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Effects of Temperature and Trophic State on Degradation of Environmental DNA in Lake Water

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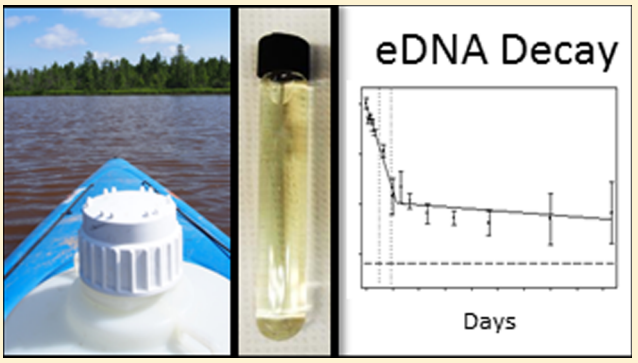
Supporting Information

ABSTRACT: Degradation of environmental DNA (eDNA) in aquatic habitats can affect the interpretation of eDNA data and the ability to detect aquatic organisms. The effect of temperature and trophic state on the decay of Common Carp (*Cyprinus carpio*) eDNA was evaluated using lake water microcosms and quantitative PCR for a Common Carp-specific genetic marker in two experiments. The first experiment tested the effect of temperature on Common Carp eDNA decay. Common Carp eDNA exhibited exponential decay that increased with temperature. The slowest decay rate was observed at 5 °C, with a T_{90} value (time to 90% reduction from initial concentration) of 6.6 days, as opposed to ∼1 day at higher temperatures. In a second experiment, decay was compared across waters from lakes of different trophic states. In this experiment, Common Carp eDNA exhibited biphasic exponential decay, characterized by rapid decay for 3−8 days followed by slow decay. Decay rate was slowest in dystrophic water and fastest in oligotrophic water, and decay rate was negatively correlated to dissolved organic carbon concentration. The overall rapid decay of eDNA and the effects of temperature and water quality should be considered in protocols for water sample storage and field sampling design.



INTRODUCTION

Environmental DNA (eDNA), the genetic material released by an organism into the environment through excreted mucus, feces, or other material, is increasingly being used to survey and detect both invertebrate[1] and vertebrate animals in aquatic habitats.[2,3] The advantage of this approach is that an organism can be detected without the need for capture or visual identification, making eDNA a valuable tool in the study of rare or reclusive species.[4−6] The use of eDNA for detection of aquatic vertebrates is still a relatively new technique, so there are numerous unresolved questions, such as optimal sampling methodology and how to accurately interpret data.[7−9] In addition, factors that affect eDNA degradation (i.e., the physical breakdown of eDNA over time) in the environment, and whether they slow or hasten decay, are not well-understood.[3] Improved understanding of eDNA degradation would help researchers and managers choose how frequently to sample, when to sample, and how to refine sample storage methods.

The detectability of eDNA in water has been shown to decline dramatically following removal of an organism from stocked tanks or ponds.[6,10−12] One week following removal of Burmese pythons (*Python bivattatus*) from tanks, the eDNA detection rate decreased from 100 to 40%.[6] Bullfrog (*Rana* [*Lithobates*] *catesbeiana*) eDNA persisted up to 20 days in tanks, whereas sturgeon (*Acipenser baerii*) eDNA persisted only 2 weeks in ponds.[10] The decrease in eDNA concentration over time can be used to calculate a decay rate, and reported eDNA decay rates for aquatic vertebrates range widely, from 0.05 to 17.9 day^{-1} (Table 1).[12−16] Environmental conditions, such as temperature, pH, and light, have been shown to affect eDNA decay.[12−14]

Despite the important impact of eDNA degradation on probability of species detection, there is a dearth of information on how factors such as temperature and water chemistry influence eDNA decay rates in natural fresh waters. Many studies to date have used tap, well, or store-bought spring water, which might not reflect natural fresh waters, as there are differences between these water types which can affect degradation (e.g., microbial load, nutrient availability, dissolved organic matter, and suspended particulates). Moreover, these factors typically covary across the range of trophic states (i.e., from oligotrophic to eutrophic). Studies of bacterial persistence have shown higher microbial load acts to hasten decay, whereas suspended humic material and clays can significantly slow decay.[17,18] In sterile water, microbial DNA persists longer due to decreased predation by other microbes and utilization of DNA as a food source.[19] Clays and humic material strongly

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Table 1. Previously Published eDNA Decay Rates for Aquatic Vertebrates

organism	scientific name	water source	light regime (LL:DD)[a]	temp (°C)	k[b] (day^{-1})	T_{90}[c] (days)	T_{99}[d] (days)	reference
American bullfrog	*Lithobates catesbeianus*	tap water mixed with a mild salt solution	varied	5−35	0.05−0.34	6.8−46	13.5−92	12
Common Carp	*Cyprinus carpio*	50:50 mixture of well water and pond water	14:10	25	2.43−17.9	0.1−0.9	0.3−1.9	13
Bluegill Sunfish	*Lepomis macrochirus*	not specified	12:12	20	2.5	0.9	1.8	16
European Flounder	*Platichthys flesus*	seawater	12:12	15	0.32	7.2	14.4	15
Idaho giant salamander	*Dicamptodon aterrimus*	store-bought spring water	12:12	11−25	1.89[f]	1.2	2.4	14
Idaho giant salamander	*Dicamptodon aterrimus*	store-bought spring water	00:24	13−20	1.64[f]	1.4	2.8	14
Three-spined stickleback	*Gasterosteus aculeatus*	seawater	12:12	15	0.70	3.3	6.6	15

[a]The hours of light (LL) versus hours of darkness (DD) within a 24 h period. [b]Decay rate. [c]Time for initial concentration to decrease by 90%. [d]Time for initial concentration to decrease by 99%. [f]Decay rates were estimated from graphical data.

binds to DNA, protecting it from enzymatic breakdown.[17,18] Consequently, dystrophic waters, which have a large amount of dissolved humic material, could have significantly slower eDNA degradation than oligotrophic or eutrophic waters. There is also a need to establish a baseline of decay rates in natural freshwaters, since previous studies have reported a wide range of values.

Fishes, cyprinids in particular, have been widely used in eDNA research.[4,13,20,21] This focus is likely due to the use of eDNA for monitoring of Bigheaded Carps (*Hypophthalmichthys* spp.) in the Mississippi and Illinois Rivers. In a previous study, we found that the distribution of Common Carp (*Cyprinus carpio*, hereafter "Carp") eDNA in a small eutrophic lake was extremely patchy.[22] We hypothesized that rapid degradation was one of the primary factors that contributed to the patchy distribution of eDNA. Although few studies have examined the degradation rate of cyprinid eDNA, Barnes et al.[13] found that the decay rate of Carp eDNA in well water was rapid, with most eDNA being degraded within 1 d. Decay rate was correlated with a suite of covarying factors, such as oxygen demand, pH, chlorophyll *a*, and total DNA, that were associated with the number of fish stocked in each tank.[13]

The goal of this study was to measure the effects of temperature and trophic state on the decay rate of Carp eDNA in lake waters. Specifically, we aimed to answer the following questions: (1) How does temperature, which ranges considerably in temperate lakes from near freezing to over 30 °C, affect the Carp eDNA decay rate in lake water?, and (2) What is the effect of lake trophic state on Carp eDNA decay rate, and how does it compare to well water, which is typically used in studies of eDNA degradation? Understanding the effect of temperature would help elucidate how seasonal changes affect eDNA persistence and how low-temperature storage may affect sample integrity. Comparing degradation of eDNA across lakes with different trophic states would establish an expected range of eDNA decay rates for aquatic animals in fresh waters.

MATERIALS AND METHODS

Water Collection. Water was collected from study lakes using 40 L HDPE carboys (Nalgene, Rochester, NY) which had previously been soaked in 10% bleach for 30 min to remove residual DNA and rinsed thoroughly with deionized (DI) water. Water was collected from the surface of the limnetic zone of each site. For the first experiment, which tested the effect of temperature, water was sampled from a eutrophic lake. For the second experiment, which examined the effect of trophic state, three lakes were sampled: a eutrophic lake, an oligotrophic lake, and a dystrophic lake. Well water was also included in the trophic state experiment for comparison. The density of Common Carp in the eutrophic lake was approximately 170 carp/ha.[22] The density has not been measured in the other study lakes. The names, locations, and sampling dates of study lakes are shown in Supporting Information (SI) Table S1. Water quality parameters of study lakes are shown in SI Table S2.

Addition of Carp eDNA. Carp eDNA was added to the water by adding live Common Carp directly to the water to be tested. Carp eDNA was added to the waters tested because we anticipated that the concentration of eDNA would likely be close to or below our limit of detection. To confirm this assumption, four replicate 50 mL water samples were taken from each water type prior to Carp eDNA addition (denoted as "Pre" samples). To increase the initial amount of Carp eDNA, 10 live Common Carp (25 g each) were added to 30 L of water in 40 L aerated glass aquaria for 1−2 h. The exposure time was limited to minimize the potential impact on water quality. After removing fish, the water was poured through a polyethylene terephthalate (PET) woven mesh spacer (EMD Millipore, Germany) with a pore size of ∼1 mm to remove large debris. This process was repeated for every water type tested. Fish were held in accordance with the University of Minnesota's Institutional Animal Care and Use Committee (IACUC) (Protocol: 1407-31659A).

Experimental Setup. After Carp eDNA was added, the water was placed into a large carboy that was mixed with a magnetic stir bar to ensure even distribution of DNA while water was aliquoted into 50 mL glass microcosms with no headspace. The microcosms were silanized to reduce binding of DNA to tube walls over the course of the experiment.[23] The oxygen concentration was monitored over the course of the experiment to ensure anoxic conditions did not develop. In the first experiment, eutrophic lake water was incubated at different temperatures. Temperatures were chosen to span the typical seasonal range of temperate lakes. Microcosms were incubated at 5, 15, 25, and 35 °C in the dark in growth chambers. In a second experiment, dystrophic lake water, eutrophic lake water, oligotrophic lake water, and well water were incubated at a constant temperature (15 °C) in the dark in a growth chamber.

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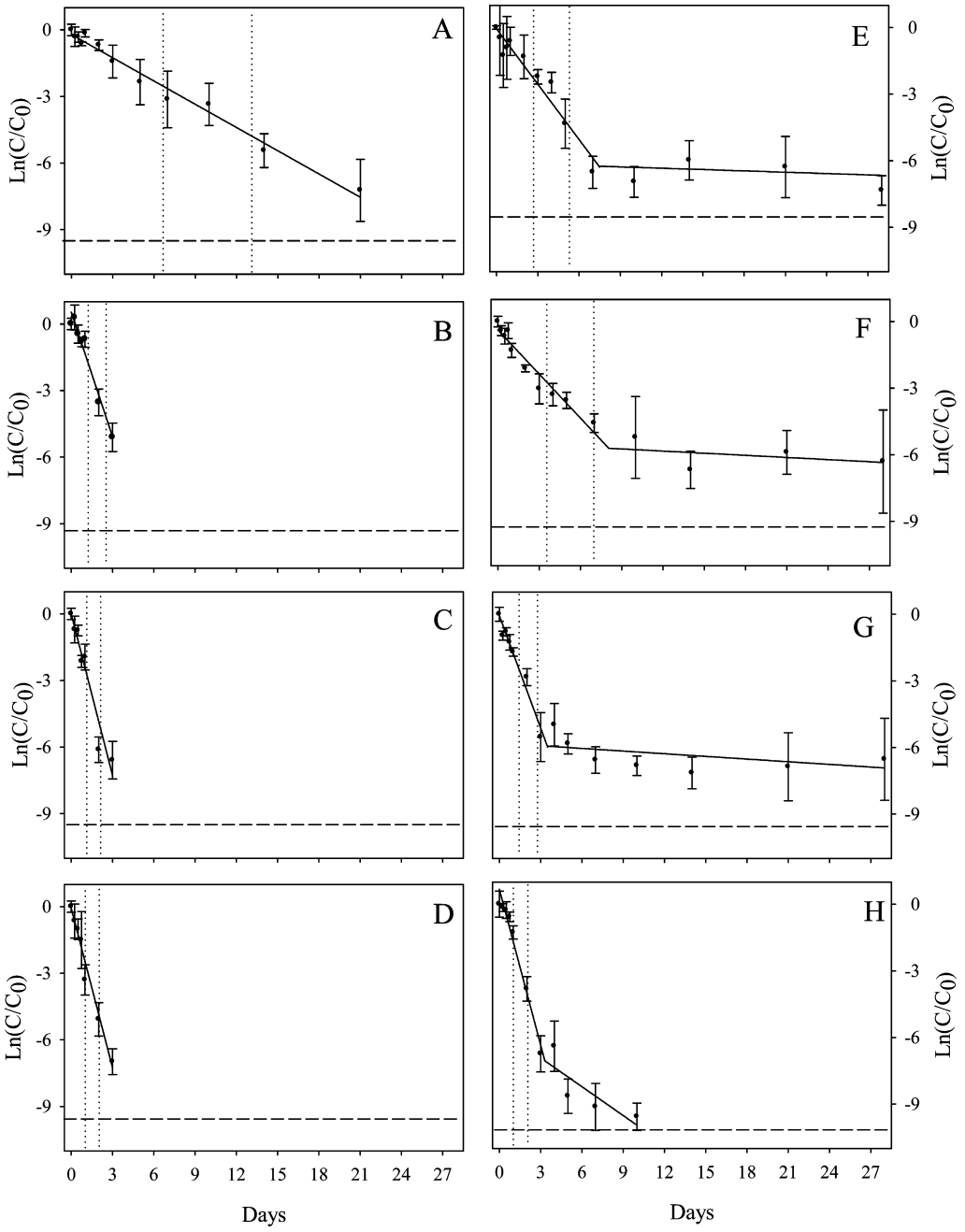

Figure 1. Decay curves for the temperature experiment (A–D) and trophic state experiment (E–H). Decay curves are shown for eutrophic lake water at 5 °C (A), 15 °C (B), 25 °C (C), and 35 °C (D) and for well water (E), dystrophic lake water (F), eutrophic lake water (G), and oligotrophic lake water (H) at 15 °C. Error bars show 95% confidence intervals of the mean, and the solid line shows the fitted decay curve. Dotted lines show the T_{90} (time to 90% decay; left dotted line) and T_{99} (time to 99% decay; right dotted line) time points, and the dashed line shows the limit of detection (LOD). Time points with concentrations below the LOD are not shown.

Eutrophic lake water was from the same lake as the temperature experiment; however, the lake was sampled later in the year during an algal bloom. This enabled a comparison of decay within a single lake between early and late summer (~2 mo. difference) to examine seasonal effects.

Microcosm Sampling. Upon sampling, microcosms were randomly chosen from the incubation chambers, and they were destructively sampled at 14 time points: Pre, 0, 0.25, 0.5, 0.75, 1, 2, 3, 5, 7, 10, 14, 21, and 28 days. In the trophic state experiment, an additional time point was taken at 4 days. "Pre" samples indicate lake water prior to Carp eDNA addition. At each time point, 50 mL of water was filtered through a 0.2 μm polycarbonate filter membrane (EMD Millipore, Germany). Four microcosms were sampled per time point, and microcosms were placed on ice after removal from growth chambers until filtration. Filters were folded, and stored at −80 °C until DNA extraction. The time from microcosm removal to storage was under 1 h. Details regarding sampling and analysis for dissolved oxygen (DO) can be found in SI.

Molecular Analysis. Prior to DNA extraction, 50 ng of salmon sperm DNA (Life Technologies, Grand Island, NY) was added to the extraction buffer to serve as an extraction control and check for complete inhibition of the PCR reaction.[22,24] DNA was extracted using the PowerSoil DNA Isolation Kit

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(MoBio Laboratories Inc., Carlsbad, CA) which produces consistent yield and high quality of DNA from water samples containing Carp eDNA.[25] The extraction protocol was modified to include an additional wash with buffer C5, and DNA was eluted in a total volume of 50 μL (25 μL buffer C6, 5 min incubation at room temperature, centrifuge, repeat), and stored at −20 °C.

Multiplex quantitative PCR (qPCR) for Carp eDNA and the extraction control were performed as previously described.[22] A synthetic dsDNA fragment (gBlocks Gene Fragments, Integrated DNA Technologies Inc., Coralville, IA) containing the two target genes separated by 5 Thymine bases was used for the qPCR standard. Each run contained triplicate reactions of no template controls and 30, 300, 3000, 30 000, and 300 000 copy standards. The assay limit of detection (LOD) is defined as the copy number at which 95% of replicate standard successfully amplify,[26] and LOD was determined to be 30 copies/reaction. See SI Table S3 for a summary of calibration curves. Samples were diluted 1:10 and run in duplicate, and samples were rerun if the standard deviation of the Cq values (quantification cycle value) exceeded 0.3. If samples were below the assay LOD, the sample was run undiluted. The sample LOD (6 copies/mL) was calculated using the volume of sample filtered, extract elution volume, the proportion of extract run per assay, and the assay LOD.

To check for partial inhibition of the qPCR reaction, DNA extracts from lake and well water were spiked with a known copy number. Cq values were compared to a reference that contained nuclease free water rather than DNA extract. A total of 5 μL of DNA extract was used for comparison, and comparison samples consisted of the Pre samples of each water type. Each replicate extract was run in duplicate, for a total of eight reactions per water type including the nuclease free reference samples. Ten thousand copies of Common Carp eDNA marker was added to each reaction, and the reaction was considered inhibited if the Cq value increased by 3 or more.[27]

Statistical Analysis. A one phase exponential decay model was used to examine eDNA decay, as it has shown to provide good fit of data in previous studies:[12,13,15]

$$C = C_0 e^{-kt} \quad (1)$$

where C is the concentration of genetic markers at time (t), C_0 is the initial concentration of markers (SI Table S4), and k is the decay rate. The decay rate is a constant, and is thus independent of C_0. The data were fitted to a two phase (biphasic) exponential decay model if a single phase model exhibited poor fit, as indicated by residual plots, and there was sufficient data to determine a breakpoint between two distinct phases of decay:

$$C = C_0 e^{-k_1 t'} e^{-k_2 t - t'} \quad (2)$$

where k_1 is an initial rapid decay phase until a breakpoint at time (t') occurs when the decay dynamics shift into a second phase of slower decay (k_2).

The breakpoints for the biphasic decay model were determined by the package "segmented" in R (R: A Language and Environment for Statistical Computing, R Core Team, R Foundation for Statistical Computing, Vienna, Austria, 2014, http://www.R-project.org). A linear model was used to test effects of temperature and water type. If a significant effect was observed ($p \leq 0.05$), contrasts were used to compare decay rates. Comparisons were adjusted for multiple comparisons using Bonferroni correction. The lengths of time for 90% (T_{90}; SI eq S1) and 99% (T_{99}; SI eq S2) reduction of initial concentration were also calculated.

For comparisons to previously published studies that did not report decay rates, decay rates were calculated from graphical data using Web Plot Digitizer.[28] The persistence of Carp eDNA in lake water at natural concentrations was calculated for several lakes in which the concentration of Carp mitochondrial eDNA was reported (SI eq S3). Additional description of the calculation can be found in the SI. One-way ANOVA was used to examine the effect of water type, temperature, and sampling day on the extraction efficiency (percent recovery of the extraction control DNA). Pairwise correlations of decay rates at 15 °C and water quality parameters (SI Table S2) were used to examine which factors likely influence decay rates. ANOVA and correlations were done in JMP, Version 10 (SAS Institute Inc., Cary, NC).

RESULTS AND DISCUSSION

The Effect of Temperature on eDNA Decay Rate. Temperature affected the persistence time and decay rate of Carp eDNA in eutrophic lake water. Carp eDNA was below detection in lake water prior to its addition (SI Table S4). The concentration of eDNA was 2.42×10^5 copies/mL at the start of the experiment (SI Table S4), and it remained above the LOD (6 copies/mL) for 21 days at 5 °C and for 3 days at 15, 25, and 35 °C. Carp eDNA exhibited good fit to a one phase exponential decay model for all temperatures (Figure 1, Table 2), confirmed by an even and symmetrical distribution of residual plots (SI Figure S1). Decay was slowest at 5 °C ($p < 0.05$) relative to all other temperatures, with a T_{90} value of 6.6 days. T_{90} values at or above 15 °C were approximately 1 day. At greater temperatures, the only significant difference ($p = 0.04$) in the decay rate was observed between 15 and 25 °C, although the difference between 15 and 35 °C was significant at $\alpha = 0.1$. DO was 6.4 mg/L at the start of the experiment and declined over time, but DO did not fall below 4 mg/L prior to reaching T_{99} (SI Figure S2). Therefore, oxic conditions were maintained throughout the period of time used to calculate decay rate, which is important, as anoxic conditions promote preservation of extracellular DNA.[29−31]

Table 2. Characteristics of Decay Curves for the Temperature Experiment

temp	intercept ± SE	k^a ± SE^a (days^{-1})	R^2	$T_{90}{}^b$ (days)	$T_{99}{}^c$ (days)
5 °C	−0.21 ± 0.14	0.35 ± 0.02	0.90	6.6	13.2
15 °C	0.53 ± 0.16	1.87 ± 0.11	0.92	1.2	2.5
25 °C	−0.01 ± 0.22	2.42 ± 0.15	0.91	1.0	1.9
35 °C	−0.08 ± 0.22	2.39 ± 0.15	0.91	1.0	1.9

[a]The decay rate. [b]Time for initial concentration to decrease by 90%. [c]Time for initial concentration to decrease by 99%.

The extraction control DNA amplified in all samples, indicating no incidence of complete inhibition of the PCR reaction. The average extraction efficiency (percent recovery of the extraction control) was 7.7%. While temperature did not affect extraction efficiency ($p = 0.40$), sampling time point did affect extraction efficiency ($p < 0.0001$). Tukey posthoc test showed that samples taken at 6 h had slightly lower extraction efficiency than other samples taken prior to day 5, but all other sampling time points had similar extraction efficiencies. On

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Table 3. Characteristics of Decay Curves for the Trophic State Experiment

water type	intercept ± SE	k_1[a] ± SE (day^{-1})	k_2[b] ± SE (day^{-1})	break point ± SE (days)	R^2[c]	T_{90}[d] (days)	T_{99}[e] (days)
well water	−0.06 ± 0.21	0.83 ± 0.06	0.02 ± 0.03	7.50 ± 0.64	0.80	2.8	5.5
dystrophic	−0.38 ± 0.11	0.66 ± 0.06	0.03 ± 0.03	8.02 ± 0.83	0.91	3.5	7.0
eutrophic	−0.06 ± 0.15	1.69 ± 0.16	0.04 ± 0.02	3.51 ± 0.30	0.92	1.4	2.7
oligotrophic	0.68 ± 0.16	2.34 ± 0.16	0.43 ± 0.09	3.30 ± 0.28	0.95	1.0	2.0

[a]The decay rate prior to breakpoint. [b]Decay rate after breakpoint. [c]R^2 for first phase of decay. [d]Time for initial concentration to decrease by 90%. [e]Time for initial concentration to decrease by 99%.

average, values corrected for extraction efficiency reduced the coefficient of variation (CV) by 7.8%. No partial inhibition was observed in DNA extract from Pre samples. The difference between Cq values of the Pre samples and controls spiked with standard DNA was 0.23, below the analytical variation of the real-time instrument (~0.3).

Although Carp eDNA decayed more slowly at 5 °C, decay was still relatively rapid, and over 90% of the Carp eDNA was degraded within 1 week. Similarly, Pilliod et al.[14] observed rapid exponential decay of salamander eDNA (*Dicamptodon aterrimus*) stored at 4 °C in the dark. In the only other study to examine the effect of temperature on the decay rate of aquatic vertebrate eDNA, Strickler et al.[12] also found that decay of bullfrog eDNA was slower at 5 °C than at 20 and 35 °C. The slow degradation of DNA at low temperatures is thought to result from lower enzymatic activity.[31−33] Low temperature also slows decay of microbial DNA.[34−36] Water sampling and storage protocols in eDNA studies typically recommend transport of samples on ice and attempt to limit the time samples are kept at 4 °C prior to analysis.[37] However, storage of water samples at 4 °C for up to 24 h did not affect the detectability of salamander (*D. aterrimus*) eDNA.[38] Alternatively, samples can be filtered in the field[38] and preserved in buffer at room temperature for several weeks.[39] For such studies where field filtration and preservation is not possible, our results indicate that water samples should be cooled as rapidly as possible after collection, and sample storage time prior to eDNA capture and extraction should be minimized. Failure to cool and store samples properly will lead to a decrease in eDNA concentration and may possibly lead to a failure to detect eDNA as a results of degradation.

There appears be a trade-off between the rate of eDNA shedding, or production from an organism, and the rate of eDNA decay. At colder temperatures, eDNA release rates may be lower due to the slower metabolism of some organisms,[40,41] but the slower decay of eDNA may enhance persistence. Conversely, the implication for sampling warmer waters is that eDNA is likely only detectable for a short time following the presence of an organism. Temperature was found to have no effect on the concentration of eDNA in laboratory tanks stocked with fish, indicating that the release rate and decay rate may respond proportionately to temperature.[42,43] Sampling of eDNA from Bigheaded Carp in rivers,[44] salamanders in streams,[45] and Common Carp in a lake[22] has shown that eDNA detection rates are temporally and spatially variable. Our results indicate that the variability of eDNA detection may partially be a result of rapid degradation of eDNA in the environment; therefore, eDNA sampling should be done at frequent intervals. In addition, interpretation of eDNA results should have a seasonal component. For instance, summer sampling may reflect organism presence or movement within the previous day or hours, whereas winter sampling may reflect movement or presence within the previous week. Sites with spatial variation in temperature within a season may have interesting patterns in eDNA distribution. For example, Carp eDNA concentration was correlated to temperature in a freshwater lagoon.[42] Although there was no difference in eDNA detection or concentration between surface and subsurface water samples in an unstratified lake,[22] the vertical distribution of eDNA in stratified lakes has never been characterized.

The Effect of Trophic State on eDNA Decay Rate. Trophic state affected the persistence of Carp eDNA. Carp eDNA was detectable until 10 days for oligotrophic lake water and until 28 days for dystrophic lake water, eutrophic lake water, and well water. Carp eDNA was absent from the test waters prior to its addition. After addition of Carp eDNA, the concentration of eDNA ranged less than an order of magnitude among water types, from 4.89×10^5 to 1.73×10^6 copies/mL (SI Table S4).

Complete PCR inhibition was not observed, as the extraction control DNA amplified in all samples. The average extraction efficiency was 4.1%. Water source significantly affected extraction efficiency (One-Way ANOVA, $p < 0.001$); however, the average extraction efficiencies had a small range (3.0−5.7%) with well water having the highest efficiency. Among lake waters, the eutrophic lake water had 1.0% lower extraction efficiency than the dystrophic lake water. However, as concentrations used to calculate decay rates are scaled to initial concentration, the difference in extraction efficiency among water types should not affect the decay rate calculation. Sampling time point also significantly affected extraction efficiency (One-Way ANOVA, $p < 0.001$), with slightly lower extraction efficiencies at time points after 10 days. On average, values corrected for extraction efficiency reduced the CV by 2.5%. Partial inhibition was not observed in any of the water types. The difference between Cq values of the Pre samples and controls spiked with DNA standard ranged from 0.15 to 0.31, which is similar or below the analytical variation of the real-time instrument (~0.3).

A one phase exponential model of decay did not fit the data from the trophic state experiment; residual plots indicated that the model underestimated the initial and final values and overestimated the middle range of the data (SI Figure S1). Instead, decay of Carp eDNA was biphasic, with a period of rapid exponential decay followed by a period of slower decay (Figure 1). Biphasic decay could also have been present in in the temperature experiment; however, the data fell below the LOD relatively early in the experiment, perhaps due to low starting concentration of eDNA relative to the second experiment. Although the method of adding Carp eDNA was the same for each water type, variation in release rate of eDNA led to differences in starting concentration. The release rate of eDNA can be extremely variable within the first several days following fish introduction into a tank, and it is likely due to stress from handling.[42,43]

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Biphasic decay has also been observed in microbes and incubations of free DNA and RNA.[46−48] Biphasic decay indicates that a portion of the material being degraded is labile and degraded quickly in the first phase of decay, and the rest of the material is recalcitrant and resistant to degradation, degrading slowly in the second decay phase.[49] Segmented regression showed that the break point between the two phases of decay occurred at approximately 3 d for eutrophic and oligotrophic lake water, and the break point was at approximately 8 days for well and dystrophic lake water (Table 3). The decay rate in the second phase of decay was 2−18% that of the initial phase. For all water types, the T_{99} value occurred within the first phase of decay, indicating that the second decay phase is of much less importance with respect to decay dynamics. Therefore, the slightly lower extraction efficiency following day 10 should not substantially affect decay rate calculation. DO did not fall below 3 mg/L prior to reaching the T_{99} value (SI Figure S2).

Trophic state significantly affected the initial decay rate (k_1). The eDNA decay rates of well water and dystrophic lake water were not different ($p = 0.08$); however, the decay rates among lakes were ($p < 0.01$). The T_{90} values of well water and dystrophic lake water were approximately 3 d, as opposed to eutrophic and oligotrophic lake waters, with T_{90} values of 1.4 and 1.0 days, respectively. As we hypothesized, Carp eDNA decayed more slowly in well water than in oligotrophic and eutrophic lake water. Bacterial DNA has been shown to persist for longer periods of time in sterile water or in water where the microbial population has been considerably reduced.[18,50] The nutrients, bacteria, and oxygen demand in well water were lower than in the lake waters tested (SI Table S2), so the slower decay may be a reflection of less microbial activity as well. Interestingly, the replicate time point samples from 0 to 3 days from well water were more variable (wider 95% confidence interval of the mean) than any of the lake waters. This variability may reflect more heterogeneous distribution of bacteria relative to lake water.

Of the lake waters tested, Carp eDNA decay was slowest in the dystrophic lake water. The heterotrophic bacterial community of dystrophic bog lakes, such as the site used in this study, dominates the ecosystem energy flow.[51,52] Therefore, slow decay did not result from a sparse population of bacteria, as the bacterial load was similar to the eutrophic lake (SI Table S2). The dystrophic lake had the highest amount of dissolved organic carbon (DOC) of all lakes tested, at 13.8 mg/L compared to 5.83 and 9.46 mg/L in oligotrophic and eutrophic lakes, respectively. Free DNA binds readily and strongly to humic substances, which contribute to the DOC pool in aquatic environments, and binding protects DNA from enzymatic degradation.[17,53,54] The concentration of DNase required to fully degrade DNA bound to humic substances is 100 to 1000 times that required to degrade free DNA.[17,53] Although humic substances may inhibit PCR, we found no such inhibition in our samples. Therefore, the presence of humic substances most likely slowed degradation of Carp eDNA.

To further understand the factors which affect eDNA decay, correlations were done between water quality parameters (SI Table S2) and eDNA decay rates for all experiments done at 15 °C. For all water types, no single variable was correlated ($p > 0.05$) with decay rates. When well water was excluded from the analysis, decay rates were negatively correlated with DOC ($r = −0.997$, $p = 0.049$), but rates were not correlated to any other variable ($p > 0.05$). Although DOC was lowest in the well water (0.97 m/L), suggesting that decay rates should be high, the low level of nutrients and low oxygen demand (O^2 did not significantly decrease over the course of the experiment; SI Figure S2) may have led to a longer persistence of eDNA. Although we hypothesized that degradation would be slower in the oligotrophic lake water, the decay rate of eDNA in oligotrophic lake water was 40% greater than in eutrophic lake water. Interestingly, the total organic carbon was 38% lower in the oligotrophic lake water compared to the eutrophic lake water. Further research is needed to confirm the role of organic carbon in slowing the decay of aquatic vertebrate eDNA. Specifically, future studies should examine how composition of DOC, which is a heterogeneous mixture of humic and fulvic acids, carbohydrates, and amino acids,[55,56] effects degradation.

Comparison of Early and Late Summer Decay Rates within a Eutrophic Lake. The eutrophic lake water used for the temperature experiment was collected in June, prior to the period of high algal productivity. In comparison of lake trophic state, water from the same lake was collected in August, when the water was visibly green due to high algal productivity. Despite observable differences in the water collected at these times, the decay rates at 15 °C were not different ($p = 0.18$). However, there were differences in the decay dynamics between these periods. In June, decay exhibited only one phase, and fell below detection after 3 days, whereas in August, decay was biphasic, shifting into a slow decay phase after 3.5 days. This difference is likely due to the higher initial amount of eDNA in the trophic state experiment (an order of magnitude), which allowed eDNA to remain above the LOD after the initial phase of rapid decay.

Persistence of eDNA in Lakes. Several studies have shown that eDNA is detectable in water for a week or more following removal of an organism from a tank or pond.[6,10] In a tank containing a fish carcass, eDNA was detectable for at least one month.[57] However, as in this study, the biomass used at the start of these experiments was often several orders of magnitude greater than natural densities. The concentration of eDNA is positively related to organism biomass,[11,43,58] and, consequently, eDNA persists for longer in tanks with a higher initial biomass.[10,59] For example, New Zealand mudsnail eDNA was detectable 3 days after removal from aquariums stocked at five densities.[1] At 21 days, eDNA was only detectable at the two highest density treatments.[1] Therefore, in order to determine a realistic estimate of eDNA persistence, initial eDNA concentration must reflect field estimates. For example, incubation of seawater that contained eDNA from two fish species (at natural concentrations) showed that eDNA was detectable for a maximum of 1 week.[15]

The concentration of Carp mitochondrial eDNA in natural lakes has been determined for several water bodies in previous studies.[20,22,42] We calculated the persistence time in days if all Carp were removed or migrated (Table 4). In spring and fall, Carp eDNA would not persist past 3 days, whereas Carp eDNA would persist in winter for approximately 12 days. Although the average concentration of eDNA was found to be lower in winter than in spring,[20] the longer persistence of eDNA in winter may be advantageous for sampling.

Comparison to Previously Published Decay Rates. The range of Carp eDNA decay rates observed in the current study (0.35−2.42 day^{-1}) falls within the range previously reported for other aquatic vertebrates (0.05−17.9 day^{-1}, Table 1). The decay rate of Carp eDNA at 5 °C was much more rapid than the range of decay rates reported for bullfrog eDNA at the same

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Table 4. Estimated Persistence of Common Carp eDNA in Natural Waters

name	location	season	persistence (days)	reference
Iba-naiko Lagoon	Japan	winter	12.2	42
St. Mary's Lake	Notre Dame, IN	spring	2.8	20
St. Mary's Lake	Notre Dame, IN	winter	11.4	20
Staring Lake	Eden Prairie, MN	fall	2.6	22

temperature. The difference in decay rates may be due to species, as bullfrog eDNA decay never exceeded 0.34 day^{-1}, even at 35 °C.[12] The slowest decay rate in our study was similar to European Flounder (*Platichthys flesus*) eDNA in seawater.[15] The Flounder eDNA was incubated at 15 °C, but the decay rate was much slower than Carp eDNA in all water types tested at 15 °C in the present study. Most likely, higher salinity slowed the decay of eDNA, as salinity has been similarly shown to slow decay of bacterial DNA.[60,61]

The average decay rate previously reported for Common Carp eDNA (6.3 day^{-1}) was over twice that observed for eutrophic lake water incubated at the same temperature (25 °C) in the present study.[13] Barnes et al.[13] found that the tanks in which Carp eDNA decayed the slowest had higher biological oxygen demand (BOD), chlorophyll *a*, pH, and total DNA, factors which were correlated. Some of these factors were likely lower in the present study, resulting in slower degradation of eDNA. Exposure to light may also have contributed to the faster degradation of eDNA in the Barnes et al.[13] study, as our microcosms were incubated in the dark. Light exposure can increase decay rate; however, the magnitude of the effect is dependent on temperature and pH.[12] Strickler et al.[12] found that at neutral pH at 20 °C, conditions most similar to the Barnes et al.[13] study, light exposure increased the decay rate of bullfrog eDNA by only 14%.[12] A similar magnitude of effect for light exposure (15%) was observed for salamander eDNA.[14] Consequently, the chemical characteristics of the water likely resulted in the discrepancy between our results and those of Barnes et al.[13]

Finally, it is important to note that we did not include negative control microcosms in our study. Cross-contamination has been observed in other microcosm studies of eDNA decay where individual tanks were repeatedly sampled over the course of the experiment.[12,13,16] Our experimental study, however, most closely resembles that of Pilliod et al.[14] where microcosms consisted of capped jars or bottles that were destructively sampled at each time point. Pilliod et al.[14] did not observe any contamination of negative control microcosms, indicating that destructive sampling of capped containers might be preferable to reduce potential contamination in studies of this type. It should also be noted that contamination level is typically low and sporadic in nature and can therefore be considered as a source of random error.[12,16] While future studies on eDNA degradation should include negative microcosm controls into their experimental design, it is also important to take replicate samples at each time point at frequent intervals to mitigate error due to incidental contamination.

Another consideration for future studies is the use of exogenous DNA to determine and correct for differences in extraction efficiencies among sample replicates. In the current study, the percent recovery of extraction control DNA was substantially lower (~47% difference) than what we previously found using the same extraction kit.[25] We hypothesize that this is due to lower total DNA in the current study, as the uncorrected concentration of Carp eDNA was nearly 20 times lower. Saturation of soils with nucleic acids prior to extraction increases recovery of target DNA.[62] The slight decrease in recovery of Carp eDNA in the trophic state experiment after 10 days might also be a result of the same phenomenon. Although the extraction efficiencies in this study are low, they consistent with other studies.[23,63] Extraction controls can also be used to reduce variation among sample replicates.[23,25,64]

Implications. Despite increasing interest in the use of eDNA as a research and monitoring tool, there is still limited understanding of the factors that control eDNA decay. Previous research that examined eDNA degradation has shown that light exposure, temperature, and water quality affect the rate of eDNA decay.[12−14] Results of the current study have confirmed that temperature affects eDNA decay in lake water, and lake trophic state affects eDNA decay. Future research is needed to elucidate the effect of humic material on persistence of eDNA. Although the understanding of the factors that affect eDNA decay is currently limited, the consensus of studies suggest that decay is rapid, and, thus, an important factor to consider in the design and implementation of future studies.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.5b05672.

Calculation of T_{90} and T_{99} values; Nutrient analysis of water samples; Analysis for total bacteria; Calculation of the persistence of Carp eDNA in lakes; Residual plots for one phase exponential decay model; Dissolved oxygen over the course of experiments; Names and locations of sampling sites; Water quality of sampling sites; Summary of qPCR calibration curve data; Carp eDNA concentration at start of experiment (PDF)

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Notes

The authors declare no competing financial interest.

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