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Development of a Quantitative PCR (qPCR) Based Method for Studying Temporal DNA Degradation in Waterlogged Bone

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Abstract

Human activities are often centered around the presence of water, thus it is not surprising that there are many water-related human deaths. Accumulated degree days (ADD), and other aquatic variables may affect DNA retrieval from waterlogged bone. Calcium and collagen in bone can inhibit the PCR necessary to produce an STR profile; the current solution is a time-consuming organic extraction. While there are examples of research on DNA degradation in terrestrial bone over time, there has been little work done on submerged bone samples and they are usually limited to case studies. The major aim of this study was to measure host DNA quality and quantity in porcine waterlogged bones over time/ADD. It was accomplished by 1) attempting to optimize qPCR protocol for host DNA quantification and degradation index (DI) estimation, 2) determining the best extraction method (ChargeSwitch® gDNA Plant Kit v organic phenol-chloroform), and best bone type (between rib or scapula) for host DNA recovery in freshwater environment using a qPCR based method, and 3) identifying the variance of host DNA recovery in different bone types and water bodies. A SYBR based quantitative PCR protocol was developed for quantification host DNA using two target DNA loci (larger fragment target: 274-314bp and small fragment target: 93-127bp). The protocol was highly effective with the chosen STR primers, with the organic method obtaining the highest quantity with the lake samples, specifically in the ribs. There was evidence of a significant difference in degradation index over time, and the scapulae having the higher degradation index between bone samples. Individually, there was no significant difference in bone, method or location when it came to DNA quantity; combined interactions were required to find significance. Overall, scientists now have the opportunity to implement a more streamlined, efficient workflow from sample prep to profile development, which is pivotal in identification matters where time and resources are of the essence.

Keywords: forensic science, bone, waterlogged bone, quantitation, qPCR, degradation, DNA extraction

Introduction

Decomposition in water varies from that on land, as it encompasses the taphonomic process of remains transitioning from freshly submerged, to floating, to sinking. Soft tissue can completely disappear over time, leaving behind only bone, which takes considerably longer to weather and biodegrade. Haglund (1993), one of the earliest to research decomposition in aquatic environments, created a system to compare the number of days the body spent underwater to the amount/percentage of disarticulation in the joints of the body; since the suspension of the body in water would allow it to move in multiple directions, this resulted in the physical breakdown of soft tissue, occurring simultaneously with decomposition. Haglund's research demonstrated that the torso, the ribs and scapulae together with the vertebral column, remain an articulated unit the longest.

Despite its highly mineralized composition, bone may be more susceptible to an accelerated degradation in water than on land. Bone dissolution is the initial process through which water enters the pores in bone, destroying it and exposing the DNA contained within the osteocytes. The DNA attracts water molecules, resulting in deamination, depurination and depyrimidination (Latham and Madonna, 2013). This does not occur in living bone due to the inorganic, hydroxyapatite portion in bone that protects the DNA from degradation (Gotherstrom et al., 2002). Because this process results in the exposure of the DNA, along with other inhibitors, DNA extraction is the first and most important step in recovering optimal DNA samples.

Previous studies have examined DNA extraction methods from bone, though they were mainly focused on bones recovered from terrestrial environments. Mundorff et al., (2013) and Vass et

al., 2013) explored DNA retrieval from terrestrial bones, with the goal of comparing and empirically ranking which bones will provide more DNA, as well as which bones last for extended periods of time.

Marshall et al. (2014) studied fresh, terrestrial bone and compared organic versus solid-phase extraction using a "Hi-Flow" silica column. It showed comparable DNA quantity extracted between the two methods, though the Hi-Flow can extract higher volumes with less tube transfer steps. Iyavoo et al. (2013) compared five extraction methods on terrestrial bone, including the ChargeSwitch® protocol used in this study. Studies measuring DNA yield, extraction and degradation in waterlogged bone over time are few. A case study done by Crainic et al., (2002) provided a case report where skeletal remains and soft tissue remains that had been submerged in water for up to three years still retained extractable DNA in bone; this highlights a prospective timespan for how long DNA, and thus degradation might be measured in waterlogged bone. In another pilot study in aquatic system, Cartozzo et al., 2017 observed that organic extraction method resulted in more DNA yield and the ChargeSwitch DNA extraction method was the most effective silica-based method.

Organic extractions are known to be useful for obtaining high-molecular weight DNA yields, specifically when it comes to more 'difficult' samples (Butler 2011). Rucinski et al. (2012) cite a 95% recovery rate in their research using an organic extraction method. However, this method is extremely time consuming, requires many tube transfer steps, which increases the chances of contamination and product loss, and the chemicals required are hazardous. Solid-Phase methods, like the ChargeSwitch® kit, utilize magnetic beads that selectively bind the DNA while impurities are removed (Butler 2011). It is an easier and safer method than the organic method due to the lack of hazardous chemicals, and the ability to be automated. The major benefit is that solid-phase methods can remove PCR inhibitors, which is relevant because calcium and collagen from bone inhibit PCR amplification (Iyavoo et al. 2013; Desmyter et al. 2017).

DNA quality can be measured by the degradation index (DI), which is the ratio of smaller DNA fragments to larger DNA fragments (Lackey, 2018). The DI for intact DNA is less than or equal to one. If the DI is higher than one, then it suggests degradation or potential inhibition. Inhibition may mimic degradation due to the larger amplicon failing to amplify at the same rate as the smaller one. Calcium and collagen are common inhibitors when dealing with DNA amplification from bone samples. Noting inhibition is important to determining which extraction method is more effective, though both organic and ChargeSwitch have been noted to remove these reliably. Vernarecci et al. (2015) studied the capability of Quantifiler Trio to determine degradation, in conjunction with GlobalFiler PCR Amplification Kit, using a linear regression model. They found Quantifiler to be effective when characterizing degradation and potentially useful to predict how well a sample would amplify STRs. Gouveia et al. (2017) did a similar study, finding that a degraded sample with a higher DNA concentration yielded better STR results than less concentrated degraded samples.

This research addresses two critical issues identified at the Forensic Science Technology Working Group's (TWG) 2014 and 2016 meetings. Specifically, the goals set were to increase the success rate of obtaining DNA profiles from compromised (damaged) DNA evidence, and create methodologies with processes that maximize DNA lysis and recovery at the elution and/or extraction steps for best downstream DNA analysis results (TWG 2016, 2018). Although some research has addressed the degradation of DNA in bones and other tissues in terrestrial contexts as explained in previous paragraphs (Iyavoo et al. 2013; Marshall et al.,2014; Mundorff et al. 2014; Vass et al. 2014), not much is known on bone DNA degradation in aquatic system. This is mainly due to the duration of fieldwork required and difficulty in obtaining permission to introduce hundreds of samples in a single aquatic location. Research on human samples is even more prohibitive, thus most reports of water decomposition are based on case-studies.

This research will impact work conducted in forensic DNA laboratories, in multiple ways. Using Real-Time qPCR and optimized primers to test which extraction method can be used to maximize DNA recovery, while removing inhibitors that could affect downstream amplification, ensures that the cleanest DNA will be available for testing. It will also inform forensic scientists which bone, ribs or scapulae, provides higher DNA quantity and quality over an extended period of decomposition time or accumulated degree days (ADD), saving time in determining if a bone is worth sampling. It can even improve the likelihood of retrieving an STR profile by determining a DI threshold.

Methods

Fresh rib and scapula bones from domestic pigs (*Sus scrofa*) were selected based on Haglund's (1993) sequence of aquatic disarticulation, which suggests that the torso, including these skeletal elements, remains intact longest throughout the aquatic decomposition process. These bones were submerged in both the James River and Henley Lake, with the water temperature and quality measured every ~250 accumulated degree days (ADD), using a 0 °C as a base temperature for ADD calculation. This study focused on a subsample of bones between the baseline and 4000 ADD (30 ribs and 29 scapulae) from the original study (Cartozzo et al, 2019). To note, there is no way to tell if these bones are from a singular source or from different sources.

DNA Extraction:

Genomic DNA was extracted by Cartozzo et al. (2019) using ChargeSwitch® gDNA Plant Kit (as described in CST Protocol for Extracting gDNA from Bone Samples (Invitrogen, 2009) and organic phenol-chloroform method (as described in Iyavoo et al., 2013). For both extraction methods, starting bone powder weight (0.1g) and final elution volume (100 μL) was same for all samples. To test for PCR inhibitors, variable region four (V4) of 16S rRNA gene was amplified from all DNA extracts using primers (V4_515F 5’

AATGATACGGCGACCACCGAGATCTACACXXXXXXXXTATGGTAATTGTGTGYCAGCMGCCGCGGTAA- 3') and (V4_806R 5'-CAAGCAGAAGACGGCATACGAGATXXXXXXXXAGTCAGTCAGCCGGACTACNVGG_GTWTCTAAT-3') and PCR protocol as described in Kozich et al. (2013). DNA extracts that failed to amplify the 16S rRNA gene were cleaned using Qiagen's DNeasy Power Clean Pro Clean Up Kit (Qiagen Inc. USA) following the manufacturer's protocol.

DNA Quantitation:

DNA was quantitated using both TaqMan (KLF9) and SYBR Green (SW240 and FH1733) methods on ABI 7500 Real-Time PCR Instrument. Data analysis was performed using 7500 System Sequence Detection System (SDS) software, V 1.4.

TaqMan Method

All extracted DNA from baseline to 4500 ADD (every 500 ADD) was quantified using half reaction volume (Total volume=10 uL), KLF9 (FAM) primers (62bp) (ThermoFisher Scientific Inc., USA), and by following Applied Biosystems TaqMan Universal Master Mix II protocol (ThermoFisher Scientific Inc., USA) with no UNG step. Standard curves were developed from 50 ng, 12.5 ng, 3.125 ng, 0.781 ng, 0.195 ng, 0.049 ng, 0.012 ng, and 0.003 ng of porcine genomic DNA (Novagen Inc. USA). DNA extracts (obtained using organic extraction method) from baseline (0 ADD) and 500 ADD samples were diluted for qPCR quantification. Many samples didn't amplify using TaqMan primers, and hence a new SYBR Green based quantitation method and primers were chosen.

SYBR Green Method

Subsets (baseline, 1000 ADD, 2000 ADD, 3000 ADD, 4000 ADD) of extracted DNA from both extraction methods (n=59; Lake=29 and River=30) were quantitated using SYBR Green based quantitative PCR (qPCR) method. Standard curves were developed from 50 ng, 12.5 ng, 3.125 ng, 0.781

ng, 0.195 ng, 0.049 ng, 0.012 ng, and 0.003 ng of porcine genomic DNA (Novagen Inc. USA) using qPCR protocol as described in Seashols-Williams et al. (2018) (except number of cycle was changed from 35 to 38) and using two pairs of pig specific primers SW240 (SW240F: 5'-AGA AAT TAG TGC CTC AAA TTG G-3', SW240R= 5'-AAA CCA TTA AGT CCC TAG CAA A-3'; target fragment size range=93-127bp) and FH1733 (FH1733F 5'-AAG CCT CAA ACT CCT CAT CTC A-3' and FH1733R 5'-ACC AAA GGC ATA CTA GGG CTA A-3'; target fragment size range = 274-314bp.)

Data Analysis

Because the goal of this project was to compare two DNA extraction methods for temporal variation in DNA quantity and quality in different bone types and at different locations, DNA concentration values from each sample were recorded using qPCR. The baseline samples were removed as they were outliers, deviating significantly from the remainder of the samples. The overall DNA concentration dataset contained 59 Bone samples organized into groups. There was one amplification per extraction method (118 samples), and each of those was done once per primer (236 samples). After the baseline samples were removed, a total of 192 samples remained for data analysis.

DNA yield was determined from each sample using both small fragment (SW240) and large fragment (FH1733) primer pairs. An analysis of variance (ANOVA) and general linear model (GLM) were run using SAS v 9.4 software, used to test for the presence of a significant difference in means between the methods, and the significance of that difference in a regression, respectively. Both raw quant values and log transformed quant values from each sample were used for the generation of individual regression models in RStudio V 1.2.1335 to compare DNA concentrations at each location (lake versus river) and bone type (rib versus scapula). Finally, a Tukey's Honest Significant Difference (HSD) was run using RStudio to specify which means were different.

Temporal changes in DNA quality for each extraction method was calculated using the degradation index (DI) approach. The Degradation Index dataset contained 78 samples. DI values that were undefined or zero could not be used in the ratio. For DI calculation, following equation was used.

$$DI = \frac{DNA\ yield\ from\ small\ fragment\ (SW240)\ primer\ pair}{DNA\ yield\ from\ large\ fragment\ (FH1733) primer\ pair}$$

An ANOVA and GLM were run on the DI data using SAS v 9.4 software. The graphs produced showed three outlier samples (HLS2C8CS; HLS3C15CSCU, HLS4C15CS), which were removed from the following analyses. Individual regression models measuring the degradation index over ADD were run using RStudio, comparing both extraction methods at each location and bone type. Both raw DI values and log transformed DI values from each sample was used for generation of individual regression models in RStudio to compare DNA concentrations at each location (lake versus river) and bone type (rib versus scapula). Finally, a Tukey's Honest Significant Difference (HSD) was run using RStudio to specify which means were different.

Results

Results are organized under three subheadings: Assay validation, DNA quantity, and DNA quality.

Assay validation

Four independent plates were run using SYBR Green based method for the quantification of DNA extracted from two extraction methods (i.e., ChargeSwitch and Organic) using two pairs of primers (i.e., SW240 (small fragment) and FH1733 (large fragment). Average amplification efficiency for small fragment primer pair (i.e., SW240) and large fragment primer pair (i.e., FH1733) were 93% (average slope -3.502) and 86% (average slope -3.715), respectively (Table 9). Average R^2 values were 0.993±0.004 across all four independent runs. Similarly, for the TaqMan method, average amplification

efficiency for KLF9 was 91% (average slope -3.559). Average R^2 values were .986± .013 across five independent runs.

DNA Quantity

DNA quantity calculated using both of the SYBR Green primer pairs differed significantly in the analysis of variance (ANOVA) test (p=0.0427, Table 1) but didn't differ significantly in generalized linear model (GLM) test (p=0.8366, Table 2). Both ANOVA and GLM test indicated that DNA quantity obtained using combined SYBR Green primers differed significantly between lake and river environments for both bone types (i.e., rib and scapula) (Tables 1 and 2). Both DNA extraction methods (i.e., ChargeSwitch and Organic) affected DNA quantity in certain interactions (Tables 1 and 2). Average DNA quantity obtained from rib samples using organic extraction method in a lake environment was significantly higher than all other samples (Table 3). DNA quantity obtained using organic method in a lake environment at 1000ADD was significantly higher than all other DNA extracts (Table 3).

The SW240 dataset compared the extraction methods with 48 samples from Henley's Lake and 48 from the James River. The R^2 value for the ANOVA was .557. The ANOVA showed significant difference between the bone types and locations (table 1), as well as different interactions between ADD, method, bone type and locations. The 1000ADD-Henley Lake-Organic interaction samples had a significantly higher mean DNA concentration than the other DNA extracts (Table 3). The FH1733 dataset compared the same dataset as SW240. The R^2 value for the ANOVA was 0.558. The ANOVA also showed significant difference between the bone types and locations, as well as the interaction between the two (table 1). The R^2 value for the GLM was 0.390, and indicated that the interaction between ADD*Method*Location has an effect on DNA concentration over time (table 2). In general, the SW240 dataset showed a decrease over time/ADD in all samples excluding the Henley Lake (ChargeSwitch) bone samples (Figure 1). The FH1733 dataset showed a decrease over time/ADD in all samples excluding the Henley Lake (organic) scapula samples (Figure 2).

The KLF9 TaqMan data compared the same 95 samples used in the SW240 and FH1733 datasets (missing one outlier). The R^2 value was .737. Fourteen of the 15 variables and combinations were significantly different (table 6). While the TaqMan probe shows a much higher amount of significance, due to costs and the inefficacy of the large primer with amplification, this method was abandoned and no further data analysis was run.

DNA Quality

The R^2 value of degradation index (DI) for combined ANOVA/GLM test was 0.764 (Table 7). Degradation Index (DI) values differ significantly between bone types (rib versus scapula) and ADD's (1000-4000) but not between DNA extraction methods (ChargeSwitch versus Organic) and aquatic environments (Lake versus River). In general, scapula had significantly high average DI than rib samples, and DI values increased with time/ADD in all samples except rib (organic) and scapula (ChargeSwitch) samples from the lake, where it decreased over time/ADD (Figure 3; Table 8).

Discussion

The original aim of this project was to measure host DNA quality and quantity in porcine waterlogged bones over time/ADD. This was to be accomplished by (1) attempting to optimize a qPCR protocol for host DNA quantification and degradation index estimation; (2) determining the better extraction method (between organic and solid-phase), the better of two bone types (rib and scapula) in two different freshwater environments with qPCR; and (3) identifying the variance of host DNA recovery from those environments. The original hypothesis was that the DNA concentration would decrease over ADD and that, in response, the degradation index (DI) would increase over ADD. From these results, it was expected that a preferred method and bone type could be identified, and the lake vs river environment impact on degradation determined.

The Williams et al. (2018) qPCR protocol with SYBR Green helped to provide stable assays with high R^2 values (>.98). The lower than average efficiency in the FH1733 runs may result from the larger fragment size of the primer (274-314bp) in comparison to the smaller SYBR Green SW240 and KLF9 TaqMan primer. The efficiency may also have been artificially increased by the amount of inhibitors in the sample; given that these were bone samples, the purity of the samples could have impacted the efficiencies. The targets used in this study were STR loci, meaning that instead of having one specific target there was a range of base pairs that were counted. This method was deemed acceptable because the ranges for both loci (SW240- 93-127bp; FH1733-274-314bp) did not overlap, allowing for differentiation of the two datasets.

As shown in the Tukey HSD tests, while a difference in DNA quantity existed between the bone types, the difference was not significant. There was no significant difference in DNA concentration or degradation found between either the extraction methods or the location. Thus, a bone or method or location apparently has no impact on DNA quantity and is not to be preferred over another on an individual variable basis. However, certain variable interactions were correlated to significant differences in quantity. The organic extraction method always produced a higher mean DNA concentration, and obtained the highest DNA quantity in the Henley Lake samples, specifically in the ribs. Overall, the DNA quality samples showed degradation over time, with the 4000 ADD samples having the highest index. As shown by a higher degradation index, scapular DNA degraded more than rib DNA. While the Henley Lake location showed the highest DNA quantity, it also showed the highest degradation index in the interaction between Bone*Location as well as the interaction among ADD*Bone*Method*Location. In sum, the scapula, using ChargeSwitch extraction, exhibited a higher degradation index.

While this experiment gave preliminary answers to the questions posed, the low R^2 values in the ANOVA (<.80) and GLM (<.40) analyses suggest that there are other as yet unidentified variables

affecting the data, and these need to be identified and explained for greater accuracy of prediction. One way to improve the accuracy would be to use the same bone in every ADD for a more consistent base measurement, rather than having bones from different individuals from each collection. While this doesn't have an effect on quantifying, this does specifically have an effect on creating a degradation index. This random assortment of individuals may have influenced the distribution and may have potentially created 'outliers'. A sample from the 4000 ADD data may have originally yielded a higher DNA concentration than some others; thus, consistency of sampling bones form the same individual over time would have provided better contextual information for interpretation for the results.

Maintaining the same sample size throughout the experiment might also improve the results as it might have eliminated missing data. A major issue in the DNA Quality Tukey's HSD testing, though partly compensated for in RStudio, was that many of the samples could not be used in the calculations due to having a value of "zero" or "undefined". This means that the only samples that could be compared were the ones that amplified, which certainly affected the ability to measure change over time with accuracy (e.g. with a dataset of 0-4000 ADD, if 1000 ADD and 3000 ADD had a measurable DI that showed increase but 2000 ADD did not have samples that could be used, it would show in the data that there was a decrease in that time period that might not be accurate). Finally, having a larger sample size (Marshall et al. 2014) might have allowed for more of the variation to be explained by the models.

Conclusion

While pitfalls in the experiment exist, there are still strong implications for its results. One irrefutable result is that DNA is still present and can be amplified if a bone is in water for at least 4000 ADD, meaning that there is still value in testing a waterlogged bone sample that's been submerged for a year under similar environmental conditions. There is also no evidence for a significant difference in methods; while the organic extraction method was seen to yield greater DNA quantity, the

ChargeSwitch method did not perform significantly worse, particularly as it removed inhibitors with much less effort than required for the organic samples. This is consistent with the conclusions of Iyavoo et al. (2013), who found that while the phenol-chloroform method did provide a higher DNA yield, the ability of solid-phase methods to remove inhibitors cannot be understated. Marshall et al. (2014) seconded this, finding that comparable quantities of DNA can be found in solid-phase methods versus organic. If there is no significant difference in DNA quantity in methodology, scientists can save time and labor by choosing the shorter solid-phase ChargeSwitch method versus the strenuous, currently utilized organic method, and ensure amplification with the qPCR method described.

The results of this study have the potential to impact the operational protocols for dealing with bone samples recovered from water. The option of the shorter extraction method allows for more casework involving waterlogged bone samples to be finished than before, with the added bonus of knowing that the samples will amplify during a certain time frame. These profiles will benefit from the cleaner extracted samples, resulting in profiles with less artifacts and cleaner peaks. This research offers a more efficient, optimized workflow from sample prep to profile development, which can be especially beneficial for forensic scientists in situations of emergency mass identification where time and labor is of the essence.

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Vita

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EDUCATION

Virginia Commonwealth University (VCU), Richmond, VA — May 2020
Master of Science in Forensic Science, FEPAC Accredited — GPA: 3.5/4.0
Concentration: Forensic Biology

Kent State University, Kent, OH — May 2018
Bachelor of Science in Biology, *cum laude* — GPA: 3.5/4.0
Concentration: Molecular & Cellular Biology
Minor: Forensic Anthropology

ACADEMIC EMPLOYMENT

Biology Teaching Assistant, **Department of Biology**, VCU, January 2019-present. Responsibilities include: teaching core biological concepts including cell structure, cellular metabolism, cell division, DNA replication, gene expression and genetics; Creating and teaching lectures over new topics weekly; Helping to design individual projects*;* Setting up the lab equipment and reagents.

PRESENTATIONS

Thornton, I., Mays, D.P.; Singh, B., Cartozzo, C., Simmons, T. Creating a Real Time Quantitative Polymerase Chain Reaction (qPCR)- Based Method for Studying Temporal DNA Degradation in Waterlogged Bone. 72nd AAFS Annual Scientific Meeting, Anaheim, CA, 20 February 2020.

Appendix

Table 1.

ANOVA of the combined SW240/FH1733, SW240, and FH1733 DNA Concentration data

	Source	Pr > F
Combined SW240/FH1733		
	Bone	***<.0001***
	Location	***0.0009***
	Bone*Location	***0.0016***
	ADD*Method*Bone*Location	***0.0076***
	ADD*Method*Location	***0.0083***
	ADD*Method	***0.0084***
	ADD*Method*Bone	***0.0125***
	ADD*Location	***0.0167***
	ADD*Bone*Location	***0.0194***
	ADD	***0.0328***
	Primer	***0.0427***
	*ADD*Bone*	*0.0554*
	*Primer*Bone*	*0.091*
	*Primer*Location*	*0.1876*
	*Primer*ADD*Method*	*0.2595*
	*Primer*Bone*Location*	*0.2599*
	*Primer*ADD*Method*Bone*Location*	*0.2886*
	*Primer*ADD*Location*	*0.3023*
	*Primer*ADD*Method*Location*	*0.3093*
	*Primer*ADD*Method*Bone*	*0.3257*
	*Primer*ADD*Bone*Location*	*0.3257*
	*Primer*ADD*	*0.3597*
	*Primer*ADD*Bone*	*0.4738*
	*Primer*Method*Bone*Location*	*0.6164*
	*Primer*Method*Location*	*0.6723*
	*Method*Bone*Location*	*0.6787*
	*Method*Location*	*0.7435*
	*Method*Bone*	*0.8667*
	Method	*0.8784*
	*Primer*Method*	*0.8952*
	*Primer*Method*Bone*	*0.9001*
SW240		
	Bone	***0.0023***
	Location	***0.0147***
	Bone*Location	***0.0242***

	ADD*Method	***0.0453***
	ADD*Method*Bone*Location	***0.0469***
	*ADD*Method*Location*	*0.0506*
	*ADD*Method*Bone*	*0.0637*
	*ADD*Location*	*0.0736*
	*ADD*Bone*Location*	*0.0837*
	ADD	*0.1108*
	*ADD*Bone*	*0.1711*
	*Method*Bone*Location*	*0.6295*
	*Method*Location*	*0.692*
	*Method*Bone*	*0.9833*
	Method	*0.9911*
FH1733		
	Bone	***0.0002***
	Location	***0.0023***
	Bone*Location	***0.0023***
	*ADD*Bone*Location*	*0.1149*
	*ADD*Location*	*0.1201*
	*ADD*Method*Location*	*0.1294*
	*ADD*Method*Bone*Location*	*0.1313*
	*ADD*Method*Bone*	*0.1573*
	*ADD*Method*	*0.1626*
	ADD	*0.1773*
	*ADD*Bone*	*0.1813*
	*Method*Bone*	*0.6583*
	Method	*0.6651*
	*Method*Location*	*0.8841*
	*Method*Bone*Location*	*0.8931*

Table 2.

General Linear Model for the combined SW240/FH1733, SW240, and FH1733 DNA Concentration data with significant values at α<.05

	Source	***Pr > |t|***
Combined SW240/FH1733		
	Method*Location	***0.0005***
	ADD*Method*Location	***0.0005***
	Method*Bone*Location	***0.0122***
	ADD*Method*Bone*Location	***0.0133***
	*Primer*Method*Location*	*0.0557*
	*Primer*ADD*Method*Location*	*0.0693*
	*Primer*Method*Bone*Location*	*0.1727*
	*Primer*ADD*Method*Bone*Location*	*0.1994*
	Location	*0.399*
	*Bone*Location*	*0.5774*
	Bone	*0.7378*
	Primer	*0.8366*
	*ADD*Method*	*0.8402*
	*Primer*Location*	*0.845*
	*ADD*Location*	*0.857*
	*Primer*ADD*Method*	*0.891*
	*Primer*Bone*	*0.892*
	*Method*Bone*	*0.9077*
	*ADD*Bone*Location*	*0.9091*
	*Primer*Bone*Location*	*0.9116*
	*Primer*ADD*Location*	*0.913*
	*ADD*Method*Bone*	*0.9409*
	*Primer*ADD*Bone*Location*	*0.946*
	*Primer*ADD*	*0.9483*
	*Primer*Method*Bone*	*0.9544*
	Method	*0.9596*
	*Primer*ADD*Method*Bone*	*0.9606*
	*Primer*ADD*Bone*	*0.9608*
	*ADD*Bone*	*0.9851*
	ADD	*0.9858*
	*Primer*Method*	*0.9974*
SW240		
	Method*Location	***0.0093***
	ADD*Method*Location	***0.0095***
	*Method*Bone*Location*	*0.0618*

	*ADD*Method*Bone*Location*	*0.065*
	Location	*0.529*
	*Bone*Location*	*0.6775*
	Bone	*0.8027*
	*ADD*Method*	*0.8803*
	*ADD*Location*	*0.893*
	*Method*Bone*	*0.9311*
	*ADD*Bone*Location*	*0.9321*
	*ADD*Method*Bone*	*0.9559*
	Method	*0.9699*
	*ADD*Bone*	*0.9889*
	ADD	*0.9894*
FH1733		
	ADD*Method*Location	***0.0402***
	*Method*Location*	*0.0715*
	*ADD*Method*Bone*Location*	*0.1425*
	*Method*Bone*Location*	*0.1974*
	Location	*0.2193*
	*Bone*Location*	*0.3853*
	Bone	*0.7564*
	ADD	*0.8725*
	*ADD*Bone*	*0.912*
	Method	*0.9203*
	*Method*Bone*	*0.9393*
	*ADD*Location*	*0.9555*
	*ADD*Bone*Location*	*0.9683*
	*ADD*Method*	*0.9864*
	*ADD*Method*Bone*	*0.9926*

Table 3.

Tukey's HSD results of the DNA Quantity interactions, with significance at α > .05

		Variable/Interaction	*# of combos*	*Highest Mean*	*p adj*
Combined SW240/FH1733					
		Method*Bone*Location	***8***	*Organic-Rib-Henley Lake*	*0.0183932*
		ADD*Method*Location	***16***	*1000ADD-Organic- Henley Lake*	*0.0018776*
		ADD*Bone*Method*Location	***32***	*1000ADD-Rib-Organic-Henley Lake*	*<.0000001*
SW240					
		ADD*Method*Location	***16***	*1000ADD-Organic-Henley Lake*	*0.0333878*

Table 4.

*General Linear Model of the SW240 DNA Concentration Data Method*Location interactions between Bone and Accumulated Degree Days (ADD)*

	Parameter	Estimate	Standard Error	t Value	Pr > |t|
SW240, ChargeSwitch, James River					
	Intercept	0.765821667	0.51339502	1.49	0.1514
	ADD	1.00207E-05	0.00018747	0.05	0.9579
	Bone	-0.72949	0.7260502	-1	0.327
	ADD*Bone	-1.481E-05	0.00026512	-0.06	0.956
SW240, Organic, James River					
	Intercept	0.6555	0.23317152	2.81	0.0108
	ADD	-0.00015047	0.00008514	-1.77	0.0924
	Bone	-0.3724	0.32975432	-1.13	0.2721
	ADD*Bone	6.85933E-05	0.00012041	0.57	0.5752
SW240, ChargeSwitch, Henley Lake					
	Intercept	2.605678333	1.34188509	1.94	0.0664
	ADD	0.000153406	0.00048999	0.31	0.7575
	Bone	-2.44721833	1.89771209	-1.29	0.2119
	ADD*Bone	-0.00014326	0.00069295	-0.21	0.8383
SW240, Organic, Henley Lake					
	Intercept	13.46248333	3.84907102	3.5	0.0023
	ADD	-0.00399915	0.00140548	-2.85	0.01
	Bone	-13.1158	5.44340843	-2.41	0.0257
	ADD*Bone	0.00391676	0.00198765	1.97	0.0628

Table 5.

*General Linear Model of the FH1733 DNA Concentration Data Method*Location interactions between Bone and ADD*

	Parameter	Estimate	Standard Error	t Value	Pr > |t|
FH1733, ChargeSwitch, James River					
	Intercept	0.316326667	0.14267616	2.22	0.0384
	ADD	-4.15313E-05	0.0000521	-0.8	0.4347
	Bone	-0.311143333	0.20177457	-1.54	0.1387
	ADD*Bone	4.04507E-05	0.00007368	0.55	0.5891
FH1733, Organic, James River					
	Intercept	0.21595	0.09004416	2.4	0.0263
	ADD	-0.00004779	0.00003288	-1.45	0.1616
	Bone	-0.203136667	0.12734167	-1.6	0.1263
	ADD*Bone	4.52757E-05	0.0000465	0.97	0.3418
FH1733, ChargeSwitch, Henley Lake					
	Intercept	1.553768333	0.77263263	2.01	0.058
	ADD	-0.000021101	0.00028213	-0.07	0.9411
	Bone	-1.545046667	1.09266755	-1.41	0.1727
	ADD*Bone	0.000019863	0.00039899	0.05	0.9608
FH1733, Organic, Henley Lake					
	Intercept	4.034683333	1.17142933	3.44	0.0026
	ADD	-0.001103545	0.00042775	-2.58	0.0179
	Bone	-4.034973333	1.65665125	-2.44	0.0243
	ADD*Bone	0.001105747	0.00060492	1.83	0.0825

Table 6.

A GLM of the KLF9 TaqMan probe-DNA Concentration data

Source	***Pr > |t|***
Bone	***<.0001***
Method*Bone	***<.0001***
Method	***<.0001***
ADD*Location	***0.0032***
ADD	***0.0041***
Method*ADD*Bone*Location	***0.0076***
Method*Bone*Location	***0.0098***
Method*ADD*Location	***0.0112***
Location	***0.0135***
Method*ADD*Bone	***0.0169***
Method*Location	***0.0204***
Method*ADD	***0.0216***
ADD*Bone*Location	***0.0221***
ADD*Bone	***0.0275***
*Bone*Location*	*0.178*

Table 7.

An ANOVA of the Degradation Index data measuring DNA Quality.

Source	***Pr>F***
Bone	***<0.0001***
Bone*Location	***<0.0001***
ADD*Bone	***0.0007***
ADD*Method*Bone*Location	***0.0026***
ADD	***0.0051***
*Method*Bone*Location*	*0.065*
*ADD*Method*	*0.0979*
*ADD*Method*Bone*	*0.1765*
*ADD*Location*	*0.1922*
*ADD*Bone*Location*	*0.5617*
*ADD*Method*Location*	*0.7361*
Location	*0.8162*
*Method*Bone*	*0.8451*
Method	*0.9495*
*Method*Location*	*0.9673*

Table 8.

Tukey's HSD results of the variables and interaction from the DNA Quality data; significance at α < .05

Variable/Interaction	***# of combos***	***Highest Mean***	***p adj***
Bone	***2***	*Scapula*	*0.016516*
ADD	***5***	*4000ADD*	*0.0420746*
Bone*Location	***4***	*Scapula-Henley Lake*	*0.0093717*
ADD*Bone	***10***	*4000ADD-Scapula*	*0.011617*
ADD*Bone*Method*Location	***40***	*4000ADD*Scapula*ChargeSwitch-Henley Lake*	*0.0373003*

Table 9.

Standard curves of each of the qPCR plates from each quantitation method.

	Plate	R^2	Slope	Intercept
SYBR Green				
	SW240-CS	*0.989*	*-3.649*	*26.326*
	SW240-O	*0.992*	*-3.355*	*30.867*
	FH1733-O	*0.993*	*-3.774*	*27.162*
	FH1733-CS	*0.998*	*-3.656*	*27.349*
TaqMan				
	KLF9-5	*0.964*	*-3.936*	*26.472*
	KLF9-2	*0.988*	*-3.357*	*26.459*
	KLF9-4	*0.991*	*-3.532*	*26.681*
	KLF9-1	*0.993*	*-3.417*	*28.303*
	KLF9-3	*0.997*	*-3.553*	*26.314*

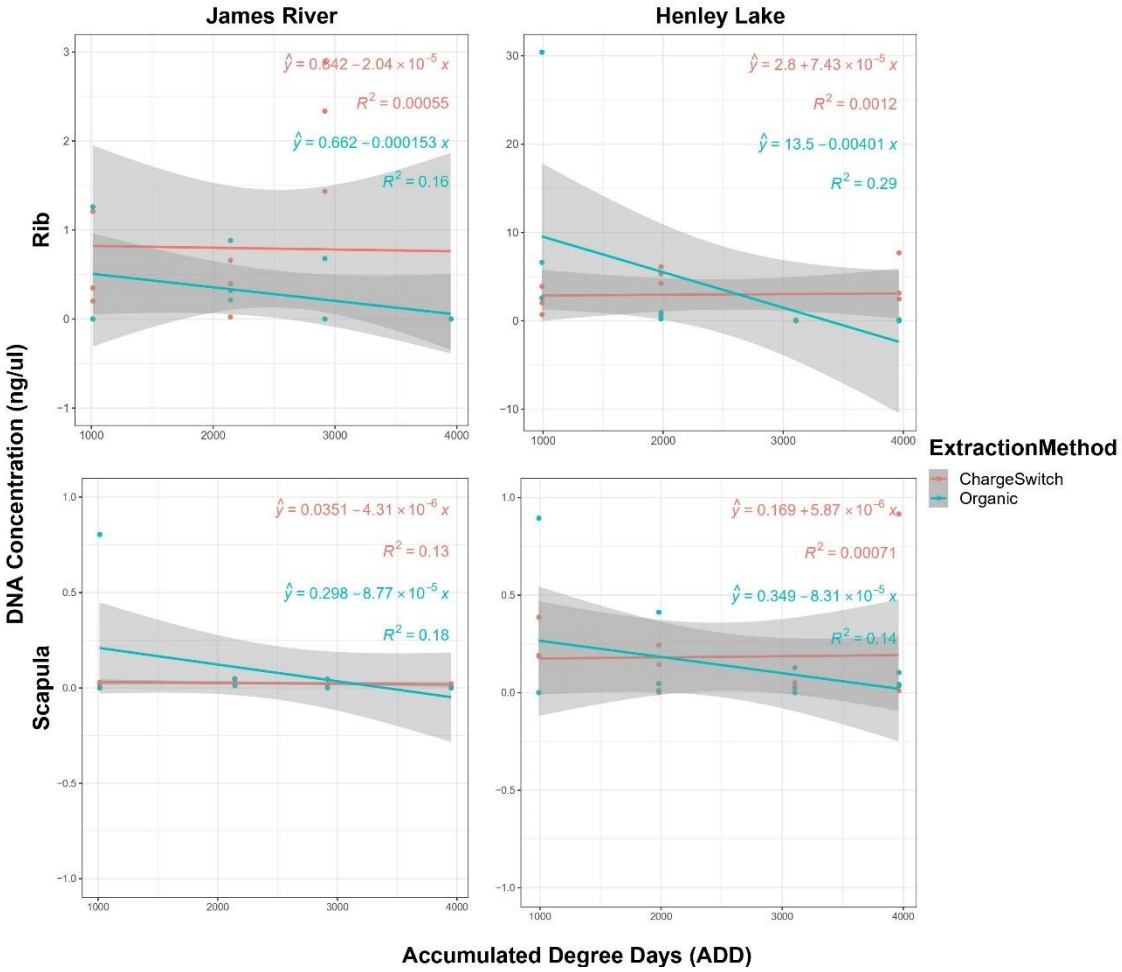

Figure 1.

Temporal changes in DNA quantity data of rib (top panel) and scapula (bottom panel) samples from river (left panel) and lake (right panel) environments. DNA quantitation was performed using small fragment primer pair (SW240). Scales are not the same for each figure.

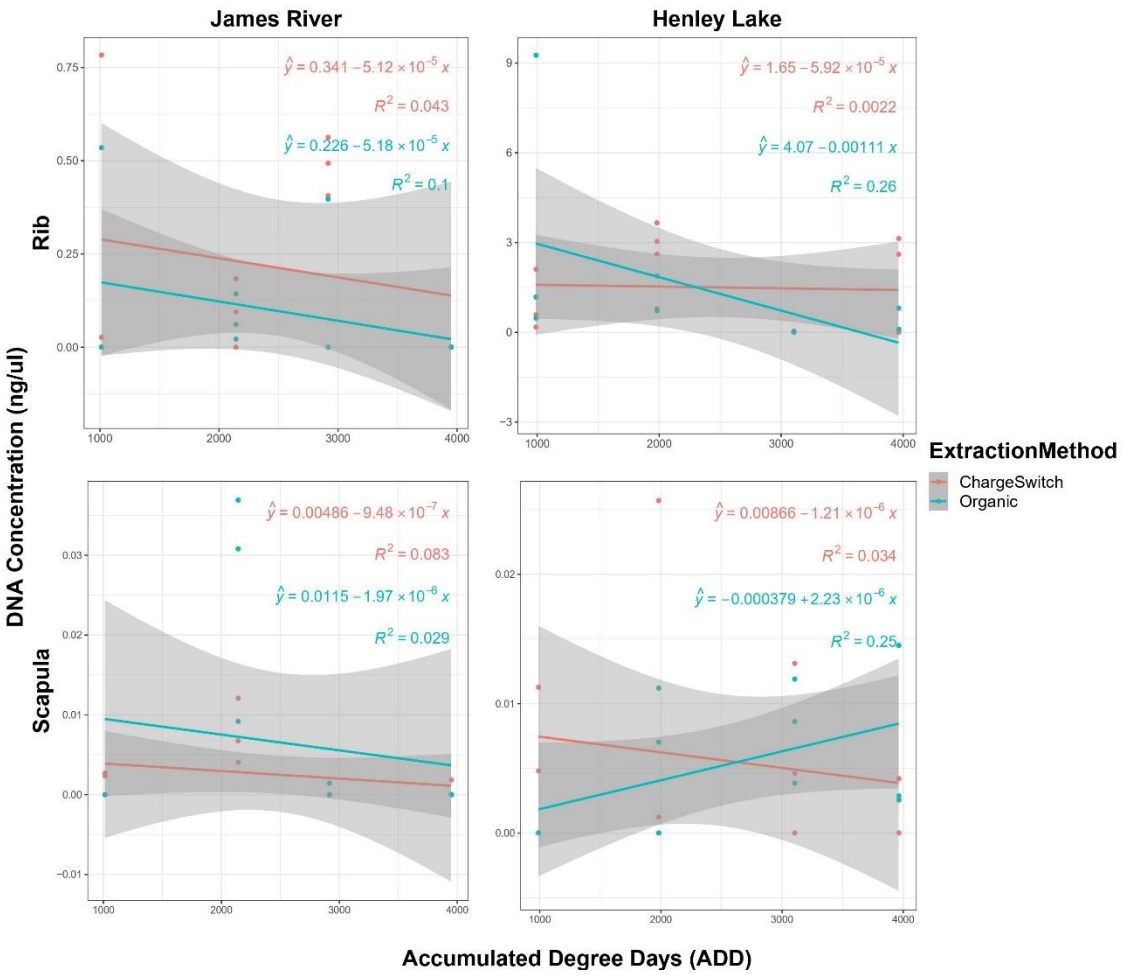

Figure 2.

Temporal changes in DNA quantity data of rib (top panel) and scapula (bottom panel) samples from river (left panel) and lake (right panel) environments. DNA quantitation was performed using large fragment primer pair (FH1733). Scales are not the same for each figure.

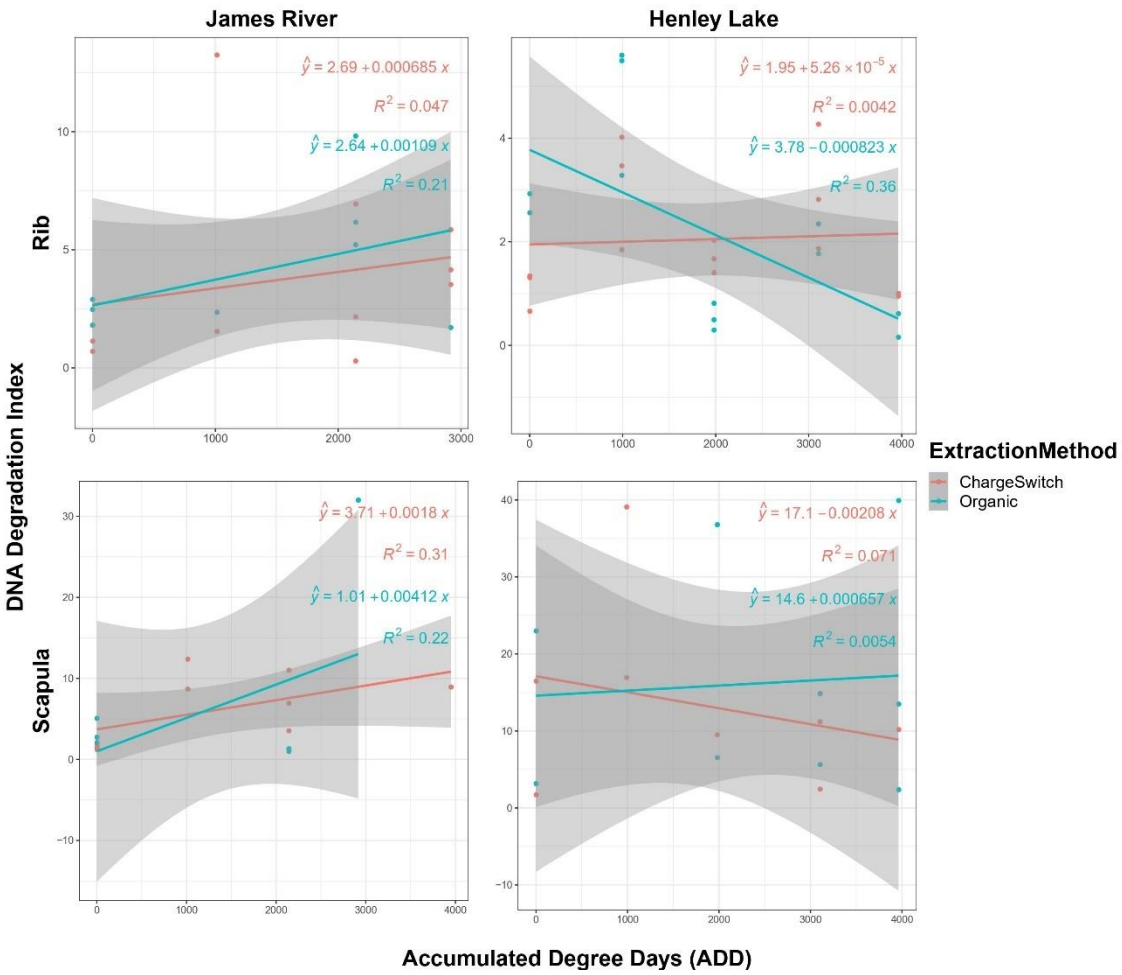

Figure 3.

Temporal changes in DNA quality data of rib (top panel) and scapula (bottom panel) samples from river (left panel) and lake (right panel) environments. Degradation Index was calculated using small fragment primer pair (SW240) and large fragment primer pair (FH1733). Scales are not the same for each figure.