copies using the median double-stranded molecular weight of the 95% consensus 1022-bp amplicon sequence from all Silver Carp mitogenomes on GenBank (635 518 g mole^{-1}) as calculated by OligoCalc version 3.26 (Kibbe, 2007). Each qPCR plate included a five-point standard curve from 3×10^4 copies reaction^{-1} down to 3 copies reaction^{-1}. The fluorescence threshold for each plate and the fluorescence baseline for each reaction were determined using default settings of the Eppendorf realplex software version 2.2 (Noiseband and Automatic Baseline, respectively). Every amplification profile was

visually examined to confirm exponential amplification. To provide additional verification of qPCR assay specificity, beyond the in silico (NCBI GenBank), in vitro (tissue-derived DNA from non-target species), and in situ (eDNA from sites with target and non-target species) testing described in Turner et al. (in press), we purified (ExoSAP-IT, USB Corporation) and Sanger sequenced (ABI 3730xl, Applied Biosystems) qPCR product from at least one water sample and one sediment sample for every site.

We tested every eDNA extract for PCR inhibition using an internal positive control (Universal Exogenous qPCR Positive Control for TaqMan Assays, Yakima Yellow-BHQ-1 Probe Kit, Eurogentec, San Diego, California, USA). This internal positive control (IPC) assay was used in duplex with the bigheaded carp assay by including 2 μL of IPC mix and 0.4 μL of IPC DNA in the 20-μL reactions. Prior to its application in this study we conducted tests confirming the absence of cross-reactivity between IPC and bigheaded carp assays and the stability of Cq values when the two assays were run separately or in duplex. qPCR amplification of the IPC DNA was measured with fluorescence data collection using the VIC filter (550 nm) during the 1 min 60 °C thermocycling step. For each qPCR plate we used the average IPC quantification cycle (Cq) from the reference reactions (standard curve and NTCs) as the expected IPC Cq in order to calculate an IPC ΔCq value (IPC ΔCq = expected IPC Cq – observed IPC Cq) for every eDNA reaction. Following the protocol of Hartman et al. (2005), we used an IPC ΔCq value of three cycles as the threshold defining PCR inhibition. This threshold was supported by our observation that the maximum range of IPC Cq values in the reference reactions on any qPCR plate was 2.8 cycles.

2.7. Data analysis

Following the recommendation of Ellison et al. (2006) for qPCR with low level DNA, we calculated concentrations for each reaction, assigning zero concentration to non-detect reactions and averaging concentration across the six technical replicates for each eDNA extract. In three reactions the measured reaction copy number was slightly below one, so we rounded all reaction copy numbers up to the next largest integer. Final aqueous eDNA concentrations were expressed in copies mL^{-1}, and final sedimentary eDNA concentrations were expressed in copies g^{-1}. To test for a significant difference in concentration between sedimentary and aqueous eDNA, we used the Wilcoxon signed-rank test because the data exhibited non-normal error distribution. To test for a significant relationship between sedimentary and aqueous eDNA concentrations we used a generalized linear model (GLM). Because the data were positive-only with positively-skewed errors we used the Gamma distribution and log link function (Crawley, 2005; Zuur et al., 2010). To compare detection probability (i.e., diagnostic sensitivity) between sedimentary and aqueous eDNA, we calculated the proportion of true positive samples and the associated 95% confidence interval for a binomial probability using the Wilson score method (Newcombe, 1998). All statistical analyses used an alpha level of 0.05 and were performed in R version 3.0.1 (R Core Team, 2014).

3. Results

Aqueous eDNA from pilot sampling showed no evidence of PCR inhibition but sedimentary eDNA extracts required 10-fold dilution before the pGEM-IPC amplified. For primary sampling all aqueous eDNA samples produced an IPC ΔCq value <3 cycles whereas 13 of the initial 27 sedimentary eDNA extracts produced an IPC ΔCq value ⩾3 cycles (range: 3.2–16.5). Purification of sedimentary eDNA extracts using the OneStep Inhibitor Removal Kit reduced all IPC ΔCq values to <3 cycles (range: −1.3 to 1.4), demonstrating that our modified versions of the Coyne et al. (2005, 2006, 2001) protocol for eDNA extraction effectively removed PCR inhibitors from both sediment and water (Burnet et al., 2012; Hartman et al., 2005). Our experimental, field, and laboratory controls showed no contamination. No bigheaded carp eDNA was detected in sediment or water from the KUFS negative control pond (Pond 311; no fish present), indicating that pond maintenance and sampling protocols successfully prevented cross-contamination among experimental ponds. No bigheaded carp eDNA was detected in corer negative controls or collection negative controls,

Table 1
qPCR-measured concentrations of bigheaded Asian carp (*Hypophthalmichthys* spp.) eDNA in sediment and water samples.

Site	Sedimentary eDNA samples					Aqueous eDNA samples					
	n	*n* positive[b]	Mean concentration (copies g^{-1})[c]	SE	RSE	*n*	*n* positive[b]	Mean concentration (copies mL^{-1})[c]	SE	RSE	Ratio of [sedimentary eDNA] to [aqueous eDNA]
Pond 311[a]	3	0	0	0	0	3	0	0	0	0	n/a [d]
Pond 313	3	1	19	19	100%	3	0	0	0	0	n/a [e]
Pond 316	3	3	1025	463	45%	3	1	1	1	100%	1846
Pond 317	3	2	140	108	77%	3	2	2	2	64%	58
Pond 321[a]	3	1	17	17	100%	3	0	0	0	0	n/a [e]
Pond 322	3	3	901	150	17%	3	2	2	1	52%	572
Pond 326	3	2	9425	8477	90%	3	3	49	25	52%	194
Pond 331	3	3	150	64	43%	3	1	1	1	100%	231
Pond 332	3	3	544	99	18%	3	2	4	2	51%	154
Pond 333	3	3	711	432	61%	3	3	27	8	32%	26
Pond 335	3	3	564	113	20%	3	3	13	8	61%	44
Wabash River	3	3	14,544	6829	47%	3	3	185	43	23%	79
Kansas River	3	3	418	208	50%	3	3	51	16	31%	8
Wakarusa River	2	2	1578	1348	85%	3	3	8	1	10%	198

n = number of eDNA samples from a site. SE = standard error of the mean. RSE = SE/mean.

[a] Pond sites were experimental ponds at the University of Kansas Field Station (KUFS) that had been stocked with at least one bigheaded carp 132–138 days before sample collection. Pond 311 was a negative control pond not stocked with fish. Pond 321 contained only one bigheaded carp for 5 days before it died and was removed (132 days before sample collection).

[b] A sample was positive if at least one qPCR amplified.

[c] Mean concentration was calculated using all samples for a site, including negative samples (zero concentration). All aqueous eDNA samples from a site were collected prior to all sedimentary eDNA samples from a site, thus no within-site pairing of water and sediment samples is possible.

[d] Not applicable because Pond 311 contained no fish and bigheaded carp eDNA was not detected in sediment or water.

[e] Ratio cannot be calculated because target eDNA was detected in sediment but not water.

demonstrating the effectiveness of our 4-step decontamination protocol for sediment core sampling. All extraction controls and NTCs also showed no detection of bigheaded carp eDNA, indicating that our contamination precautions were sufficient at every step, from sample collection to qPCR assay. The range of qPCR efficiency across the entire study, calculated from the slope of standard curves, was 96–104% and the range of standard curve R^2 values was 0.963–1.00. All Sanger sequenced qPCR products matched the target amplicon from bigheaded carp.

Pilot sampling of four renovated CERC ponds with no bigheaded carp presence in the preceding three months yielded no detection of aqueous eDNA but did detect sedimentary eDNA in two ponds – one containing water and one that was empty. These results informed the primary sampling design.

At every primary sampling site, bigheaded carp eDNA was more concentrated per g of sediment than per mL of water (8–1846 fold; Table 1) and overall sedimentary eDNA concentration was significantly higher than aqueous eDNA concentration ($P = 0.0002$). By using the conventional units of weight for sediment and volume for water these concentration comparisons implicitly assume the equivalence of 1 mL and 1 g of water. All sediment samples were collected with a 5-mL scoop thus sedimentary eDNA concentrations could also be expressed per mL of sediment. However, sediment sample weight ranged from 5.5 to 10.9 g so the use of volume would only produce higher sedimentary eDNA concentrations, leaving the overall results unchanged.

Sedimentary and aqueous eDNA concentration were positively correlated when analyzed across all primary sampling sites ($P = 0.001$; Fig. 1) and within the KUFS ponds ($P = 0.002$; Fig. 1). A correlation test was not performed on the natural sites alone because only three were sampled. Aqueous eDNA sampling failed to detect the presence of the bigheaded carp in one experimental pond, whereas sedimentary eDNA sampling never failed to detect site presence (Table 1). Average detection probability (i.e., diagnostic sensitivity) across all primary sampling sites was 89% for sedimentary eDNA and 72% for aqueous eDNA, considering bigheaded carp present at the natural sites. This difference was not statistically significant based on overlapping 95% confidence intervals.

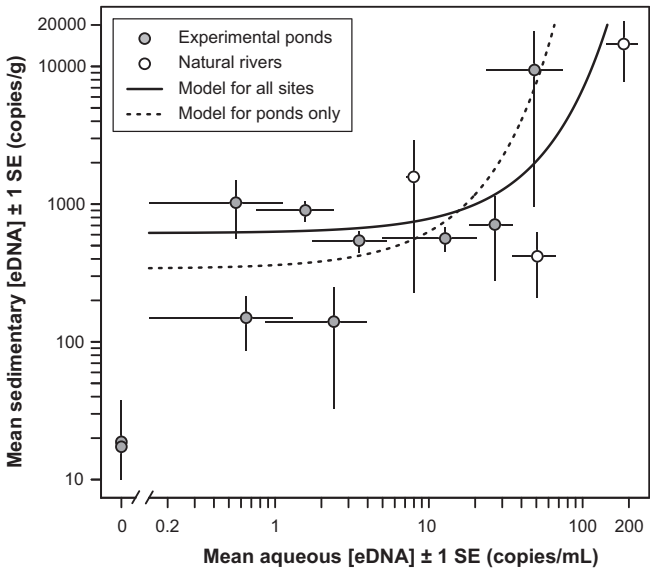

Fig. 1. Plot of sedimentary and aqueous eDNA concentration for experimental ponds ($n = 10$) and natural rivers ($n = 3$). Filled circles show experimental ponds and unfilled circles show natural rivers. Note that both axes are logarithmic and the x-axis contains a break to include zero. The solid curve shows predicted values for the best fit GLM for all sites ($P = 0.01$). The dashed curve shows predicted values for the best fit GLM for experimental ponds only ($P = 0.02$).

Bigheaded carp eDNA was detected in sediment from one experimental pond 132 days after the single bigheaded carp was removed, whereas no target eDNA was detected in the water from that pond (KUFS pond 321; Table 1).

4. Discussion

As we hypothesized, concentrations of bigheaded carp eDNA were consistently higher in sediment than water. These results concur with reported differences between sedimentary and aqueous concentration of total extracellular DNA in microbiology studies (Corinaldesi et al., 2005; Dell'Anno and Corinaldesi, 2004; Pietramellara et al., 2009). Also, a recent comparison between sedimentary and aqueous eDNA of *Cyprinid herpesvirus 3*, a DNA virus that infects Common Carp, found 46-1238 times higher concentration in sediment than in the water column (Honjo et al., 2012). Thus, aquatic sediments appear to accumulate fish eDNA, viral eDNA shed by fish, and extracellular microbial eDNA. Although we demonstrated that net accumulation (i.e., deposition minus degradation and transport) of bigheaded carp eDNA is higher in sediment than water, the respective roles of degradation and transport remain to be determined. Few paired measurements of DNA degradation rate in water and sediment exist (Pietramellara et al., 2009), and evidence exists for both faster and slower DNA degradation rates in sediment compared to water (Corinaldesi et al., 2011; Dell'Anno and Corinaldesi, 2004; England et al., 2005, 2004). The focus of previous studies on extramembranous DNA (i.e., DNA molecules not protected by cellular, organellar, or viral membranes) limits comparison with naturally occurring fish eDNA, which could exist in multiple states along a continuum from whole living organisms (e.g., larvae) down to 'free' extramembranous DNA molecules not bound to other particles. Feeding experiments by Klymus et al. (2015) implicate feces as a major source of fish eDNA. We suspect that settling of fish eDNA-bearing particles such as feces is the dominant process explaining the large accumulation of sedimentary eDNA we observed, but more research is needed to tease apart settling from degradation rate.

The other main result, that sedimentary eDNA lasted longer than aqueous eDNA, is linked to concentration differences because eDNA degradation generally follows an exponential decay pattern where higher starting concentration creates longer persistence (Barnes et al., 2014; Thomsen et al., 2012a). In pilot sampling we detected no bigheaded carp eDNA in water from three CERC ponds that were fishless for three months. Water from KUFS pond 321 also produced no detection 132 days after removal of its bigheaded carp. However, bigheaded carp eDNA was detected in sediment from ponds without bighead carp: KUFS pond 321, one of the three watered CERC ponds, and one CERC pond that was dry. These repeated observations of fish eDNA lasting 90+ days in sediment but not water are consistent with our finding that eDNA concentration was always higher in sediment than water. By comparison, previous studies of aqueous macrobial eDNA found a maximum persistence time of 25 days (Barnes et al., 2014; Dejean et al., 2011; Goldberg et al., 2013; Pilliod et al., 2014; Thomsen et al., 2012a,b). Strickler et al. (2015) reported 58-day persistence of frog eDNA in water held at 5 °C. The persistence we observed is not unusual in the context of literature on the fate of extraorganismal eDNA in soils and sediments (Pietramellara et al., 2009). For example, unfrozen lake sediment cores yielded fish eDNA from 3600 yr before present (BP; Matisoo-Smith et al., 2008) and mammal eDNA from 4800 yr BP (Giguet-Covex et al., 2014). Given the DNA-preserving properties of aquatic sediments we suspect the temporal window for surficial sedimentary eDNA extends much further than 132 days, which was the longest our study could observe. Sediments reduce biologically driven DNA degradation by adsorbing

both DNases and DNA molecules (Levy-Booth et al., 2007; Pietramellara et al., 2009). Chemically driven DNA degradation (e.g., depurination) also appears to be reduced in aquatic sediments compared to terrestrial environments (Corinaldesi et al., 2008). Experiments measuring the degradation rate of aquatic macrofaunal eDNA in surficial sediments are needed to estimate the temporal window of eDNA persistence for diverse species and environments.

The concentration and persistence results collectively suggest a strategy for how aqueous and sedimentary eDNA from aquatic macrofauna should be used for biological conservation. First, sediment sampling may provide higher detection probability if water sampling – which is far easier to conduct – proves inadequate. A recent eDNA survey for invasive crayfish sampled mixtures of intentionally resuspended sediment and water, suggesting that more convenient surface water sampling failed for this benthic species (Tréguier et al., 2014). Interestingly, in our data the relative standard error (RSE = SE/mean) of within-site eDNA concentration estimates was high for both sediment and water in spite of the significantly higher average concentrations in sediment (Table 1). As discussed by Pilliod et al. (2013), RSE values over 20% indicate high spatial heterogeneity for both types of eDNA and recommend the use of larger amounts of water/sediment per sample and/or more than three samples per site. Taberlet et al. (2012) describe a spatially-integrated approach for sampling terrestrial soil eDNA that may be transferable to aquatic sediments. However, our persistence results present a major caution for sedimentary eDNA sampling in many conservation applications: surficial sediment provides detection of current-or-past occupancy (at least 132 days) whereas surface water provides detection of current-or-recent occupancy (up to 25 days). Conservation programs requiring data on recent occupancy should avoid sediments in favor of water sampling. Aqueous eDNA capture methods such as portable filtration (Goldberg et al., 2011; Pilliod et al., 2013) or continuous flow centrifugation (Zuckerman and Tzipori, 2006) can increase water sampling effort (i.e., water volume processed) relatively easily, which may mitigate the slightly lower detection probability we observed from water compared with sediment.

The high concentration and long persistence of fish eDNA in surficial sediments creates an opportunity for sediment resuspension to influence both the temporal and spatial scales of inference from aqueous eDNA (Bloesch, 1995; Douville et al., 2007; Graf and Rosenberg, 1997; Leff et al., 1992). We saw no evidence of resuspension-derived aqueous eDNA in the three CERC ponds where it could have been identified after dewatering and refilling. However, since resuspension occurs when shear stress on the sediment bed exceeds a critical threshold value (Vanoni, 2006), studies monitoring for very recent occupancy should avoid sampling shallow lakes or ponds experiencing high bed shear stress conditions. For example, increased resuspension of sediment particles has been observed during or immediately after high wind events (Evans, 1994), in areas with significant wave action (Mian and Yanful, 2004), and in areas with substantial bed loads (the surficial sediments that are transported along the bed; Debnath et al., 2007). Similarly, results from a natural stream experiment with tracer bacteria seeded in sediments (Jamieson et al., 2005) suggest that water sampling in rivers during unusually high flow events would increase the chance of collecting old eDNA resuspended from sediment beds that are stable at lower flow. Testing this resuspension hypothesis may be useful when monitoring seasonally occurring species, such as anadromous salmon, in rivers with large seasonal flow variation (Laramie et al., 2015). Human disturbance and transport of aquatic sediments should also be considered, including that caused inadvertently by scientists collecting eDNA samples. For example, cleaning mud from gear and footwear between sites appears to be particularly important given high sedimentary eDNA concentration and the detection of wetsuit-derived amphibian DNA reported by Spear et al. (2015).

The low temporal resolution of sedimentary eDNA may be appropriate for conservation programs that can use information about current-or-past occupancy, such as retrospective genetic monitoring (Schwartz et al., 2007) of aquatic macrofauna for spatial distribution and historical range studies concerned with long-term site occupancy (Fernández et al., 2010; Provan et al., 2008; Wandeler et al., 2007). The abundance and persistence of sedimentary eDNA could benefit monitoring for species introductions or range shifts where the target species has no prior occupancy (Tréguier et al., 2014). Retrospective monitoring of macrofauna using sedimentary eDNA is well established for terrestrial sediments (Andersen et al., 2012; Haile et al., 2009), but analysis of aquatic sediments for macrobial eDNA has largely been limited to plants (Anderson-Carpenter et al., 2011; Boessenkool et al., 2013; but see Giguet-Covex et al., 2014; Matisoo-Smith et al., 2008; Naviaux et al., 2005). Our results suggest that fish eDNA in aquatic sediments may be a promising source of historical genetic material, although further research is needed to evaluate the generality of our small study. Monitoring and managing biodiversity during the course of human population growth and ecosystem modification is a central goal of conservation biology, and aquatic animals are particularly vulnerable to extinction and difficult to monitor (Xenopoulos et al., 2005). The different temporal windows provided by sedimentary and aqueous DNA should facilitate, for example, determination of historical native range from sediment and seasonal occupancy from water, thereby enabling more effective conservation actions.

The positive correlation between sedimentary and aqueous eDNA likely represents a relationship with both downward and upward processes (Fig. 2). Suspended particles (i.e., aqueous eDNA) may eventually sink downward and accumulate in the surficial sediment layer (i.e., sedimentary eDNA). Alternatively, large pieces of DNA-rich fish ejecta (e.g., feces; Caldwell et al., 2011; Corse et al., 2010) which have rapidly settled to the substratum (Robison and Bailey, 1981; Saba and Steinberg, 2012), may slowly disintegrate (Wotton and Malmqvist, 2001) and release smaller DNA-containing particles upward back into the water column through resuspension by turbulent flow and bioturbation (Bloesch, 1995;

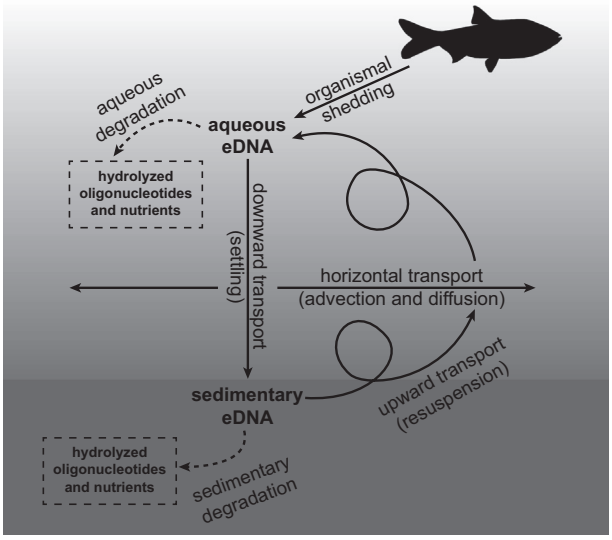

Fig. 2. Conceptual diagram of the processes affecting eDNA released into the water column by aquatic macrofauna. Because sedimentary eDNA persists longer than aqueous eDNA resuspension of sediments could influence the temporal resolution of inferences about organism presence made from aqueous eDNA. Horizontal transport of resuspended sediments could also influence the spatial resolution of inferences from aqueous eDNA.

Graf and Rosenberg, 1997; Leff et al., 1992). These processes are common for many aquatic particles but have not yet been specifically studied with respect to eDNA. Factors such as water flow, substrate, wind, depth, stratification, and biota likely determine whether downward or upward processes dominate the link between aqueous and sedimentary eDNA. Better understanding of how these processes influence eDNA is needed if research continues to pursue aqueous eDNA concentration as an indicator of organism abundance or proximity (Pilliod et al., 2013; Takahara et al., 2012; Thomsen et al., 2012b).

Importantly, many of the sedimentary eDNA concentrations we measured would have been erroneous without use of a quantitative IPC assay sensitive to partial PCR inhibition. Even the use of commercial soil extraction and qPCR reagent kits specifically designed to mitigate inhibition did not guarantee success. Measuring partial inhibition with IPC ΔCq led us to add the One-Step Inhibitor Removal Kit, which simply and affordably removed remaining inhibitors. Mckee et al. (2015) found the performance of this kit to compare favorably with alternative inhibitor mitigation methods. Other studies have also shown the effect that eDNA collection, storage, capture, extraction, and assay protocols have on results (Deiner et al., 2015; Takahara et al., 2015; Turner et al., in press). Although the small-volume aqueous eDNA samples in this study showed no inhibition after CTAB extraction we have observed it for other samples, particularly from large volumes or water with high concentrations of algae or suspended sediment (CRT, unpublished data). In agreement with the MIQE guidelines (Bustin et al., 2009) and recent reviews (Hedman and Rådström, 2013) we recommend application of a quantitative IPC assay for all eDNA studies, especially when eDNA quantification is attempted.

In conclusion, we adapted a simple, low-cost extraction method to recover inhibitor-free eDNA from both sediment and water samples and showed that bigheaded carp eDNA is more concentrated in sediment. Sedimentary eDNA was a slightly more sensitive detector of site occupancy, but in at least three sites it remained detectable months after the target species was no longer present. eDNA-based monitoring to conserve rare species or prevent establishment of invasive species should consider how the relatively high concentration and long persistence of sedimentary eDNA can influence the spatiotemporal resolution of eDNA-based inferences. However, more research is needed before sedimentary eDNA can be routinely used to study contemporary populations. Future studies on the degradation of sedimentary eDNA and the processes moving eDNA between water and sediment would improve our understanding of how to use these reservoirs as a proxy for directly observing organisms.

5. Glossary

- **Environmental DNA (eDNA):** DNA extracted from bulk environmental samples (e.g., soil, water, air) without isolating target organisms or their parts from the sample. eDNA can exist in multiple states along a continuum from whole living organisms (e.g., macrobial larvae or single-celled microbes) to 'free' extramembranous DNA molecules not bound to other particles.
- **Intraorganismal eDNA:** eDNA contained in whole living organisms, such as microbes, meiofauna, or macrobial larvae, where it is protected, active, and can replicate.
- **Extraorganismal eDNA:** eDNA outside of living organisms, such as cellular DNA in shed tissue, shed macrobial cells, and 'free' DNA molecules from unicellular or multicellular organisms, where it is less protected, inactive, and cannot replicate.
- **Extramembranous DNA:** DNA not bound by cellular, organellar, or viral membranes; synonymous with the term extracellular DNA in microbial literature.
- **Internal positive control assay (IPC assay):** A qPCR assay that detects and quantifies the IPC DNA.
- **Internal positive control DNA (IPC DNA):** An exogenous DNA molecule that is spiked into a qPCR at a known and standardized concentration, then detected and quantified to determine if PCR inhibition has occurred.
- **PCR inhibition:** interference with the polymerase chain reaction caused by an excess of non-target DNA molecules, or by non-DNA substances inadvertently extracted with the DNA sample. Complete PCR inhibition causes failure to detect target DNA, and partial PCR inhibition biases quantification of target DNA.
- **Quantification cycle (Cq):** The fractional number of qPCR thermocycles at which the reporter dye fluorescence exceeds a standardized threshold.
- **Quantitative real-time polymerase chain reaction (qPCR):** a thermocycled chemical reaction used for targeted detection and quantification of specific nucleic acids during the reaction (in 'real time'), based on their nucleotide sequence and a fluorescent reporter dye.

Author contributions

Conceived and designed the experiment: CRT RCE KLU. Developed field methods: RCE CRT. Developed lab methods: CRT KLU. Performed the experiment: CRT RCE KLU. Analyzed the data: CRT. Contributed reagents/materials: CRT RCE. Wrote the paper: CRT RCE KLU.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biocon.2014.11.017.

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