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Experimental observations on the decay of environmental DNA from bighead and silver carps

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Editor's note:
This study was first presented at the 19th International Conference on Aquatic Invasive Species held in Winnipeg, Canada, April 10–14, 2016 (http://www.icais.org/html/previous19.html). This conference has provided a venue for the exchange of information on various aspects of aquatic invasive species since its inception in 1990. The conference continues to provide an opportunity for dialog between academia, industry and environmental regulators.

Abstract

Interest in the field of environmental DNA (eDNA) is growing rapidly and eDNA surveys are becoming an important consideration for aquatic resource managers dealing with invasive species. However, in order for eDNA monitoring to mature as a research and management tool, there are several critical knowledge gaps that must be filled. One such gap is the fate of eDNA materials in the aquatic environment. Understanding the environmental factors that influence the decay of eDNA and how these factors impact detection probabilities over time and space could have significant implications for eDNA survey design and data interpretation. Here we experimentally explore decay of eDNA associated with bighead carp (*Hypophthalmichthys nobilis)* biological waste collected from an aquaculture filtration system and with sperm collected from captive silver carp (*H. molitrix)*, and how decay may be influenced by differing levels of water turbulence, temperature, microbial load, and pH. We found that the decay patterns of eDNA associated with both *H. nobilis* biological waste and *H. molitrix* milt significantly fit monophasic exponential decay curves. Secondly, we observed that the highest temperature we tested resulted in a decay half-life as much as 5.5× more rapid than the lowest temperature we tested. When we suppressed microbial loads in eDNA samples, we observed that overall losses of eDNA were reduced by about 2.5×. When we amended eDNA samples with pond water the half-life of eDNA was reduced by about 2.25×, despite relatively little apparent increase in the overall microbial load. This pattern indicated that species constituency of the microbial community, in addition to microbial load, might play a critical role in eDNA degradation. A shift in pH from 6.5 to 8.0 in the samples resulted in a 1.6× reduction in eDNA half-life. Water turbulence in our study had no apparent effect on eDNA decay. When we combined different temperature, pH, and microbial load treatments to create a rapid decay condition and a slow decay condition, and tracked eDNA decay over 91 days, we observed a 5.0× greater loss of eDNA by Day 5 under rapid decay conditions than under slow decay conditions. At the end of the trials, the differences in eDNA loss between the rapid decay and baseline and slow decay conditions were 0.1× and 3.3×, respectively. Our results strongly demonstrate the potential for environmental factors to influence eDNA fate and, thus, the interpretation of eDNA survey results.

Key words: eDNA, *Hypophthalmichthys nobilis*, *Hypophthalmichthys molitrix*, environmental factors, microbial load, pH, water temperature

Introduction

The type of sample now commonly referred to in the literature as environmental DNA, or eDNA, refers to the DNA of macrobiotic organisms extracted and isolated from water, soil or air samples (Ficetola et al. 2008; Goldberg et al. 2011). Early use of the same term also occurred in the field of microbial environmental genetics (e.g. Ogram et al. 1987; Wintzingerode et al. 1997). Following the demonstration by Ficetola et al. (2008) that the DNA of invasive American bullfrogs (*Lithobates catesbeianus* Shaw, 1802) could be detected in pond water, the growth in interest in eDNA monitoring for other aquatic macrobiota has been substantial. Since Ficetola et al. (2008), dozens of papers describing aquatic eDNA survey efforts, development of eDNA resources and methodological advances, the role of environmental factors on eDNA detection, and probabilistic framework for interpreting eDNA data have been published (Barnes et al. 2014; Darling and Mahon 2011; Dejean et al. 2011; Farrington et al. 2015; Goldberg et al. 2011; Jerde et al. 2011; Schmidt et al. 2013; Schultz and Lance 2015). The breadth of taxa targeted by aquatic eDNA surveys is continually expanding and includes, among others, frogs (Ficetola et al. 2008; Goldberg et al. 2011), freshwater snails (Goldberg et al. 2013), salamanders (Goldberg et al. 2011; Olson et al. 2012), fish (Jerde et al. 2011; Olsen et al. 2015; Takahara et al. 2012), marine mammals (Foote et al. 2012) and invertebrates (Lance and Carr 2012; Deiner and Altermatt 2014). Environmental DNA has also generated attention through symposia at major scientific conferences (26th International Congress for Conservation Biology, 143rd Annual Meeting of the American Fisheries Society) and has had a central role in important legal issues (e.g. Michigan et al. vs. United States Army Corps of Engineers et al., US Supreme Court Case No. 11-541).

Despite the growing interest in and burgeoning use of eDNA in survey and monitoring programs, the nature of eDNA (i.e., its physical state in the environment and how long it persists) is in many ways unknown and limits the inferential power of eDNA data (Barnes and Turner 2015). For example, eDNA may exist in the environment as molecules encapsulated by cell and/or mitochondrial membranes, as a free molecule in solution, as molecules adhered or adsorbed to environmental particles (e.g. sediment), or some mixture of forms (Turner et al. 2014; Turner et al. 2015). The degree to which eDNA is bound to or aggregated with other particles is typically unknown, and consequently, the degree to which eDNA is available for microbial breakdown or chemical degradation is unknown. All of these factors affect the degradation of eDNA and influence the abundance of detectable eDNA for a given species at any location. These uncertainties limit the spatio-temporal inferences that might be drawn from eDNA data and thus limit its utility as a monitoring tool.

One of the first steps towards a quantitative understanding of the fate of eDNA in water is to examine the decay of eDNA "signal" (i.e. concentration of intact copies of target DNA loci) over time and how different factors impact decay rates. To date there have been several published studies that report on simple decay patterns in eDNA signal (Dejean et al. 2011; Thomsen et al. 2011; Thomsen et al. 2012; Maruyama et al. 2014) including a few that report observations or experimental results regarding the influence of environmental factors on eDNA decay (Barnes et al. 2014; Pilliod et al. 2014; Strickler et al. 2015). Early studies reported that, after removal of organisms, eDNA persisted in artificial systems and outdoor mesocosms for as long as 2–7 weeks (Barnes et al. 2014; Dejean et al. 2011; Goldberg et al. 2013). As a general trend, eDNA signal rapidly declines and then asymptotically approaches zero concentration. However, different studies have also reported conflicting observations on the influences of environmental factors on eDNA decay. For example, Pilliod et al. (2014) found no effect of exposure to sunlight on changes in eDNA concentration in a river, but Barnes et al. (2014) surmised that reduced penetration of sunlight into mesocosm waters, due to high algal densities, contributed to longer persistence times for eDNA. Strickler et al. (2015) found that eDNA decayed more rapidly with increasing exposure to UV-B light. Strickler et al. (2015) also observed slower eDNA decay in colder temperatures and, to a lesser degree, under alkaline conditions. They surmised that conditions under which decay was greater (warmer temps, neutral pH and moderate UV-B) were also conditions favorable to microbial growth and activity (Strickler et al. 2015). Barnes et al. (2014) also point to microbial activity as a primary driver of eDNA decay patterns. Eichmiller et al. (2016) observed a complicated interaction between microbial communities and eDNA decay patterns, with dystrophic and eutrophic lake waters having similarly high microbial abundances, but very different decay patterns, and microbe-poor oligotrophic lake water showing the most rapid eDNA decay. In this case, the authors surmise eDNA sorption to humic substances was an important confounding factor in comparisons of lake waters. The significant influence of microbial extracellular enzymes on the fate of extracellular DNA is well known (Nielsen et al. 2007), while the fate of encapsulated eDNA (e.g. free mitochondria), which can constitute a significant

fraction of eDNA in aquatic systems (Turner et al. 2014; Wilcox et al. 2015), is less well-characterized. It seems likely that the degradation of cell and organelle membranes, and the encapsulated DNA, is also closely tied to microbial activity.

Continuing experimentation, observation, and data accumulation will be key to clarifying the influences of different factors on the fate of eDNA in aquatic environments and to incorporating environmental complexity into data interpretation and decision making. To that end, we undertook several related experiments. First, we characterized simple decay patterns in eDNA derived from two sources, bighead carp (*Hypophthalmichthys nobilis* Richardson, 1845) waste materials and milt from silver carp (*H. molitrix* Valenciennes, 1844). Biological waste (e.g., feces, urine, sloughed epidermis; termed here "biowaste") and gametes (especially from broadcast spawners like *H. molitrix*) are two significant sources of eDNA in aquatic systems (Ficetola et al. 2008; Klymus et al. 2015). We observed relative decay patterns in eDNA derived from bighead carp biowaste under varying levels of several environmental factors (pH, temperature, turbulence, and microbial load). There is currently significant interest in the potential for invasive species spawning events to be detected via seasonal fluctuations in a species' eDNA (Erickson et al. 2016), thus we also examined the decay of eDNA signal from silver carp milt. A need to better understand the results from surveys for *H. nobilis and H. molitrix* eDNA in the Chicago Area Waterway System (CAWS; Illinois and Indiana, USA) was the basis for funding of this study, and the levels of water temperature and pH in our experiments were influenced by the recorded ranges of these factors in the CAWS. As a final experiment, we compared decay rates of *H. nobilis* eDNA under conditions where combined factors strongly favored either eDNA persistence or decay.

Materials and methods

eDNA sources

Carp biowaste

For experiments utilizing biowaste as a source of eDNA we created a concentrated solution of slime, feces, and other materials that were collected from filtration units serving several tanks that held juvenile and subadult *H. nobilis*. Tanks holding *H. nobilis* were located at the US Army Engineering Research and Development Center (ERDC) in Vicksburg, MS, USA, which is the same installation where laboratory facilities used for subsequent experiments with biowaste took place and where DNA laboratory work was conducted. The tank facility is located nearly 1.5 km away from the research labs and DNA laboratory, and the potential for DNA contamination from the tank facility was negligible. A stock of concentrated carp biowaste was prepared by mixing 3.0 grams of wet biowaste in 50.0 ml of deionized water. Working solutions of "carp slurry" for the various experiments were then created by adding 2.0 ml of concentrated biowaste stock to 12.0 ml deionized water in a 15 ml polypropylene screw-top centrifuge tube. The final working concentration in the carp slurry was therefore 8.6 mg biowaste/ml water. For each new experiment we collected new batches of biowaste and prepared new carp slurry solutions. This was done primarily because adequate volumes of carp biowaste for the entire set of trials could not be obtained in a single or small number of collections. Also we were concerned that repeated freezing and thawing of stored biowaste could result in DNA damage and an additional uncontrolled factor in our trials. We expected that using new batches of biowaste and new carp slurry solutions for each experiment would result in some variation among experiments, including variance in starting concentrations of carp eDNA and somewhat different rates of decay due to variations in relative proportions of different components in the biowaste. With these factors in mind, our primary focus was on comparing eDNA decay rates and patterns among different levels within treatments, as opposed to rates and patterns across different types of treatments.

Carp milt

The sperm eDNA decay experiments were conducted at the United States Geological Survey's Columbia Environmental Research Center in Columbia, MO, USA. The sperm came from a mixture of milt collected from eight male silver carp (*H. molitrix*) in 2011 and stored at −80 °C.

Carp biowaste

Basic decay trial

To characterize baseline degradation of eDNA from carp biowaste, 80 samples (i.e. 14 ml of carp slurry in a 15 tube as described previously) and 10 negative control samples (14 ml of deionized water in 15 ml tube) were placed on rotating shakers at 66 revolutions per minute (RPM) in the dark and at room temperature (20 °C). Following immediate removal of eight randomly selected samples (Day 0) and one negative control, eight additional randomly selected samples and one negative control were removed at each sampling point, which corresponded to Days 1, 2, 3, 5, 7, 10, 14, 21, and 28. Immediately after

being removed, samples were centrifuged at 4000 RPM for 15 minutes at 4 °C, after which the supernatant in each tube was carefully decanted. The pelleted material from each sample was then stored at −20 °C until DNA extraction (always less than one week after collection). DNA from each sample and negative control was extracted using a modified cetyltrimethyl ammonium bromide (CTAB) method (Doyle and Doyle 1987) and eluted with 100 µl of commercial DNAse-free purified water (Ambion®, Thermo Fisher Scientific, Waltham, MA, USA). Three replicate quantitative polymerase chain reactions (qPCR) were run for each sample and the mean copy number calculated. Water blank negative control qPCRs were run in conjunction with sample qPCRs. Each qPCR contained the following: 1X TaqMan® Environmental Master Mix 2.0 (Thermo Fisher Scientific), 500, 500, and 125 nM of forward primer, reverse primer, and TaqMan® probe respectively, 1 µl DNA extract, and enough sterile molecular-grade water to bring the solution to 20 µl. The qPCR primers and probe were those described for *H. nobilis* in Coulter et al. (2013). The qPCR thermocycler program used an initial denaturation step of 95 °C for 10 min., followed by 40 cycles of 95 °C for 15 sec. and 60 °C for 1 min. Reactions were run on a ViiA™ 7 Real-Time PCR System (Thermo Fisher Scientific). Plasmids containing the target locus DNA sequence were created using TOPO® TA Cloning Kit for Sequencing (Thermo Fisher Scientific), isolated and purified using QIAprep Spin Miniprep Kits (Qiagen Inc., Valencia, CA, USA), and then used in the qPCR to generate standard curves (10^0–10^7 DNA copies) for estimating DNA copy number. The number of plasmids in solution was calculated using the combined DNA base pair length of the plasmid and insert, a standard DNA base-to-Daltons conversion for double-stranded DNA (650 Daltons/base), a Daltons-to-nanograms conversion, and DNA mass quantification using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific). Pre-PCR (DNA extraction) steps, qPCR set-up, and qPCR were conducted in separate dedicated rooms with strict anti-contamination protocols, including the use of a biological hood with HEPA filters and a UV sterilization lamp. All surfaces were cleaned with a commercial DNA solvent or 20% bleach solution prior to use. Metrics of qPCR performance (R^2, slope, and efficiency (E)) for all study trials are provided in Appendix 2.

Environmental factors: turbulence

We prepared 256 samples of carp slurry and 32 negative controls using methods described previously. Samples and negative controls were randomly and evenly distributed among four treatment groups (n = 64 samples, 8 negative controls each). Each set was placed onto a different Lab Companion© SK-300 orbital shaker (Jeio Tech, Inc., Daejeon, KR) and run at 0 RPM, 66 RPM, 132 RPM or 200 RPM, respectively. Samples were shaken at room temperature in the dark for 14 days. Subsets of eight samples and one negative control were taken from each RPM class at Days 0, 1, 2, 3, 5, 7, 10, and 14. Samples were stored and processed (through qPCR) as described above.

Environmental factors: temperature

We prepared 256 samples of carp slurry and 32 negative controls using methods described previously. Samples and negative controls were randomly and evenly distributed among four different temperature classes: 4 °C, 12 °C, 20 °C and 30 °C. Sample temperatures were maintained by placing tubes in temperature-controlled rooms or chambers and allowing samples to equilibrate with room temperatures. In each room or chamber, tubes were placed on orbital shakers and continuously shaken at 66 rpm in the dark for 14 days. Subsets of eight samples and one negative control were taken from each temperature class at Days 0, 1, 2, 3, 5, 7, 10, and 14. Samples were stored and processed (through qPCR) as described above.

Environmental factors: microbial load and source

A preliminary trial was conducted to assess the viability of microbial cells present within carp slurry from biowaste that had been stored a −20 °C. First, frozen bighead carp biowaste was used to create slurry, as described previously, after which serial dilutions of slurry, ranging from 10^0 to 10^{-5} were created. Then 10 µL from each slurry dilution class were spread onto tryptic soy agar (TSA) plates and incubated at 30 °C for 24 hours. Additionally, liquid cultures were prepared for each dilution class by inoculating 10 ml of tryptic soy broth with 100 µl of the diluted slurry and incubating at 30 °C with shaking for 24 hours. A second trial was performed to verify that treatment of carp slurry with an antibiotic cocktail could reduce overall bacterial loads without negatively impacting carp eDNA integrity. Six sample tubes of carp slurry were prepared by diluting 2 ml of concentrated carp biowaste into 12 ml of sterile molecular-grade water. A 15 µL volume of antibiotic cocktail (1g ampicillin and 1 g kanamycin in 10 ml of sterile water) was filtered through a 0.22 µm syringe filter and then added to three of the slurry tubes (treatment group). The remaining three tubes

were used as a non-treatment control group. Both groups were incubated at room temperature for six hours. A serial dilution (10^0 to 10^{-3}) was prepared from each sample tube, after which 50 µL was spread onto TSA media to be incubated at 30 °C for 24 hours. Samples were stored and processed (through qPCR) as described above.

We then prepared 256 samples of carp slurry and 32 negative controls as described previously. Samples and negative controls were randomly and evenly distributed among four different treatment classes: a baseline microbial load, a low microbial load, a minimal microbial load, and an augmented microbial load. The untreated baseline microbial load experimental units (n = 64) were created in the same fashion as described earlier for other experiments, and contained only microbes endogenous to the carp tanks and carp biowaste. To create a low microbial load treatment, we administered an antibiotic serum to the carp slurry. The antimicrobial serum was created by adding 1 g of ampicillin and 1 g of kanamycin to 10 ml of deionized water, filtering the concentrated serum through a 0.22 µM syringe filter, and then diluting 10 ml of the antibiotic solution in 1 L of deionized water (final concentration = 1 mg combined antibiotics per ml water). Then 2 ml of concentrated biowaste solution was added to 12 ml of the water containing the antibiotic serum to create carp slurry with low microbial loads (0.86 mg combined antibiotic/ml concentrated biowaste = 1× treatment). The minimal microbial load samples (n = 64) were created in the same manner, but with the concentrated carp biowaste being treated with a 2× antibiotic serum (= 1.71 mg combined antibiotic/ml working slurry). The augmented microbial load experimental units (n = 64) were created by adding 2 ml of the concentrated carp biowaste to 12 ml of pond water. Pond water was collected from a nearby mesotrophic pond on the ERDC installation. The pond is inhabited and frequented by a variety of taxa including fish, turtles, waterfowl, and wading birds, but not *Hypophthalmichthys* carp. All samples (N = 256) and negative controls (N=32) for all four treatments in the experiment were placed on orbital shakers and shaken at 66 rpm in the dark for 14 days. Subsets of eight samples and one negative control were taken from each microbial load class at Days 0, 1, 2, 3, 5, 7, 10, and 14. Samples were stored and processed (through qPCR) as described above.

In order to characterize the general states of the microbial loads in each treatment class over the course of the 14-day experiment, 50 µl aliquots of carp slurry were taken from each of three randomly selected samples from each of the four treatment groups at each of the sampling points (N = 8) and plated in serial dilutions (10^0–10^{-4}) onto TSA media. Bacterial colonies were then counted after 24 hour incubation periods at 30 °C. Colony counts were recorded as colony forming units (CFU) and the average CFU/ml of each sample was calculated.

Environmental factors: pH

A fairly narrow range of pH was selected in order to test levels of pH similar to those observed in the 2011 water quality data for the CAWS (MWRD 2011). Untreated carp slurry exhibited a pH ≅ 6.5. In order to prepare the higher pH treatment classes, we divided concentrated carp biowaste into four large flasks and then diluted the stock with purified deionized water to the carp slurry concentration (1:6 biowaste:water). The pH in each of three flasks was adjusted to the target pH using increasing volumes of 1M NaOH. Tubes of carp slurry (14 ml per tube; N = 69 tubes) and deionized water controls (14 ml per tube; N = 8) were prepared for each of four pH treatment classes: 6.5, 7.0, 7.5 and 8.0. Because pH above 6.5 was unstable in the carp slurry and because measuring and adjusting pH for each sample on a frequent basis was both infeasible and a source of considerable risk for DNA cross-contamination between samples, five randomly-selected samples within each treatment class were designated as "sentinel samples." The pH levels of each sentinel sample would be measured daily and recorded, and then adjusted to the target pH using 1M NaOH. The mean volume of NaOH required to adjust the sentinel samples (typically 30–80 µl) within each treatment class was then applied to each sample in that same class. Subsets of eight samples and one negative control were taken from each pH class at Days 0, 1, 2, 3, 5, 7, 10, and 14. Samples were stored and processed (through qPCR) as described above.

Environmental factors: combined treatment

Following the experiments with individual environmental factors, we conducted a final experiment to assess the relative eDNA decay under three different combined treatment classes: 1) rapid decay: 30 °C, pH 8.0, and augmented microbial load, 2) no treatment: room temperature (20 °C), native pH, untreated microbial load, and 3) slow decay: 4 °C, pH 6.5, minimal microbial load. Concentrated carp biowaste was prepared in three large flasks designated, respectively, as the "rapid decay" flask, the "no treatment" flask, the "slow decay" flask. The biowaste was then diluted to carp slurry concentrations (1:6 biowaste:water) with either deionized water (no treatment, slow decay classes) or pond water from the same pond as described earlier (rapid decay class).

The pH in all three flasks was measured and the pH in the rapid decay treatment flask adjusted to pH 8.0 using increasing volumes of 1M NaOH. The slow decay class slurry was then treated with a 2× combined antibiotic solution (1.71 mg antibiotic/ml slurry) as described above. Slurry from each of the flasks was then used to prepare 69 samples and eight negative controls per treatment class. Five samples within each treatment class were then randomly designated as pH sentinel samples as described previously. All tubes were placed in appropriate temperature-controlled rooms and pH was adjusted in the same fashion as described earlier. The pH level in each treatment class eventually stabilized, at which point pH monitoring was conducted every two days instead of daily. The pH level in the untreated class was tracked, but never adjusted. Subsets of eight samples and one negative control were taken from each treatment class at Days 0, 1, 2, 3, 5, 7, 10, 14, 21, 28, 35, 42, 49, 56, 63, 77, and 91. Samples were stored and processed (through qPCR) as described above, with the exception of a change in the qPCR marker. For qPCR in this experiment we used a newly developed TaqMan® marker, BHTM-1 (Farrington et al. 2015). This marker, designed at ERDC, consistently produced higher DNA estimates and exhibited lower limits of detection than the marker used in the earlier experiments.

Carp milt

For the sperm eDNA decay trials, we added 200 µl of silver carp milt into each of four 10 L glass jars which were filled with 5 L of well water. A fifth jar, also filled with 5 L of well water, did not have sperm added and acted as our negative control. Well water was recorded as having a pH of 8.0 at the beginning of the experiment. All experimental jars were aerated with air stones, and covered with plastic wrap to minimize cross contamination and evaporation. Jars remained in a temperature-controlled room (24 °C), and water temperature was monitored each day using a digital thermometer. Light exposure was indirect sunlight from a nearby window.

At each sampling time, two replicate 50 ml samples were taken from each jar using a clean serological pipette. Samples were taken approximately 10 cm from the bottom of the jar. Samples were initially taken before addition of milt to the jars, immediately after addition of sperm (Day 0), and then at Days 0.25, 1, 2, 3, 4, 5, 7, 14, 21, 28, 35, 42, 49, 56, 63, 70, 77, 84, and 91. Individual samples were centrifuged for 30 minutes at 5000 RCF at 4 °C. Water was decanted off and samples were left to dry for at least 10 minutes before adding 250 µl of the extraction buffer (TDS0; AutoGen Inc., Holliston, MA). Samples were then stored at −20 °C until extracted. Prior to DNA extraction, the pelleted material in each sample was digested overnight with proteinase K (AutoGen Inc. Holliston, MA) in a 55 °C water bath. DNA was then extracted using an AutoGen 245 system (AutoGen Inc. Holliston, MA), which incorporates a phenol chloroform extraction method (manufacturer's protocol), and re-suspended in 50 µl nuclease-free water.

Samples were run in a multiplex reaction that included a TaqMan® qPCR primer/probe set previously designed for *H. molitrix* (Coulter et al. 2013) along with an internal positive control (IPC). The IPC, which is used to detect PCR inhibition, was a TaqMan® qPCR primer/probe set designed to amplify a 120 base pair gBlocks® ultramer (Integrated DNA Technologies, Inc., Coralville, IA) based on the HemT gene of house mice (*Mus musculus* Linnaeus) (Appendix 1).

Each qPCR contained the following: 1X SsoFast™ Probe Supermix (Bio-Rad Laboratories, Inc., Hercules, CA, USA), 250 nM of each *H. molitrix* primer, 125 nM of each IPC primer, 94 nM of each probe, 2.5 µl of the IPC amplicon, 2.5 µl of sample DNA, and 1.5 ul of deionized water to bring the solution to 20 µl. Reactions were run on a CFX96 BioRad thermal cycler with the following conditions for 40 cycles: 2 minutes at 95 °C, 5 sec at 95 °C, 10 seconds at 58 °C. The standard curve was made from serial dilutions of a plasmid that includes the target amplicon (10^1–10^6 DNA copies). All standards and samples were run in triplicate. Each qPCR run included wells containing no DNA template that served as water blank negative controls to test for contamination. Additionally, we used these negative controls to identify a baseline cycle number at which to expect detection of the IPC. Any milt trial sample where the IPC was detected at 3 or more cycles past the baseline from the negative controls was classified as being PCR-inhibited. Inhibited samples would then be diluted 10 fold and re-run.

To characterize baseline decay of the *H. molitrix* milt the estimated DNA copy numbers for each of the two replicate samples taken from each jar at each time point were averaged. For each experimental jar, we then fit exponential models as described below for the sampling period from 6 hours to 120 hours (0.25–5.0 days). The calculated decay rates and half-lives from the four jars were then averaged together to get an average decay rate.

Decay model statistics

Prior to assessing decay patterns in our various experiments we converted all qPCR-derived estimates of marker copy number to a common metric of concentration, number of copies of target DNA per liter (*CN/L*), using the following formula:

$$CN/_L = \frac{CN_{PCR} * \frac{V_E}{V_T} * \frac{1\,L}{V_S}}{L} \qquad \text{Eq. (1)}$$

where CN_{PCR} is the estimated number of copies of the target DNA marker in the aliquot of extracted DNA used for qPCR (and the copy number estimate typically provided by qPCR analysis software), V_E is the volume of the DNA elution produced by extraction and purification of DNA from a sample, V_T is the volume of aliquot of template DNA taken from V_E and used for qPCR, and V_S is the volume of the original water sample.

Our baseline assumption was that DNA would degrade at a constant rate over time, exhibiting a monophasic exponential decay that could be modeled by the following formula:

$$N(t) = N_0 * e^{-\lambda t} \qquad \text{Eq. (2)}$$

where $N(t)$ is the estimated number of copies of eDNA at time t, N_0 is the number of copies of eDNA at time zero, λ is the decay rate (sometimes denoted as β; Thomsen et al. 2012; Maruyama et al. 2014), and t is time (i.e., the number of days following eDNA introduction into the system). However, having observed an apparent pattern in our data and in other studies (e.g., Dejean et al. 2011; Pilliod et al. 2014; Sassoubre et al. 2016; Thomsen et al. 2012) showing a very rapid initial degradation followed by a period of slower degradation we decided to also compare the fit of the monophasic exponential decay to that of a biphasic exponential decay. Biphasic exponential decay combines the decay of a rapidly decaying subset of the eDNA and the decay of a more recalcitrant, slow decaying subset and was described using the following formula:

$$N(t) = (N_{0_R} * e^{-\lambda_R t}) + (N_{0_S} * e^{-\lambda_S t}) \qquad \text{Eq. (3)}$$

where $N(t)$ is the estimated number of copies of eDNA at time t, N_{0_R} is the number of copies of rapidly decaying eDNA at time zero, N_{0_S} is the number of copies of slowly decaying eDNA at time zero, λ_R is the decay rate of the rapidly decaying eDNA, λ_S is the decay rate of the slowly decaying eDNA, and t is time (i.e., the number of days following eDNA introduction into the system). SigmaPlot 12.5 (Systat Software, San Jose, CA, USA) was used to fit both monophasic and biphasic exponential decay curves to each set of DNA copy number estimates across time points for each treatment in each experiment. We selected a weighted least squares fitting option in SigmaPlot that weighted each data point using the reciprocal of the statistical variance around the mean copy number for that sampling point. This method takes into account the non-uniform variability of the different sampling points over time. In our experiments, where earlier time points had much higher copy numbers of eDNA, variance was concomitantly larger than for later time points. Ultimately, this approach emphasized the influence of earlier, higher copy number time points on the shape of the calculated decay rate. Following Maruyama et al. (2014) and Sassoubre et al. (2016) we report decay constants (λ) and eDNA half-lives as preferred metrics of eDNA decay. Half-lives were calculated as:

$$t_{1/2} = \frac{\ln(2)}{\lambda} \qquad \text{Eq. (4)}$$

Results

For all experiments, weighted least square fitting to monophasic exponential decay models for eDNA decay outperformed (i.e., stronger coefficients of determination (R^2)) biphasic exponential models. Therefore only monophasic model decay patterns are described for each experiment.

Baseline eDNA decay

Carp biowaste

The decay of eDNA derived from *H. nobilis* biowaste under baseline conditions is displayed in Figure 1. DNA degraded quickly, with an approximate 70% reduction after one day and a 90% reduction after 2 days. The pattern of degradation exhibited a statistically significant fit to the exponential decay model, $N_c = 8{,}136{,}973 * e^{-0.877*\text{Day}}$ ($R^2 = 0.540$; $p < 0.015$). The corresponding half-life was $t_{½} = 0.78$ days or 18.7 hours.

Environmental factors: turbulence

Patterns of eDNA decay for carp biowaste under different turbulence treatments are displayed in Figure 2. All four turbulence treatments experienced approximately the same decay pattern, with significant fits to a monophasic exponential decay curve ($R^2 = 0.777$–0.828; $p < 0.01$). Decay constants and half-lives under all turbulence treatments were similar, with no apparent pattern of increasing or decreasing decay rate with increasing or decreasing turbulence (Table 1).

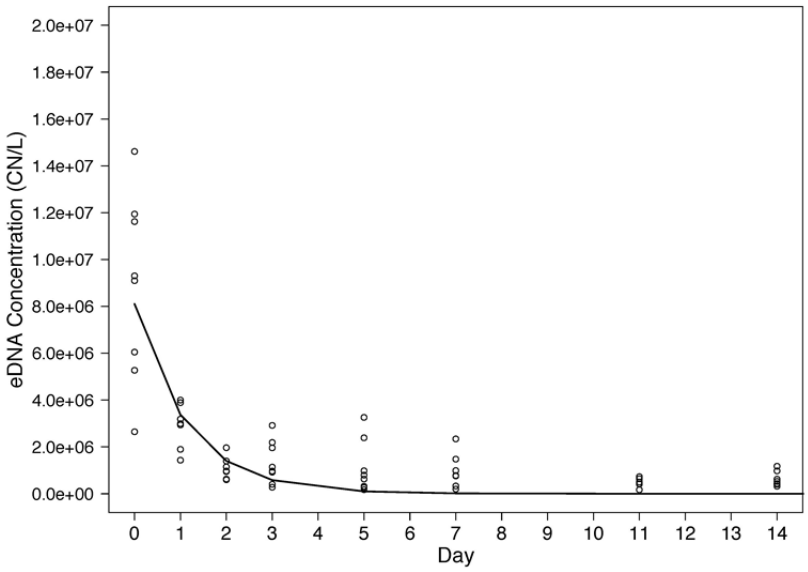

Figure 1. The decay in estimated concentration of eDNA (CN/L) remaining from *H. nobilis* biowaste over a 28-day period under baseline conditions for the study (20 °C, native pH, endogenous microbial load and richness, shaking at 66 RPM). The solid line represents the calculated monophasic exponential decay curve.

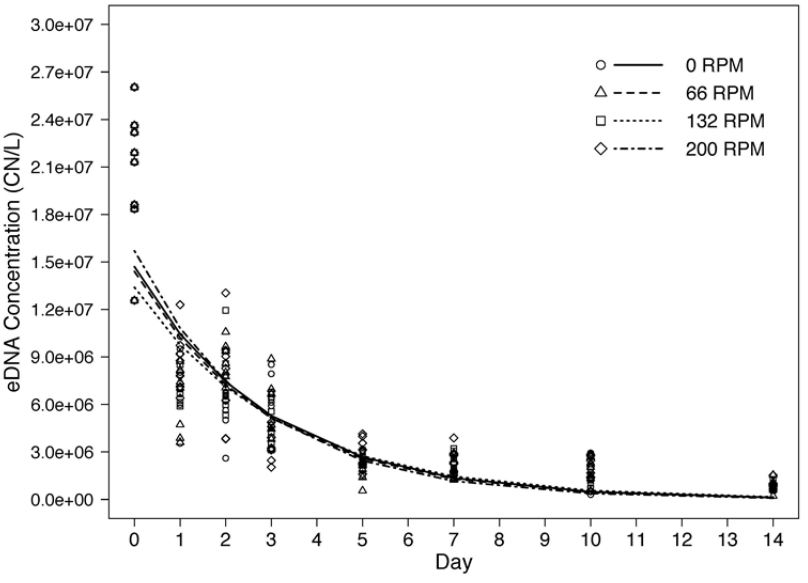

Figure 2. The decay in estimated eDNA copies per liter remaining from *H. nobilis* biowaste over a 14-day period and under four different shaking treatments: 0 RPM (no shaking), 66 RPM, 132 RPM, and 200 RPM. Lines represent calculated monophasic exponential decay curves.

Environmental factors: temperature

Patterns of eDNA decay for carp biowaste under different temperature treatments are displayed in Figure 3. Different treatments resulted in different decay rates, though patterns were in part confounded by spikes in estimated eDNA concentrations observed on Day 2, as well as Days 5, 7, and 10. These apparent and unanticipated spikes in estimated eDNA over the course of the trial are also observed in some of our other trials in this study, and are considered in the Discussion section. DNA decay under all treatments fit the monophasic exponential decay curve (R^2 = 0.518–0.780; $p < 0.05$) and exhibited more rapid decay with increasing temperature (Figure 3; Table 2). Corresponding eDNA half-lives vary considerably and are listed in Table 2.

Table 1. Decay constants (λ) and half-life ($t_{½}$) estimates for eDNA decay under different turbulence treatments.

Treatment	λ	$t_{½}$ (days)
0 RPM	0.34	2.0
66 RPM	0.34	2.0
132 RPM	0.32	2.2
200 RPM	0.37	1.9

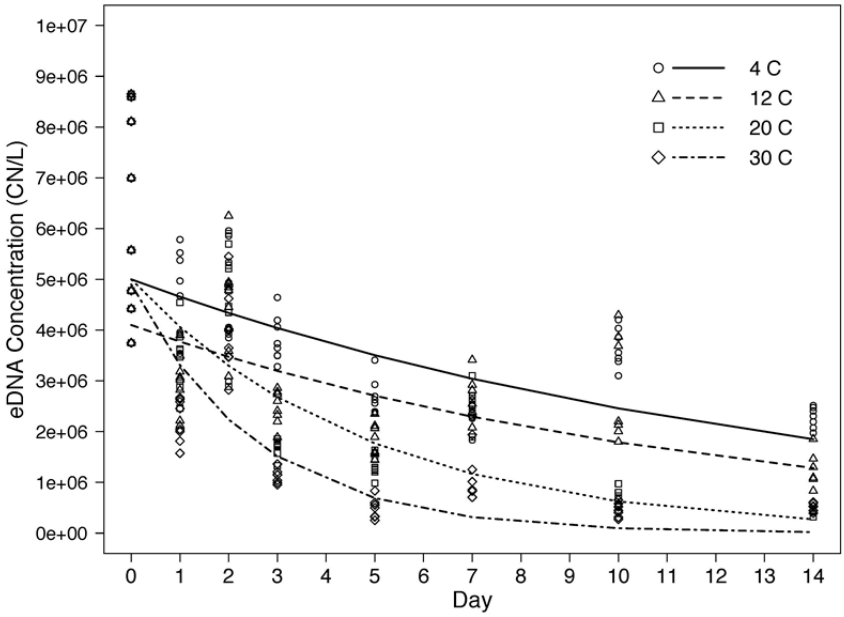

Figure 3. The decay in estimated concentration of eDNA (CN/L) remaining from *H. nobilis* biowaste over a 14-day period and under four different temperature treatments: 4 °C, 12 °C, 20 °C and 30 °C. Lines represent calculated monophasic exponential decay curves.

Notably, the amounts of eDNA in the 30 °C and 20 °C treatment groups were reduced by more than 90% relative to Day 0 estimates by the end of the experiment, the 12 °C treatment group was reduced by about 80% by Day 14, and eDNA levels in the 4 °C had diminished by only 40% at the end of the experiment. By the end of Day 3 of the experiment, when the majority of the eDNA was degraded, the mean estimate for eDNA concentration (CN/L) had declined by at least 80% in the 30 °C treatment, by at least 70% in the 20 °C treatment, by at least 55% in the 12°C treatment, and by at least 35% in the 4°C treatment.

Environmental factors: microbial load and source

The average CFU/ml for the preliminary test of microbial viability in carp slurry stored at −20 °C was calculated at 9.5×10^4 and visible growth in the liquid cultures was observed across all slurry dilutions except for the 10^{-5} class. In the preliminary test of antibiotic treatment effects on bacterial loads and eDNA, the non-treated samples had a mean microbial load of approximately 5×10^4 CFU/ml and treated samples a mean of 5×10^1 CFU/ml. All samples, both treated and untreated, showed bighead carp eDNA marker amplification between 22 and 24 qPCR cycles.

Microbial communities in the low and minimal load treatment classes were strongly suppressed by antibiotic treatments (Figure 4), and even by the end of the 14-day experiment the minimal load class never achieved equivalent microbial concentrations to the other treatment classes. The addition of pond water (augmented microbial load class) did not appear to result in large increases in overall microbial concentrations (Figure 4). The low and minimal microbial load classes exhibited slower eDNA decay (Figure 5). While the augmented microbial load and no treatment classes exhibited significant fits to monophasic exponential decay models ($R^2 = 0.674$, 0.759 respectively; $p < 0.02$; Figure 5), the low and minimal microbial load classes did not fit an exponential pattern ($R^2 < 0.1$; $p > 0.49$; Table 3). The poor fits of the low and minimal microbial load classes to exponential decay curve is due to large-scale variance in estimated eDNA copies per liter over the course of the experiment. The source of this variance is considered briefly in the Discussion section. Because of these poor fits to the exponential decay model, half-life estimates for the low and minimal microbial load classes could not be calculated.

Table 2. Decay constants (λ) and half-life ($t_{½}$) estimates for eDNA decay under different water temperature treatments.

Treatment	λ	$t_{½}$ (days)
4 °C	0.071	9.8
12 °C	0.083	8.4
20 °C	0.21	3.3
30 °C	0.39	1.8

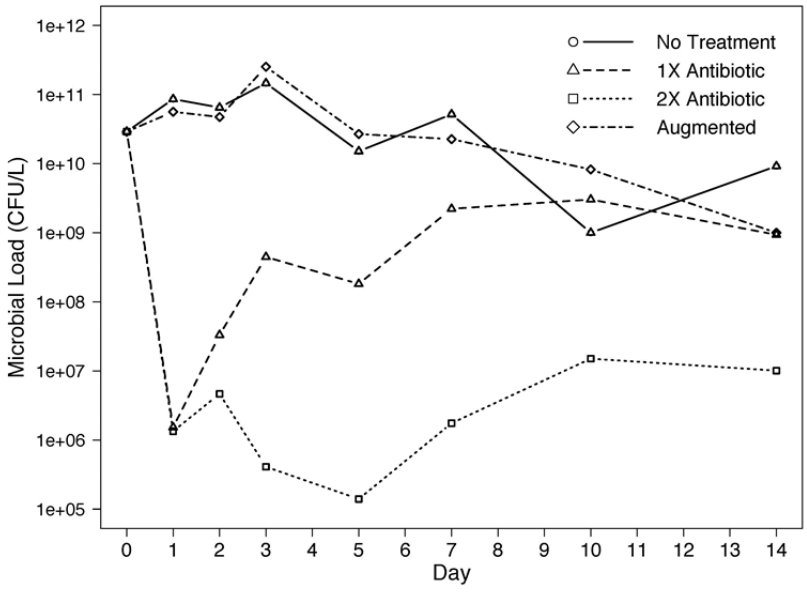

Figure 4. Microbial loads (estimated colony forming units or CFU/L) from *H. nobilis* biowaste over a 14-day period and under four different microbial load treatments: baseline (no treatment), addition of 1X antibiotic serum to carp slurry, addition of 2X antibiotic serum to carp slurry, and pond water inoculation of carp slurry (augmented). Lines track change in mean CFU over time.

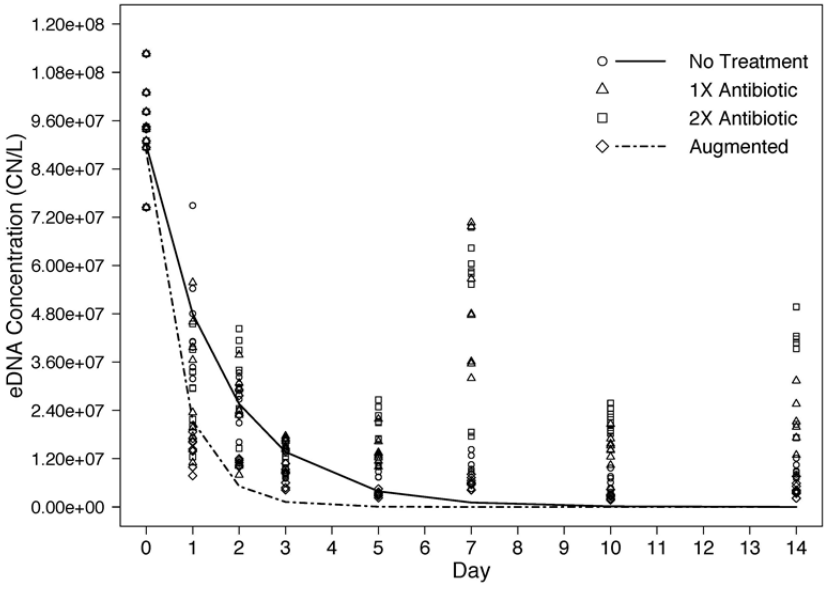

Figure 5. The decay in estimated concentration of eDNA (CN/L) remaining from *H. nobilis* biowaste over a 14-day period and under four different microbial load treatments: no treatment, low microbial load, minimal microbial load, and augmented microbial load. Lines represent calculated monophasic exponential decay curves. Significant fits to exponential decay curves did not exist for the low and minimal microbial load classes.

Despite exhibiting very similar microbial load estimates and patterns of microbial load change over time (Figure 4), the half-lives of the augmented microbial load and no treatment classes were quite different during the first 5–7 days of the experiment (Table 3). By the end of the experiment, the augmented microbial load class exhibited about 90% reduction in estimated eDNA copies per liter, the no treatment class about 85% reduction, the low microbial load class about 60% reduction, and the minimal microbial load class only about 35% reduction.

Table 3. Decay constants (λ) and half-life ($t_{½}$) estimates for eDNA decay under different microbial load treatments (NC = could not be calculated).

Treatment	λ	$t_{½}$ (days)
No treatment	0.63	1.1
Low microbial load	NC	NC
Minimal microbial load	NC	NC
Augmented microbial load	1.4	0.50

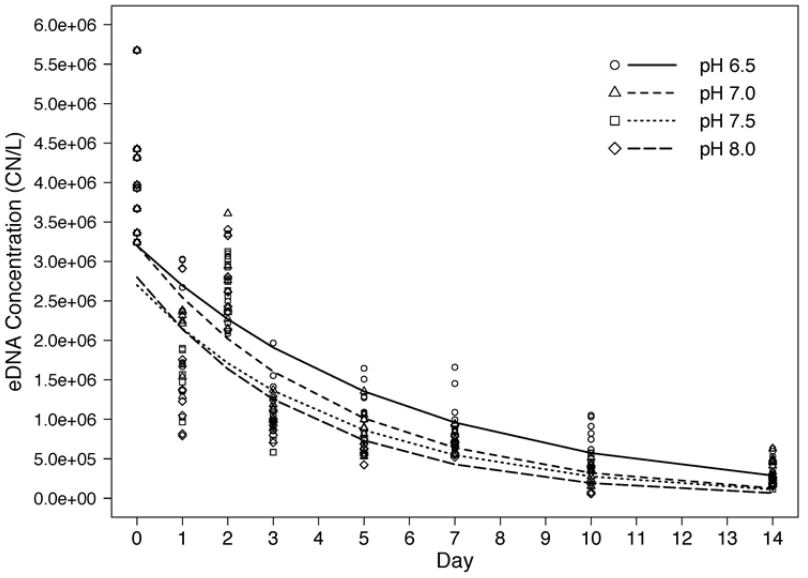

Figure 6. The decay in estimated concentration of eDNA (CN/L) remaining from *H. nobilis* biowaste over a 14-day period and under four different pH treatments: pH 6.5, 7.0, 7.5, and 8.0. Lines represent calculated monophasic exponential decay curves.

Environmental factors: pH

Patterns of eDNA decay for carp biowaste under different pH treatments are displayed in Figure 6. The pH = 6.5 treatment class required little or no amendment to achieve the target pH, while the other classes required daily amendments with NaOH. Decay of eDNA in each pH treatment exhibited a significant fit to the monophasic exponential decay model (R^2 = 0.694–0.890; $p < 0.02$). Half-life calculations (Table 4) demonstrated that decay of eDNA was relatively faster under pH 8.0, relatively slower at pH 6.5, and intermediate at pH 7.0 and 7.5. By the end of the experiment, all four treatments experienced similar total eDNA decay (approximately 85% to 95% loss).

Environmental factors: combined treatments

Patterns of eDNA decay for carp biowaste under rapid, baseline, and slow decay conditions treatments are displayed in Figure 7. The rapid decay treatment class exhibited a significant fit to a monophasic decay curve (R^2 = 0.647; $p < 0.003$) with a $t_{½}$ = 0.862 days or 20.69 hours. The no treatment class appeared to exhibit a monophasic decay pattern, though a large spike in eDNA on Day 1 resulted in lack of significant fit to that model (p = 0.948). For the slow decay treatment class eDNA, as expected, decayed relatively very slowly. Perhaps because of several sampling points exhibiting high variance (e.g. Day 1, Day 5, Day 35) the slow decay data did not exhibit a significant fit to a monophasic decay curve (R^2 = 0.087; p = 0.268).

Table 4. Decay constants (λ) and half-life ($t_{½}$) estimates for eDNA decay under different pH treatments.

Treatment	λ	$t_{½}$ (days)
pH 6.5	0.17	4.0
pH 7.0	0.23	3.0
pH 7.5	0.23	3.0
pH 8.0	0.27	2.6

By Day 5, the Rapid Decay Treatment class had exhibited a roughly 85–90% DNA loss, with the No Treatment class exhibiting about 60% loss, and the Slow Decay Treatment class exhibiting an apparent DNA loss of only about 15% (Figure 7). By the end of the experiment, on Day 91, the rapid decay treatment class had an apparent 98–100% eDNA loss, the no treatment class had an apparent loss of about 90%, and the slow decay treatment class had an apparent eDNA loss of only about 30%.

Carp milt

All sample tanks, including the negative control tank, maintained water temperatures at an average of 23.97 °C ± 0.38. No samples during the course of the 91-day experiment exhibited PCR inhibition, nor were there any indications of DNA contamination in the qPCR negative controls. No eDNA was detected in water samples taken from the experimental tanks prior to the addition of *H. molitrix* milt. On Days 1 and 2 we observed amplification of *H. molitrix* DNA (1,200 and 28,396 copies/l, respectively) in samples from our negative control tank, which was likely due

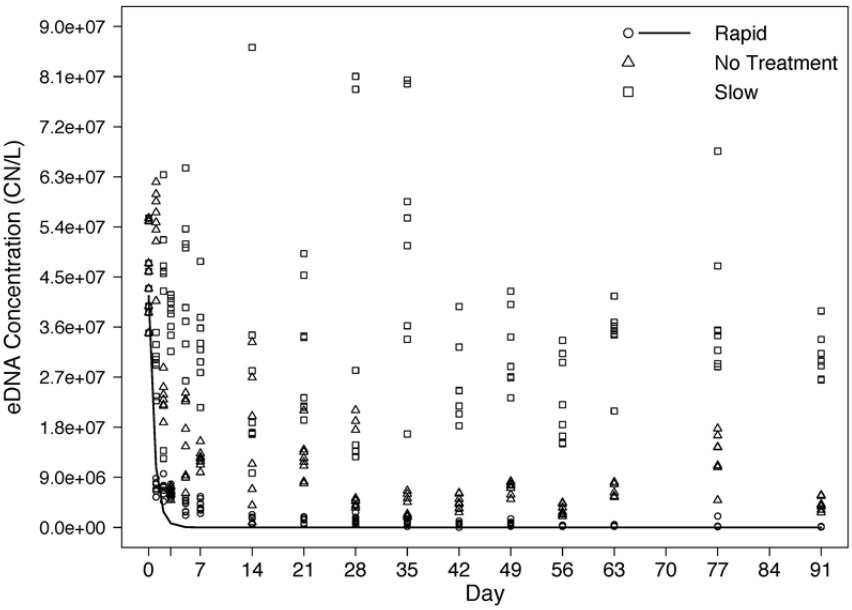

Figure 7. The decay in estimated concentration of eDNA (CN/L) remaining from *H. nobilis* biowaste over a 91-day period and under three combined treatments: rapid decay (30 °C, augmented microbial load, pH 8.0), no treatment (20 °C, no microbial treatment, no pH adjustment), and slow decay (4 °C, minimal microbial load, pH 6.5). The line represents the calculated monophasic exponential decay curve for the rapid decay treatment. The no treatment and slow decay data did not exhibit significant fits to monophasic exponential decay curves.

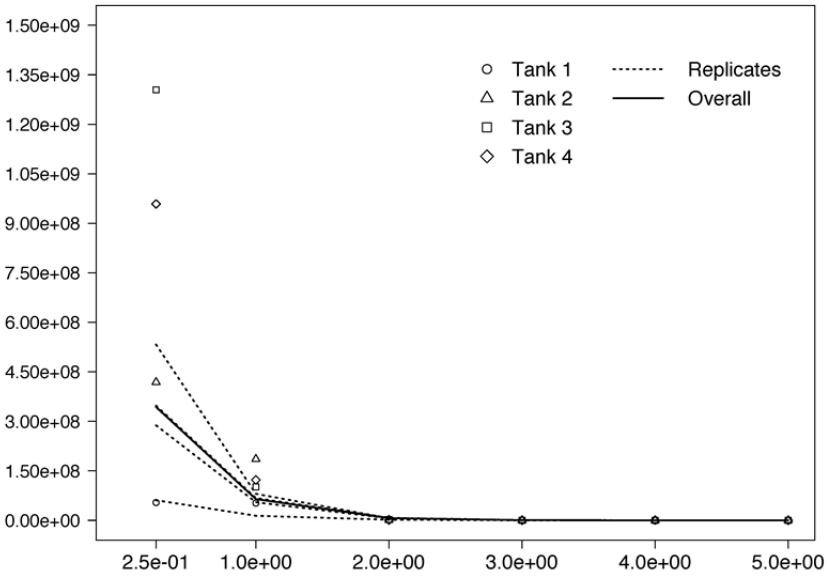

Figure 8. The decay in estimated concentration of eDNA (CN/L) remaining from *H. molitrix* milt over the first 5 days of a 91-day trial. The dashed lines represent the calculated monophasic exponential decay curves for four replicate experiments and the solid line represents the calculated monophasic exponential decay curve for the mean estimate of eDNA copies per liter over the course of the experiment.

to aerosol contamination from neighboring experimental tanks (Appendix 3). No further positive *H. molitrix* DNA detections were observed for the negative control tank after this point.

We immediately detected *H. molitrix* eDNA following milt addition (Day 0; Figure 8; Appendix 3). Six hours post milt addition (Day 0.25), the amount of *H. molitrix* eDNA increased sharply, followed by a rapid decay through Day 2 (48 hours). Decay patterns for all four tanks exhibited significant fits to monophasic exponential decay models (R^2 = 0.746–1.00; $p < 0.03$) No eDNA detections occurred beyond Day 7 (168 hours). Though the different tanks likely did not start with the same numbers of sperm cells, as reflected in the initial eDNA concentrations, decay rates and half-lives were relatively similar (Table 5) among the four tanks. Decay constants ranged between $\lambda = -3.43$ and $\lambda = -1.75$ and the corresponding half-lives ranged from $t_{½}$ = 0.2 to 0.4 days. Because of the increasing estimates of *H. molitrix* eDNA concentrations between Day 0 and Day 0.25, we did not include the Day 0 samples in our calculations.

Discussion

The eDNA decay patterns from both carp biowaste and milt largely follow an exponential decay similar to those observed in other eDNA studies (Barnes et al. 2014; Dejean et al. 2011; Maruyama et al. 2014; Pilliod et al. 2014; Strickler et al. 2015; Thomsen et al. 2012a; Thomsen et al. 2012b), though the eDNA derived from milt appeared to degrade much more rapidly than the eDNA derived from biowaste. Though there have been a growing number of studies reporting eDNA decay results, most studies do not describe decay using the same metrics, making cross-study comparisons difficult. In our study, decay constants relative to time periods of days (λ) differed considerably, even for trials in which environmental factors were not manipulated (e.g. baseline decay, 66 RPM trial, pH 6.5 trial, no treatment microbial trial, 20 °C trial), ranging from 0.17–0.88. Differences were narrower among decay constants in the sperm eDNA experiment (1.75–3.43), likely because the different trial replicates used the same batch of eDNA source material and decay was more rapid. Other reported decay constants (again relative to time periods of days) for aquatic eDNA of macro-biotic taxa include, among others, 0.322 and 0.701 for two marine fish (Thomsen et al. 2012), 1.31 for a marine crab (Forsström and Vasemägi 2016), and 0.05–0.34 for bullfrog (Strickler et al. 2015). In terms of half-lives, rates of decay for biowaste varied widely, including $t_{½}$ = 0.8–4.0 days (Tables 1–4) in the baseline decay experiment and other trials in which environmental factors were not manipulated. For the milt eDNA experiment the mean $t_{½}$ was about 0.3 days or 6.5 hours (Table 5). Maruyama et al. (2014) observed a half-life of $t_{½}$ = 6.3 hours for mesocosms from which bluegill (*Lepomis macrochirus* Rafinesque, 1819) had been removed. Clearly, eDNA decay can vary tremendously and continuing research that describes decay patterns and the factors that influence those patterns will be an important part of the maturation of eDNA as a scientific tool.

In our experiments, eDNA decay showed clear monophasic decay curves, except in cases where high levels of variance across time prevented effective curve fitting. We also observed spikes in eDNA concentrations is some of our experiments. For instance, in the carp biowaste baseline degradation trial there were slight increases in the amount of eDNA in tubes from Day 2 to Day 3, from Day 11 to Day 14, and from Day 21 to Day 28. We also observed a spike in eDNA concentrations in the milt experiment between Day 0 and six hours later at Day 0.25 (Figure 8). Increases, or spikes, in the amount of DNA over time could be a simple function of the large variances around mean copy numbers, especially in the early stages of each experiment. It is also possible that the spikes in eDNA could be caused by an initial adsorption of eDNA to the walls of the polypropylene tubes (Gaillard and Strauss 1998), followed by eventual desorption of some eDNA (Eichmiller et al. 2016). Also, the carp biowaste used in these experiments may have held some level of co-occurring PCR inhibitors, including those typically associated with fecal material (bile salts and complex polysaccharides; reviewed in Schrader et al. 2012) and apparent spikes of eDNA could result from breakdown of PCR inhibitors over time. However, qPCRs with serial dilutions of the DNA template, a common approach for overcoming PCR inhibition, failed to eliminate DNA spikes, indicating that inhibitors were likely not the source of those spikes. Furthermore, amplification of the internal positive control in the milt experiments points away from inhibition as being a cause of the spike observed within the first 6 hours of the milt experiments. Our best explanation for the unexpected spikes in estimated eDNA concentrations was that some fraction of the eDNA in each tube adhered or sorbed to the poly-propylene walls and became temporarily stabilized or protected from bacterial exonucleases. Desorption of eDNA over the course of an experiment would then account for the spikes at different time points (e.g. Days 2 and 10 of temperature experiment; Figure 3), as well as, potentially, the large variances in eDNA estimates in the microbial treatment trials.

Table 5. Decay constants (λ) and half-life ($t_{½}$) estimates for sperm eDNA decay experiments.

Treatment	λ	$t_{½}$ (days)
Tank 1	1.8	0.39
Tank 2	2.5	0.28
Tank 3	3.4	0.20
Tank 4	3.3	0.21
Combined	2.7	0.26

Another interesting pattern in our results was the persistence of a small percentage of eDNA (about 10%) through the completion of most of the biowaste trials. While it is tempting to conclude that there is, in general, a recalcitrant portion of eDNA that is very long-lived, basing such a conclusion on the data from these experiments is probably unjustified. In addition to the potential for eDNA sorbed to the polypropylene tube walls to contribute to the apparent recalcitrant portion of eDNA, our experiments did not include all the factors (e.g. complete natural microbial communities) that may

drive eDNA degradation. Also the experimental systems (closed 15-ml tubes) may have become anoxic and depressed microbial activity that may have otherwise degraded all eDNA in the sample.

Beyond the observation of spikes and biphasic decay in some of the trials, the difference in decay rates between the biowaste and milt experiments suggests that DNA derived from different tissue types may degrade differently. Additionally, the differences in experimental setup including holding water in large glass jugs rather than small plastic tubes, continuous aeration, and exposure to ambient indoor light, may have contributed to more rapid eDNA degradation of the milt eDNA. Aeration of tanks may be one important factor leading to faster decay rates observed in our milt experiments. For instance studies reporting half-lives in terms of hours similar to our milt trials also aerated their sampling tanks (Sassoubre et al. 2016; Maruyama et al. 2014) or monitored oxygen to avoid anoxic conditions (Eichmiller et al. 2016). This contrasts with our biowaste trials, which were conducted in smaller, covered containers, potentially leading to anoxic conditions and thereby reducing eDNA degradation by microbial activity.

Environmental factors

Of the all the environmental factors tested—water turbulence, water temperature, microbial loads, and water pH—only variation in water turbulence appeared to have little to no effect on decay. This is not an unexpected finding as the lengths of eDNA loci, mitochondria, and microbes are all much shorter than the Kolmogorov scale, i.e. the minimum wavelengths of shearing turbulence that can effectively propagate, which in water tend to be on the order of 0.3–2 mm (Jiménez 1997). Below the Kolmogorov scale, turbulence is rapidly damped (see also Purcell 1977). Hypothetically, turbulence could have affected adsorption and desorption of eDNA to the walls of the sample tubes, or, by keeping particulates in suspension, affected the amount of surface area available to microbes and exonucleases. However, we observed no such effects as the measures of $t_{½}$ and decay rate in the 0 RPM and 200 RPM treatment groups were approximately equal.

Not unexpectedly, when water temperatures were held at 4 °C, eDNA decay was greatly reduced, with a decay half-life ($t_{½}$) approximately 3× and 5.5× slower than when water temperatures were 20 °C or 30 °C, respectively (Table 2). By the end of the experiment, the 4 °C treatment had approximately 2× the amount of eDNA remaining as in the 30 °C treatment. While the different temperature regimes may have directly affected the thermodynamics of eDNA structural decay, different temperatures would also affect the activity of microbes and microbial exonucleases. At higher temperatures microbes would be expected to be more metabolically active and exonucleases more kinetically active, and eDNA to be more rapidly degraded. Strickler et al. (2015) found temperature differences to affect eDNA decay to a stronger degree than differences in UV-B exposure or pH, and that temperature also impacted the abundance of microbial populations in their experimental units.

In our microbial load experiments, the abundance of microbes in the sample appeared to have a very strong effect (Figure 5, Table 3). Though we could not calculate half-lives for all of the microbial load treatments due to high variation and poor fits to the exponential model in some cases, we observed that the total eDNA losses (Day 14 results) in the low and minimal treatment classes were 30% and 40% lower than in the no treatment class. In addition to microbial load, our results indicate that the constituency of the microbial community has an important role in the eDNA decay. The addition of water from a nearby pond, though having a seemingly negligible impact on microbial load (Figure 4), resulted in a decrease of eDNA half-life from $t_{1/2}$ = 1.1 hours to $t_{1/2}$ = 0.5 days (Table 3). As described by Eichmiller et al. (2016), the type of water in which eDNA is found can significantly influence decay rates, likely because different water sources host different microbial communities. The significant role of microbial communities on decay of extracellular DNA is well-recognized (reviewed in Nielsen et al. 2007), though its role in degrading mitochondrial membranes, the structures within which much of eDNA may be initially found (Turner et al. 2014), is not as well-described. In such a way, and in others, we anticipate that the role of microbial communities in eDNA fate is likely to be quite varied and complex. Along with our observation on how the source of microbial populations affects eDNA fate, Strickler et al.'s (2015) observations on pH interactions with microbial degradation of eDNA, and Barnes et al.'s (2014) study, where eDNA decayed more rapidly in mesocosms with reduced microbial activity, but where overall eDNA persistence was also greatest in those same low-activity mesocosms, provide excellent insights into this complexity.

Varying the pH of water had fairly minor effect on eDNA decay in our experiments, though there was an apparent trend of increasing decay with increasing pH (Figure 6). The half-life of eDNA decay at pH 8.0 was approximately 65% shorter than at pH 6.5 (Table 4). The relatively weak influence of different

pH levels on degradation rates may be due to the narrow range of near-neutral pHs tested. However, even with a much broader range of pH, Strickler et al. (2015) found that pH seemed to primarily have an effect on eDNA decay through interactions with other environmental factors. The binding affinities of DNA to sediment moeities such as clays, humic acids, and fulvic acids are influenced by pH, with generally greater binding affinities at lower pH (Levy-Booth et al. 2007). How pH and molecular sorption of eDNA to different water constituents and sediments affect the bioavailability and decay of eDNA bears further study and may prove useful in optimizing eDNA sampling. Likewise, studies of how the association of liberated mitochondria (from decaying cells) with organic colloids and particulates (Lead and Wilkinson 2006) affect the bioavailability and decay of eDNA may be useful.

In the combined treatments we demonstrated how eDNA decay rates can vary radically under varying conditions. While declining levels of available oxygen may have contributed to the long eDNA persistence times in the portions of this study that used carp biowaste, the differences between treatment groups were considerable even for the early sampling points. With several factors favoring decay, eDNA concentrations had dropped by about 83% by Day 2 and arrived at ≥ 90% eDNA loss by Day 14. At the other extreme, with several factors favoring persistence, eDNA concentrations had dropped by only about 5% percent, if at all, by Day 2 and had arrived at ≤ 30% loss at the close of the experiment. These patterns would lead one to expect that eDNA may persist longer in colder and/or oligotrophic waters than in warmer and/or eutrophic waters, and that eDNA sampling designs and data interpretation would differ between these types of systems. A similar extrapolation of experimental results to expectations for eDNA fate in natural waters was posited by Strickler et al. (2015). However, in a study by Eichmiller et al. (2016) eDNA actually persisted longer in waters from a eutrophic lake than in waters from an oligotrophic lake. Obviously there are a complexity of factors influencing eDNA fate that our study and other studies are only just beginning to piece together.

Sassoubre et al. (2016) recommend that future studies on eDNA degradation report decay constants as a metric of decay rather than the length of time until eDNA detection is lost. They argue that time to loss of detection is a function of the starting amount of eDNA and cannot be effectually compared across studies, whereas decay constants are independent of the starting amounts of eDNA and are more comparable across studies. We agree with this excellent recommendation. Furthermore, our study and others indicate that factors such as water temperature, microbial community, pH, and UV-B exposure can significantly influence decay constants. Therefore, in addition to reporting decay constants (and half-lives), future studies that test decay using common frameworks (starting materials, water conditions, analytical approaches, etc.), and that test various decay-influencing factors in systematic ways within that common framework, would add to the advantages of reporting these useful metrics. In fact, a consensus-building effort towards a collective, multi-laboratory, multi-study approach to understanding eDNA fate would be of tremendous benefit to the science and application of aquatic eDNA monitoring.

Conclusion

We have demonstrated that eDNA decay can be affected by differing levels of several environmental factors. Of the environmental factors tested, temperature and microbial community (abundance and constituency) had the largest effects on the general rate of eDNA decay. We have also demonstrated that both eDNA associated with bighead carp waste and silver carp sperm decay according to general monophasic decay curve. The patterns observed in this study highlight the need to take into account general eDNA decay patterns and environmental conditions when planning eDNA surveys and when interpreting eDNA data. For example the expected high decay rates in warm, highly productive waters will more strongly infer the recent presence of a source for that eDNA in the system than a similar pattern of hits in colder, less productive waters. Similarly, an effort to use eDNA to detect spawning in warmer, productive waters may benefit more from frequently repeated sampling than a similar survey in colder, less productive waters. Finally, our study addresses some general patterns in eDNA decay, but the probability of eDNA detection and the inferences that can be drawn from eDNA data are influenced by a wide range of factors, including target organism behavior and physiology, eDNA shedding rates, the forms in which eDNA is shed (e.g. within cells, within mitochondria, unencapsulated), decay factors addressed and not addressed in our study, sampling and DNA processing protocols, the presence of PCR inhibitors, water flow patterns, PCR primer efficiencies, etc. Our hope is that the results of our study will be one of many other studies that contribute to the development of an increasingly efficient, powerful, and widely-employed eDNA capability.

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Supplementary material

The following supplementary material is available for this article:

Appendix 1. Sequences for the internal positive control (IPC) primers, probe and ultramer.

Appendix 2. Standard curve metrics for eDNA decay trials.

Appendix 3. Decay of milt-derived eDNA over first five days of 91-day trial.

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