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METHODS AND RESOURCES ARTICLE

Ethanol and sodium acetate as a preservation method to delay degradation of environmental DNA

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Abstract
Environmental DNA (eDNA) samples that are collected from remote locations depend on rapid stabilization of the DNA. The degradation of eDNA in water samples is minimized when samples are stored at ≤4 °C. Developing a preservation technique to maintain eDNA integrity at room temperature would allow a wider range of locations to be sampled. We evaluated an ethanol and sodium acetate solution to maintain the integrity of the DNA samples for the time between collection and lab testing. For this evaluation, replicate water samples taken from a tank housing Asian carp were placed on ice or held at room temperature. At both temperatures, water samples were left untreated or were preserved with an ethanol and sodium acetate solution (EtOH–NaAc). Every day for 6 days following collection, a subset of the samples was removed from each preservation method and DNA was extracted and nuclear and mitochondrial markers were assayed with qPCR. Results showed comparable persistence of DNA between iced samples without the EtOH–NaAc treatment and samples that received EtOH–NaAc treatment that were kept at room temperature. We found that DNA can be amplified from preserved samples using an EtOH–NaAc solution after up to 7 days at room temperature.

Keywords Environmental DNA · eDNA preservation · Asian carp · Non-invasive genetic sampling

Introduction

Species detection via trace amounts of environmental DNA (eDNA) has become a valuable tool to monitor cryptic, imperiled, or invasive species in aquatic habitats (Bohmann et al. 2014) and aquatic organisms that evade capture gears (Jerde et al. 2011). Much of the work with eDNA has been focused on systems that are accessible. Few studies have used this tool in remote locations due to the challenges in sample preservation to ensure high quality DNA for downstream workflows. Thus, as the interest in eDNA as a detection tool continues to expand, improvements and standardization of eDNA collection techniques is necessary for its successful application.

Currently, the established method to preserve eDNA water samples dictates immediate storage on ice following collection, then the water samples are either filtered on site or promptly returned to the lab where they are filtered or centrifuged and preserved (Jane et al. 2014; Piaggio et al. 2014; Santas et al. 2013; Takahara et al. 2013). However, where these techniques are not ideal or plausible, few options for storage of eDNA water samples exist. There are various sampling protocols for preserving DNA on filters (Goldberg et al. 2013; Pilliod et al. 2013; Renshaw et al. 2015; Robertson et al. 2013) or long-term solution storage of small DNA aliquots (Robertson et al. 2013) but the intervening time between water sample collection and long-term storage has been little examined. The use of a Longmire's lysis buffer and alkyldimethylbenzylammonium chloride have been investigated as eDNA preservatives (Williams et al. 2016; Yamanaka et al. 2017). However, these solutions require specialized knowledge to prepare, and may not be ideal for remote field locations that do not have access to these reagents. Field collections become compromised when sample processing cannot be completed within short critical time periods, such as when ice or access to sample filtration capacity is unavailable. There is a need to develop an effective and simple method to preserve the DNA in samples in adverse environments.

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We tested the use of ethanol and sodium acetate (EtOH–NaAc) to prevent DNA degradation. EtOH–NaAc has been used in previous work to precipitate DNA in samples before extraction (Dejean et al. 2011; Ficetola et al. 2008; Piaggio et al. 2014; Valiere and Taberlet 2000). Instead, for this study we used EtOH–NaAc as a preservative for water samples under different temperatures for up to 1 week.

Methods

Since we did not directly use any vertebrate animals in this study, an IACUC or animal welfare protocol was not required for the study. The Asian carp were held in a recirculating aquaculture system, approved as part of a separate study (IACUC approval #AEH-12-eDNA-02). Any use of trade, product, or company name is for descriptive purposes only and does not imply endorsement by the U.S. Government.

Verification of biological productivity of water

To verify that our water samples contained microbial activity and was consistent with characteristics of environmental water samples (Albers et al. 2013; Gentry-Shields et al. 2013), three tryptic soy agar (TSA) plates were inoculated with 100 µL of water acquired from the original sampling tank collected on day 0. These plates were prepared using the spread plate technique and subsequently incubated at 37 °C for 48 h (Hartman 2011). Colony forming units (CFU) were counted under magnification for each plate.

Study design

Fifty-two water samples (15 mL) were collected from a recirculating aquaculture system housing silver carp (*Hypophthalmichthys molitrix*) located at the U.S. Geological Survey Upper Midwest Environmental Sciences Center, La Crosse, Wisconsin. We compared degradation of DNA stored in EtOH–NaAc stored at room temperature or on ice with samples simply stored on ice or incubated at room temperature without preservation. Thirty-nine samples were treated by adding 1.5 mL of 3 M sodium acetate (Amresco, Solon, Ohio, USA) and 33 mL of absolute ethanol (Fisher Scientific Waltham, Massachusetts, USA) to 15 mL of sample water, while 13 samples were left untreated to serve as controls. DNA from three treated samples and one untreated sample was extracted immediately following collection and EtOH-NaAc treatment. The remaining 36 treated and 12 untreated samples were randomly assigned and equally divided among two storage temperatures. Eighteen EtOH–NaAc-treated and six control samples were placed in a cooler of ice (4 °C) and the remaining samples were housed in an incubator and held at a constant 23.9 °C (a temperature representative of a field setting). Every 24 h for 6 days, we extracted DNA from three EtOH–NaAc-treated samples and one control sample from each temperature.

DNA extraction

Samples were centrifuged at 5000×*g* for 30 m at 20 °C. The supernatant was decanted from each sample and the remaining pellet was subjected to DNA extraction. The extraction procedure was adapted from the manufacturer's protocol of the IBI gMAX Mini Genomic DNA Kit for blood, tissue, and cultured cells (IBI Scientific, Peosta, Iowa, USA). Modifications to the established procedures included: (1) the addition of 500 µL phosphate-buffered saline before the addition of proteinase-K and (2) an increase in the volumes of GSB Buffer (reagent provided in IBI gMAX extraction kit indicated above) and ethanol from 200 to 500 µL. DNA was resuspended in 100 µL of Elution Buffer. Extractions were conducted in a room specific to this purpose. One extraction negative control was extracted alongside the samples each day, totaling seven negative control samples. Once extracted, the DNA was stored at − 80 °C until analysis.

Molecular analysis

Generally in eDNA-based studies, a mitochondrial marker is used for detection of the species of choice (Dejean et al. 2011; Jane et al. 2014; Piaggio et al. 2014; Renshaw et al. 2015; Santas et al. 2013; Takahara et al. 2013; Thomsen et al. 2012). Cells generally carry thousands of copies of mitochondrial DNA (mtDNA), versus one (diploid) nuclear genome (nDNA) with only two copies per nuclear gene. The rate of degradation or number of copies may differ among the types of DNA. Therefore, we compared the changes in copy numbers over time of the two types of DNA, mtDNA and nDNA, using qPCR. We used a silver carp mtDNA-specific primer and probe set (Merkes et al. 2014) to assess degradation of mtDNA and a custom designed nDNA primer and probe set to monitor degradation of nDNA (Table 1). The nuclear marker was designed to span the intron/exon boundaries of the 60S ribosomal protein L8 of *H. molitrix* (GenBank accession HM012534.1).

DNA was quantified using qPCR. Assays were performed in a 25 µL reaction that consisted of 1 µL of template DNA, 12.5 µL 2× SensiFAST Prob N-Rox Mix (Bioline USA Inc., Taunton, Massachusetts, USA), 500 nM of each primer and 125 nM of the FAM™ dye-labeled probe for either mtDNA or nDNA, in molecular grade water. Negative PCR controls containing only the reaction cocktail and molecular grade water were analyzed on each plate. A positive qPCR control that contained DNA from an extracted silver carp tissue was

Table 1 Primer sequences for silver carp DNA amplification with qPCR

Type of Marker	Marker Name	Direction	Sequence (5′–3′)	Amplicon length (bp)
Mitochondrial	SVC_3	Forward	GGTGGCGCAGAATGAACTA	110
		Reverse	TCACATCATTTAACCAGATGCC	
		Probe	CCATGTCCGTGAGATTCCAAGCC	
Nuclear	HM_60S	Forward	AGAGTCTGATTGGTTCCC	95
		Reverse	ATGGTTACCACCACCGAA	
		Probe	GCTGTGTGCCTTTTCTTTCTCCAGCCTGT	

also analyzed on each plate. To minimize the risk of contamination, we prepared all qPCR assays in a room separate from the DNA extraction room. Samples were amplified in duplicate on a Mastercyler® ep *realplex*^{2} with the following program: 94 °C for 2 m, followed by 45 cycles of 94 °C for 10 s, 58 °C for 15 s, and 61 °C for 15 s DNA and then a final elongation at 72 °C for 5 m. Fluorescence was read during the 61 °C elongation phase.

Statistical analysis

We used the cycle threshold (Ct) values to estimate the relative amount of target DNA per sample. A series of six two-fold dilutions of a concentrated day 0 sample were used to create a standard curve (Larionov et al. 2005) and allow us to determine assay efficiency, slope and R^2 in-line with MIQE guidelines (Bustin et al. 2009). It should be noted that the efficiency of these markers can be significantly impacted by the presence of PCR inhibitors, like humic acid, but rather represent the efficiency of the markers in the matrix of the actual samples. Ct was plotted relative to the log-transformed concentration and the best fit line was used to estimate the amount of DNA in a sample. DNA copies were estimated for all samples using the same standard curve. We then report the change in copies of DNA relative to the DNA at day 0.

To preclude a potential effect of the control data on the final analysis, data from the no ice and no EtOH–NaAc controls was removed from subsequent analyses for the mtDNA and nDNA assays. Among EtOH–NaAc only, EtOH–NaAc and ice, and ice only treatments, changes in the amount of target DNA was determined for each day relative to day 0 and then compared among treatments using a linear model. Day and treatment were incorporated as fixed effects in the models.

Results

The three inoculated TSA plates resulted in 427 ± 316 CFU/mL following incubation. The R^2, slope and efficiency for the mtDNA marker was 0.99, − 4.50 and 66.7, respectively. The R^2, slope and efficiency for the nDNA marker was 0.97, − 5.72 and 49.6, respectively. No silver carp DNA was detected in any negative control, extraction or PCR, while silver carp DNA was amplified in our positive controls.

Preserved treatments (i.e. EtOH–NaAc only, EtOH–NaAc and ice, and ice only) indicated greater DNA persistence than that of the unpreserved control (Figs. 1, 2; Tables 2, 3).

Relative change in mtDNA copy number among each technique, EtOH–NaAc only, EtOH–NaAc and ice, and ice only, decreased with time ($\beta_{Day} = -14.05$, $t = -5.11$, $P < 0.01$). When compared to EtOH–NaAc and ice ($\beta_{(EtOH–NaAc+ice)} = -30.66$, $t = -2.60$, $P = 0.01$), mtDNA was better preserved by EtOH–NaAC, however the difference was not significant relative to ice only ($\beta_{(ice)} = -14.87$, $t = -1.14$, $P = 0.26$). No significant difference was detected between EtOH–NaAc with ice and ice only ($\beta_{(ice)} = 15.79$, $t = 1.21$, $P = 0.23$).

In our supplemental analysis of nDNA, relative change in copy number was not detected among days of the experiment ($\beta_{Day} = -0.10$, $t = -0.42$, $P = 0.68$). EtOH–NaAc alone preserved nDNA better than when EtOH–NaAc was combined with ice ($\beta_{(EtOH–NaAc+ice)} = -3.14$, $t = -3.39$, $P < 0.01$) or when samples were preserved with only ice (Table 3; $\beta_{(ice)} = -9.30$, $t = -8.13$, $P < 0.01$). Using EtOH–NaAc with ice worked significantly better than ice alone ($\beta_{(EtOH–NaAc+ice)} = 6.15$, $t = 1.14$, $P < 0.01$).

Discussion

Here we demonstrated that each of the three preservation techniques (ice, EtOH–NaAc, or the combination) retained more DNA through day 3 than even the original day 0 yields of the 7-day experiment. Beginning on day 4, DNA yield remained high (> 50%) for ice only, EtOH–NaAc only, or the combination of ice with EtOH–NaAc but did not maintain the original quantity of DNA. Therefore, in remote or adverse field locations DNA levels may be maintained without the need of centrifuging or ice for up to 3 days if a EtOH–NaAc technique is applied (Fig. 1). However, after 3 days, regardless of the method, the amount of target DNA in each sample decreased which

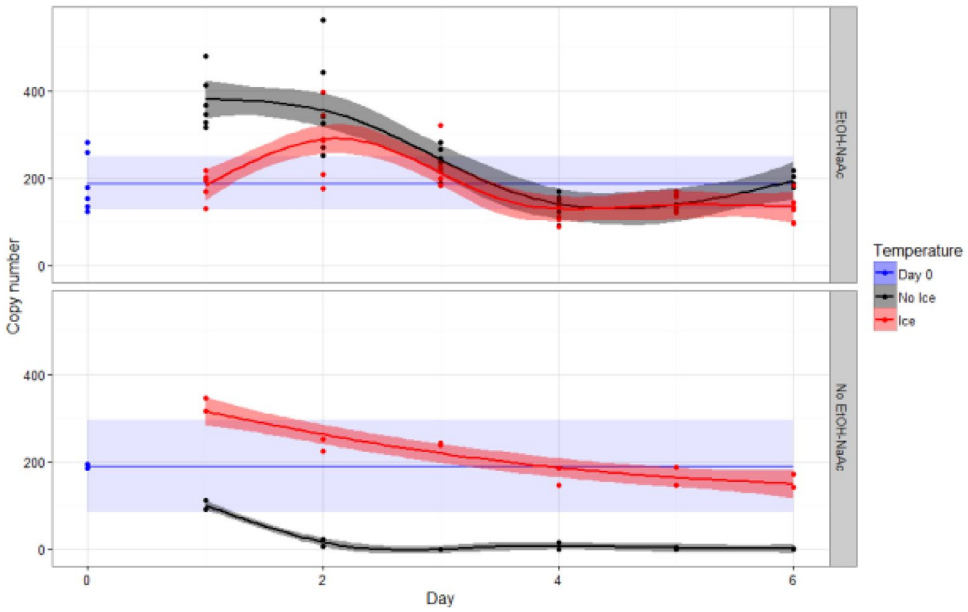

Fig. 1 Percent amount of DNA amplification with mitochondrial DNA-targeting markers among four preservation treatments over 6 days. from days one to six before adjustment by scaling day 0 to 100 copies. Color overlay represents 95% confidence interval (CI) around the mean copy number. For each temperature, day 0, with a 95% CI, was projected across all days for comparison. (Color figure online)

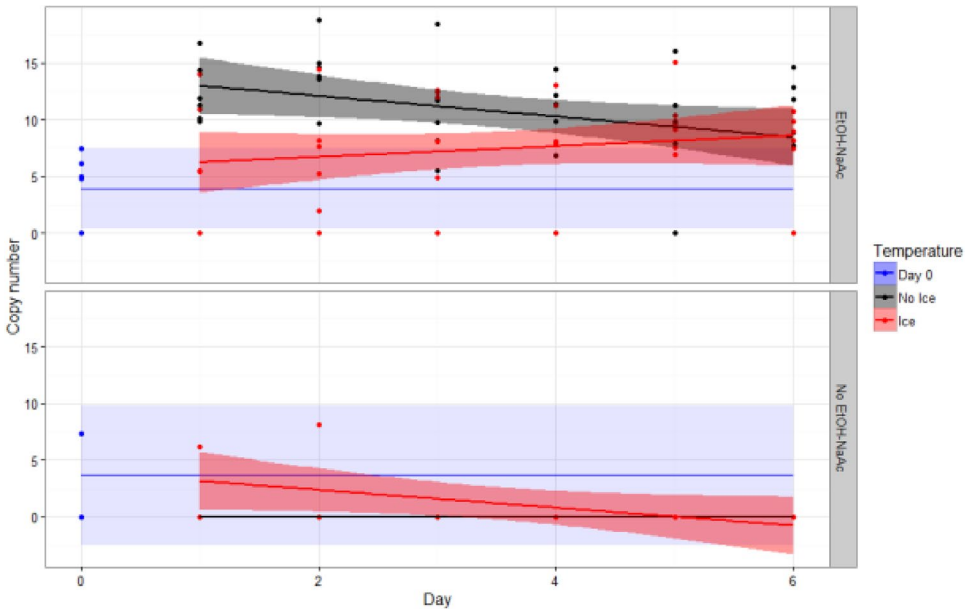

Fig. 2 Amount of DNA amplification with nuclear DNA-targeting markers among four preservation treatments from days one to six. Color overlay represents 95% confidence interval (CI) around the mean copy number. For each temperature, day 0, with a 95% CI, was projected across all days for comparison. (Color figure online)

Table 2 Relative mtDNA copies (mean ± SE) resultant of four preservation treatments for 6 days

Treatment	Day						
	0	1	2	3	4	5	6
EtOH–NaAc	100.38 ± 22.05	**199.47 ± 13.42**	**194.30 ± 37.86**	**129.65 ± 8.41**	68.98 ± 8.05	76.27 ± 2.45	**104.11 ± 1.79**
No treatment	**100.69 ± 2.31**	53.61 ± 5.06	7.72 ± 3.99	0.00 ± 0.00	3.91 ± 3.91	1.19 ± 1.19	0.39 ± 0.39
EtOH–NaAc + Ice	–	98.24 ± 6.60	150.50 ± 23.27	121.07 ± 12.53	58.66 ± 2.90	80.48 ± 3.70	69.66 ± 7.47
ice	–	175.52 ± 8.08	126.24 ± 6.77	127.36 ± 1.63	**88.04 ± 10.31**	**87.90 ± 10.96**	83.24 ± 7.85

DNA copies represent the percent increase or decrease relative to day 0; thus, day 0 copies are set at approximately 100. Highest yield per day is highlighted in bold print

Table 3 Relative nDNA copies (mean ± SE) resultant of four preservation treatments for six days

Treatment	Day						
	0	1	2	3	4	5	6
EtOH–NaAc	**3.89 ± 0.53**	**12.36 ± 1.01**	**14.25 ± 1.16**	**10.28 ± 2.10**	**9.10 ± 1.35**	9.11 ± 0.54	**9.32 ± 2.70**
No treatment	3.67 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
EtOH–NaAc + ice	–	5.98 ± 0.51	6.25 ± 2.41	8.32 ± 3.00	6.69 ± 2.73	**9.75 ± 1.21**	7.53 ± 1.91
ice	–	3.10 ± 2.52	4.05 ± 3.30	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

DNA copies are relative to day 0. Highest yield per day is highlighted in bold print

would indicate that field samples would require immediate processing to stabilize and recover remaining DNA. While using both EtOH–NaAc and ice does increase DNA preservation relative to the treatment without EtOH–NaAc and ice, it is possible this combination of treatment variables may provide a more unstable preservation environment than an ice only technique based on increased variation (Figs. 1, 2). The specific mechanism that decreases the stability of DNA when EtOH–NaAc is combined with ice is not known. Perhaps if the eDNA sample contains primarily 'free' DNA, decreasing the activity of enzymes by lowering the temperature of the sample is more important to DNA preservation than precipitating the eDNA by adding EtOH–NaAc (Eichmiller et al. 2016; Smith et al. 2001).

The amount of mtDNA detected in subsamples withdrawn after day 0 exceeded the amount detected in subsamples withdrawn on day 0 (Tables 2, 3). This finding requires further investigation (Tables 2, 3). Generally, DNA degradation occurs during storage, even when preserved (Nielsen et al. 2007). Thus, we would suspect that following environmental sampling, the amount of DNA would be equal to or less than the amount of DNA present in the initial analysis. It remains unclear why more DNA, both mtDNA and nDNA, were quantified on days 1–3 of this study than was quantified on the initial sampling day. This anomaly was also noted by Renshaw and colleagues (Renshaw et al. 2015). Other research groups have used silanized glass microcosms as they theorize DNA binds to tube walls and is slowly released into the water sample over the course of the experiment, creating a possible increase in the amount of DNA observed as the experiment progressed (Eichmiller et al. 2016). However, Williams and colleagues also studied a preservation solution and while they did not silanize their collection tubes, they also did not observe an increase of DNA from their initial sampling date (Williams et al. 2016). Their experiment used a much longer time scale albeit sampling only on days 28 and 56 post collection. It is possible that Williams and colleagues didn't observe this phenomenon because of the difference in experimental designs between their study and ours. Based on our data and those of Renshaw et al., we hypothesize that as degradation occurs, the genomic DNA becomes more available for extraction and thus is more available for amplification (Takahara et al. 2015).

The preservation method we tested used a 15 mL water sample. Though maintaining appropriate ratios of preservative (EtOH–NaAc) to sample volume would likely allow adequate preservation of larger or smaller sample volumes, future research is warranted to confirm adequate preservation of DNA in different sample volumes.

In summary, we found that after 3 days the amount of DNA after preserving water samples with EtOH–NaAc only, EtOH–NaAc and ice, and ice only were similar to the amount of DNA in water samples analyzed on day 0. The method discussed in this paper does not require the use of a laboratory (i.e. pH meter, stir plate, fume hood) to mix the DNA preservative, as in the case of Williams et al. (2016). The addition of EtOH–NaAc to water samples enhances the use of eDNA as a tool for sampling remote locations.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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