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PROOF-OF-CONCEPT OF ENVIRONMENTAL DNA TOOLS FOR ATLANTIC STURGEON MANAGEMENT

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This is to certify that the thesis prepared by Jameson E. Hinkle entitled “Proof-of-Concept of Environmental DNA tools for Atlantic Sturgeon Management” has been approved by his committee as satisfactory completion of the thesis requirement for the degree of Master of Science in Environmental Studies (M.S. ENVS)

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PROOF-OF-CONCEPT OF ENVIRONMENTAL DNA TOOLS FOR ATLANTIC STURGEON MANAGEMENT

A Thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Environmental Studies at Virginia Commonwealth University.

by

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Proof-of-Concept of Environmental DNA Tools for Atlantic Sturgeon Management

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Abstract

The Atlantic Sturgeon (*Acipenser oxyrinchus oxyrinchus, Mitchell*) is an anadromous species that spawns in tidal freshwater rivers from Canada to Florida. Overfishing, river sedimentation and alteration of the river bottom have decreased Atlantic Sturgeon populations, and NOAA lists the species as endangered. Ecologists sometimes find it difficult to locate individuals of a species that is rare, endangered or invasive. The need for methods less invasive that can create more resolution of cryptic species presence is necessary. Environmental DNA (eDNA) is a non-invasive means of detecting rare, endangered, or invasive species by isolating nuclear or mitochondrial DNA (mtDNA) from the water column. We evaluated the potential of eDNA to document the presence of Atlantic Sturgeon in the James River, Virginia. Genetic primers targeted the mitochondrial Cytochrome Oxydase I gene, and a restriction enzyme assay (*DraIII*) was developed. Positive control mesocosm and James River samples revealed a nonspecific sequence—mostly bacteria commonly seen in environmental waters. Methods more stringent to a single species was necessary. Novel qPCR primers were derived from a second region of *Cytochrome Oxydase II*, and subject to quantitative PCR. This technique correctly identified Atlantic Sturgeon DNA and differentiated among other fish taxa commonly occurring in the lower James River, Virginia. Quantitative PCR had a biomass detection limit of 32.3 ug/L and subsequent analysis of catchment of Atlantic Sturgeon from the Lower James River, Virginia from the fall of 2013 provided estimates of 264.2 ug/L Atlantic Sturgeon biomass. Quantitative PCR sensitivity analysis and incorporation of studies of the hydrology of the James River should be done to further define habitat utilization by local Atlantic Sturgeon populations.

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Introduction

The Atlantic Sturgeon (*Acipenser oxyrinchus oxyrinchus* Mitchell, Acipenseridae) is an anadromous fish that utilizes the major rivers of Eastern North America, from Canada to Florida for spawning (Bemis *et al.*, 1997). Due in large part to overfishing and habitat alteration resulting from canals constructed through the oxbows of the James River, Virginia, populations have declined (Department of Commerce, National Marine Fisheries Service, National Oceanic and Atmospheric Administration., 2012). Recent estimates suggest a return of Atlantic Sturgeon in the James River, Virginia, and there are likely multiple annual patterns of migration for spawning (Balazik *et al.*, 2012). Based on recent genetic analyses of Atlantic Sturgeon (King et al 2001), NOAA recognizes five distinct population segments, including including South Atlantic, Carolina, Gulf of Maine, New York Bight, and Chesapeake Bay. Successful management of these population units and maintenance of the species relies upon a more thorough understanding of the mechanisms that both influence population abundance and define suitable spawning habitat (Department of Commerce, National Marine Fisheries Service, National Oceanic and Atmospheric Administration., 2012).

Atlantic Sturgeon are an ancient group of anadromous fishes, the Acipenseriformes (Atlantic Sturgeon and Paddlefishes); their lineage dates back approximately 200 million years (Robles *et al.*, 2004). Individuals can grow up to 4.3 meters in length, with females attaining weights of up to 363 kilograms (Department of Commerce, National Marine Fisheries Service, National Oceanic and Atmospheric Administration., 2012). The species is largely bottom feeding whose diet consists of mollusks, crustaceans, and aquatic insects (Anderson & William, 2013). Atlantic Sturgeon growth, rate of maturity, and spawning timing vary based on seasonal

temperature variation latitudinally (Bemis and Kynard, 1997). During annual spawning eggs are deposited on rocky surfaces (Billard and Lecointre, 2001). Although Atlantic Sturgeon spawn annually, not all individuals return each year. Females return less than once a year, while male return rates range from one to six years (Bemis and Kynard, 1997). They must spawn in fresh waters as juveniles mature in these areas because of necessary food sources in estuaries (Bemis and Kynard, 1997).

Anthropogenic exploitation of Atlantic Sturgeon began as early as the Jamestown settlement, as evidence of eaten carcasses have been revealed through archaeological digs (Balazik *et al., 2010*). Present populations of Atlantic Sturgeon have been exposed to two contemporary forces against their persistence in the James River and other drainages in Eastern North America. First, the most recent decline in the Chesapeake Bay Distinct Population Segment is due to overharvesting from the fishery present from the ‘50s into the ‘90s (Department of Commerce, National Marine Fisheries Service, National Oceanic and Atmospheric Administration, 2012). Historically, a large fishery for Atlantic Sturgeon existed in the tidal James River until the mid to late 20th century for both the fish (Department of Commerce, National Marine Fisheries Service, National Oceanic and Atmospheric Administration, 2012). Secondly, habitat degradation and compartmentalization has imposed significant reductions on potential spawning habitat. In the latter half of the 20th century there was increased sediment loads in the James River which negatively impacts reproduction by reducing the ability of eggs to stick to subsurface structure. Until recently, little was known about the nature and location of spawning surfaces. Side-scanning sonar (Austin 2012) has provided more detailed information about the location and quantity of potential spawning habitat. Larger-scale historical anthropogenic riverbed-altering activities including canals cut through the oxbows of the James River downstream of Richmond have also greatly increased

sedimentation through previously suitable spawning habitat (Austin, 2012). The reduction in population size has led the National Oceanographic and Atmospheric Administration (NOAA) to list *A. oxyrinchus* as an endangered species (Department of Commerce, National Marine Fisheries Service, National Oceanic and Atmospheric Administration, 2012).

Subsequent management and conservation has largely focused on monitoring the population's behavior and recovery. Active monitoring of the James river population has continued through the use of acoustic tags (both stationary sites and real-time) and side-scanning sonar. Sampling via gill nets has continued to increase resolution of population size and return rate. Preliminary data from gill netting and tagging have estimated a population of > 3300 adult males in the James River (Balazik, unpublished). Moreover, results indicated both an increase in individuals as well as multiple patterns of migration between Fall and Spring spawning (Balazik *et al.*, 2012).

In addition to physical surveys, recent advances in molecular laboratory approaches may provide supplemental tools to monitor species such as the Atlantic Sturgeon. One method that is gaining popularity and utility is the use of environmental water samples to amplify species-specific DNA fragments. Environmental DNA (eDNA) is a non-invasive means of detecting rare, endangered, or invasive species by isolating discrete segments of nuclear or mitochondrial DNA (mtDNA) from the water column (Lodge *et al.*, 2012). Large volumes of water are collected and filtered to be used for the identification of species-specific target DNA sequences left by resident organism(s). The most common genetic markers used in this method target species-stable genetic sequences in the mitochondrial genome (Deiner *et al.,* 2014). One of the first examples of this application comes from indicating presence of American Bullfrogs (*Lithobates catesbeianus*) by Ficetola *et al.* (2008). Ficetola found that by

sampling for template DNA left behind by the invasive bullfrog, they were able to detect American Bullfrogs in controlled wetland environments. Environmental DNA can offer biologically relevant data of cryptic or endangered species, as well as invasive species without the risk of harm to that species. Goldberg *et al.* (2011) demonstrated the capability of these methods to detect known concentrations of cryptic species of salamanders and frogs in Idaho in Western North America. While Hickcox (2011) and Minamoto (2011) used eDNA methodologies to demonstrate its use with invasive species, indicating presence of carp species (*Hypophthalmichthys molitrix)* indicating presence of known concentrations and/or populations of those carp species.

One limitation of eDNA is that identification of the presence of a taxon, does not pinpoint the exact location of individuals, particularly for large and/or moving bodies of water. For example, Thomsen *et al.* (2012) indicated presence of 15 different taxa of fish from the Sound of Elsinore, Denmark, although it was difficult to pinpoint actual locations of the target organisms since the water is so well mixed in such a large dynamic volume. While the specific locations of individuals providing DNA targets in the water column remains cryptic, it is possible for eDNA techniques to estimate standing biomass. Takahara *et al.* (2011) found quantitative PCR (qPCR) with Common Carp (*Cyprinus carpio*) and predicted biomass of Common Carp well in controlled systems (regression based $R^2 = 0.96$, $p < 0.001$).

The objective of this research is to develop and test the effectiveness of using eDNA for monitoring Atlantic Sturgeon using both a captive laboratory population as well as sampling from native populations in the James River of Virginia. A set of genetic markers was developed to differentiate samples derived from Atlantic Sturgeon from other co-occurring fish taxa. Tests were designed to take eDNA methods from positive controls with tissue samples, up to

environmental water samples at known sites with Atlantic Sturgeon presence. Minimally we would hypothesize that the methods herein will indicate presence through traditional PCR and restriction enzyme digests, while the use of qPCR could allow relative abundance estimates through a biomass model as in Minamoto (2012). Finally, I discuss and provide recommendations for subsequent use of eDNA techniques for monitoring Atlantic Sturgeon and other taxa residing in large river systems.

Methods

Sampling James River Atlantic Sturgeon: Environmental samples were collected from the James River, Virginia, downstream of Richmond, a known area of spawning (Balazik *et al.*, 2012). Previous studies (Pilliod, Arkle, & Laramie, 2012) suggested that a volume of at least 1 Liter is necessary to sample enough DNA from the environment to detect target DNA sequences. Nalgene bottle(s) were sanitized with a 10% bleach solution and placed in a sterile plastic bag. After a single rinse two water samples were obtained from the surface and bottom of the water column. It was thought that these two samples would help to integrate the water column and include the area of the water column that Atlantic Sturgeon spend the majority of their time (Pilliod, Arkle, & Laramie, 2012; Balazik *et al.*, 2012). Samples were stored on ice until arrival at the lab. Samples were then filtered through 47 mm nitrocellulose (0.45 μM pore size) filters, and total DNA was extracted from half the filter, saving half for later if needed (Pilliod, Arkle, & Laramie, 2012). Field samples were collected from sites upstream and downstream of Balazik's sample sites for gill netting and radio telemetry for the spawning season. This spans from just upstream of the I - 295 bridge to VCU's Rice Center near Charles City, VA (Figure 1, Table 1). These sites were selected because they are in the known reach of an Atlantic Sturgeon "staging area" for adults before spawning events (Balazik *et al.*, 2012). Monthly samples were collected between June and August of 2013 to serve as temporal detection of the initiation of the spawning season, after which sampling was performed weekly until November. Seventy-nine samples were obtained through the study area before, during and after the spawning season for 2013. Positive control sites were from areas of active sampling and catchment of Atlantic Sturgeon individuals (Table 2). At these sites a series of water samples (n = 24) were obtained following Pilliod *et al.'*s methods. After confirmation of tagged Atlantic Sturgeon via radio telemetry or sonar gill nets were set for traditional sampling. Two

eDNA samples were also obtained from the gill netting site and were obtained a half mile above and below the gill netting site. A similarly collected set of samples were collected from a site just above the fall line in Richmond, VA (n = 2, Hugenot Bridge, 37.560550, -77.545616) and used as a negative control site since Atlantic Sturgeon are not known to migrate above the fall line for spawning.

Parameterizing eDNA detectability. Total molecular DNA was extracted from all samples using Qiagen's Blood and Tissue Kit (Qiagen Inc., Natick Massachusetts). Samples were incubated with proteinase-K at 56°C overnight. Environmental DNA is typically associated with sediments/cells suspended in the water column so, Qiagen's Qiashredder kits were used with silica balls at the beginning of extraction to remove sediments/DNA from filter. A set of genetic markers were derived from published sequences (NCBI EU524401, EU524400, EU524399, EU524398, EU523886). A stretch of the mitochondrial gene, *Cytochrome Oxidase 1* (*CO1*), was used as the initial basis of taxonomic identification. This region is commonly used in species-level genetic surveys across broad ranges of taxa and is sometimes referred to as genetic barcodes (Deiner *et al.,* 2014). Primers were derived from Ivanova (2007, FishF2t1/FishR2t1, Table 3). These primers amplify a fragment approximately 700 base pairs in length. Conditions for PCR with FishF2t1/FishR2t1 include 8.26 µL ddH_2O, 10 X PCR buffer, 50 mM $MgCl_2$, 10 µM of each primer, 10 mM dNTPs, 0.06 µL platinum Taq polymerase (5 U/µl). There was 2 µL of eDNA template per well, totalling 12.5 µL reactions. Thermocycler regimes included 1 cycle of 98°C for 1 minute, followed by 5 cycles of 94°C for 1 minute, 50°C for 40 seconds, and 72°C for 1 minute. Then there was 39 cycles of 94°C for 30 seconds, 54°C for 40 seconds, and 72°C for 1 minute. Finally, there was one cycle at 72°C for 10 minutes before a hold at 4°C until the samples were placed in either 4°C for immediate use, or -20°C for samples to be analyzed after 24 hours. These products were both cleaned up for sequencing either

directly through Qiagen's PCR cleanup kit, or samples were re-amplified and gel extracted and purified for sequencing via gel extraction clean up kits.

For higher-throughput of the samples, a restriction assay was developed to differentiate Atlantic Sturgeon from related species in the James River from which *CO1* sequences may be co-amplified. A unique cut-site not present in 16 other commonly present fishes but found in Atlantic Sturgeon was identified using the enzyme *DraIII* (Figure 2). Verification of the specificity of the *DraIII* assay was performed through the amplification and subsequent restriction of fin clip controls obtained from captive populations (Figure 3). Sequencing was completed at VCU's Nucleic Acids Research Facility, and alignments were computed through the use of Sequencher (v3.1.1). A simple BLAST (NCBI) was used to confirm the restriction enzyme assay results.

To identify the limits of detectability, aquarium samples were used as positive controls to determine the effectiveness of the markers. Water samples were taken from aquarium in 1 (n = 6) and 2 (n = 24) liter sample sizes.

Errors in identification via genotyping can happen in two ways: false positives and false negatives. To identify non-specific amplicons, fragments were gel extracted and subjected to Sanger sequencing and subsequent BLAST comparison with published nucleotide sequences. False negatives can arise when no fragments are amplified even though the target organism is present. First, given the volume of water in the James river relative to the biomass of the target species, potential hits may be rare. This error is more difficult to overcome and can be minimized by both taking lots of replicate samples and identifying the limits of detection. Second, it is unclear how water chemistry or the rich microflora/fauna of the James River would be limiting for amplification of target sequences. Many environmental factors such as temperature, sunlight, and enzyme activity can degrade DNA in water. To test the rate at which

DNA is degraded, a set of environmental water samples (n = 6) were spiked with Atlantic Sturgeon milt and were processed to test if the water chemistry or other environmental factors mentioned above could be a limiting factor for detection. The degradation time of DNA in the water column is currently thought to be 14-21 days (Dejean *et al.*, 2011).

eDNA biomass predictive curve: Two additional genomic regions Cytochrome Oxidase II (*CoxII*) and NADH Dehydrogenase I (*ND1*) were used. While not as common as *CO1*, both of these regions have also been used in other eDNA studies (Deiner *et al.*, 2014). Primers were generated from *CoxII* (NCBI accession: AF125657) and ND1 (NCBI accession: 73427201) using Primer3/Primer BLAST. The goal was to be able to differentiate between controls either by a lack of amplification, late amplification, or different melt curves. This baseline curve served as the development for a biomass curve based off linear regression for future studies. All PCR based tests contained negative (Gar fin clip, and water) and positive controls (Atlantic Sturgeon fin clip). All qPCR products were also sequenced to help confirm specificity of primers to the target amplicon. Detectability was determined by a 1:10 serial dilution of previously determined positive results (above) that were amplified using quantitative PCR (qPCR).

Conditions for qPCR included 1 µL of 5 ng/µL eDNA (or finclip) template, 10 µL of Biorad SsoAdvanced Universal Sybr Green Supermix (includes dNTPs, Sso7d fusion Polymerase, SYBR Green I, and Rox normalization dyes), 2 µL of primers (qAoxCoxIIF/R; Table 3) at 10 ng/µL, and 5 µL ddH_2O. Thermocycler regimes included 1 cycle at 98°C for 3 minutes, then 39 cycles of 98°C for 10 seconds, and 30 seconds of 61.2°C followed by a plate read (60°C) per cycle. Followed by one cycle of extension at 65°C for 30 seconds melt curve analysis included 60 cycles at 0.5 degree steps at 5 seconds per step with a plate read at every cycle, ending with a 4°C hold where necessary (if sequencing product).

Calculating Atlantic Sturgeon Biomass in James River, VA: In order to predict whether eDNA methodologies will be capable of accurately detecting Atlantic Sturgeon biomass in the James River, VA, an estimate of the biomass present during the period of study is necessary. Biomass data was gathered by Balazik (Unpublished, 2015) based on acoustic telemetry tags and catchment data from the fall of 2013 on dates of sampling for eDNA (Appendix A, Table 1). Known masses of individuals were summed for total biomass present on a given sample day. The mean of these masses per sample day were computed and then converted to grams for comparative scale with the biomass computed in *eDNA Biomass Predictive Curve*. The volume of water in the study area was retrieved from VCU's Dr. Paul Bukaveckas (P. Bukaveckas, Personal communication, April 23, 2015) based on Chesapeake Bay Program's segmentation scheme. Mean biomass values were then summed and divided by the volume to get the biomass in grams per liter of Atlantic Sturgeon in the study area. These biomass values are an underestimate of the mass present however, as not all Atlantic Sturgeon present are tagged. This value was converted to micrograms per liter to compare to values computed in *eDNA Biomass Predictive Curve*. All computations were completed with R (version 3.2.0) and RStudio (version 0.98.932; Appendix C).

Results

Sampling James River Atlantic Sturgeon: Of the 79 samples from the year of sampling in the stream reach (Figure 1, Table 1), there were 2 (2.5%) positive results identified through the restriction digest. Though neither were confirmed through subsequent Sanger sequencing. There were no positive results from the positive control test sites from sampling with Balazik.

Parameterizing eDNA detectability: Initial results of *DraIII* digested fin clips indicated enzyme specificity to only Atlantic Sturgeon when compared with 16 other negative control taxa present (Figure 3). Detectability of Atlantic Sturgeon DNA in tank water was influenced by the collection volume. No DNA product was identified in any of the 1L samples but 2L samples indicated a positive detection rate of 91% (n = 24, Figure 4). Samples spiked with milt yielded 83% positive results at a volume of two liters (n = 6, Figure 4). Remaining sampling in the project used 2L sampling volumes.

While the positive identification of Atlantic Sturgeon DNA from positive controls (fin clips) were supported by Sanger sequencing, sequencing and BLAST results of environmental samples collected from tanks indicated that the positively identified fragments belonged mostly to bacteria commonly present in water. These results suggest that while a restriction-digest approach may be appropriate for differentiating Atlantic Sturgeon from other co-occurring fish taxa, this approach is not stringent enough to differentiate among *CO1* sequences among all potential donor taxa. All tank experiments were paired with positive (Atlantic Sturgeon fin clip) and negative controls (Gar fin clip, and water), and they correctly indicate the species present in BLAST results. Given the lack of specificity in the restriction-based assay, subsequent analyses

were performed using quantitative PCR approaches.

eDNA biomass predictive curve: CoxII indicated a lower E-value in BLAST and was more consistent in trials with fin clips than *ND1* (Appendix A, Figure 1). Quantitative PCR of an Atlantic Sturgeon fin clip positive control 1:10 serially diluted (First dilution = 10 ng/µL template) as a standard curve performed as expected for qPCR analysis (Figure 5, $r^2 = 0.9724$). Tank samples of a known biomass (3.23 g/L, Table 4) were estimated within the range of this qPCR fit (Figure 5). This indicates a minimum limit of detection of 32.3 ug/L. BLAST results confirm the presence of Atlantic Sturgeon through Sanger sequencing (Dryad deposition number: XXXXXX).

Calculating Atlantic Sturgeon Biomass in James River, VA: Results indicated mean values ranging from 11 million to 75 million grams of Atlantic Sturgeon depending on the site in the study area (Appendix A, Table 1). The Chesapeake Bay data gathered from Chesapeake Bay Program website and Buckaveckas indicated a volume in the study area estimated to be 2.86 billion liters of water. A mean total biomass of approximately 75 million grams Atlantic Sturgeon was estimated from these data, approximating a biomass of 264.2 µg/L. This concentration of Atlantic Sturgeon biomass in the study area for 2013 falls on the predicted curve of detectable Atlantic Sturgeon biomass established above suggesting that this would be a viable approach for monitoring presence of Atlantic Sturgeon in the James River and predicting changes in biomass throughout their residence times for spawning.

Discussion

The most important finding of this study is that eDNA approaches are sensitive and specific enough to detect the presence of Atlantic Sturgeon DNA from environmental samples collected on large rivers such as the James River in Virginia. I suggest the most parsimonious approach should utilize quantitative PCR. While the initial *CO1* restriction based analyses were sensitive enough to differentiate among fish taxa that co-occur in the James River, they were not specific enough to differentiate Atlantic Sturgeon from other non-fish taxa resulting in erroneous false positives.

The genomic region *CO1* is used as a barcode for many different species. In our work here the use of a single restriction test was insufficiently stringent for species identification. That is not to say that barcode regions are not appropriate, rather this particular digest was not sufficiently specific, given how much DNA was available per sample. The presence of only bacteria commonly found in environmental waters from sequencing supports these data. Use of *CO1* would require cloning the fragments before sequencing, making the process more expensive in terms of time and money in comparison to more modern techniques such as qPCR or next generation sequencing. Even samples from tanks amplifying *CO1* indicated bacterial noise from tank samples after being sequenced. The motivation of a restriction enzyme digest was to reduce cost and expedite the process of identification with the intention of future implementation by programs as small as state agencies. Large-scale sequencing efforts to identify specific targets has a high cost both monetarily and logistically (e.g. correct lab conditions).Targeted amplicons to fishes seems to be more consistent historically (Deiner *et al.*, 2014). Future studies may benefit from developing 18S targets as they are accepted to be more stringent. Barcodes such as *CO1* may benefit from the design of qPCR primers. While the primers themselves may not be as stringent as necessary, the melting temperatures of

amplicons can offer means of differentiation between species (Appendix B, Figure 1).

While qPCR has indicated its usefulness for Atlantic Sturgeon in this study, it has only been used to indicate and estimate the presence of the biomass of individuals from tanks samples, not from biomass in the study area. Given the complexity of environmental samples extracted from the James River and the causal mechanisms that may result in a false negative, currently, the absence of evidence for Atlantic Sturgeon should not be considered as evidence of absence. Future efforts will focus on the collection of more water samples in conjunction with ascertaining more information on hydrology.

Another potential extension to these techniques would be the use of multiplexing. The simultaneous amplification of several species-specific targets from the amplification of individual samples, allowing the quantification of aquatic community biomasses. For small streams, community analysis is something that may be redundant due to current collection methods for fishes and other aquatic species. However, for a stream on the order of the tidal James River where electrofishing at depth is more difficult, multiplexing could be useful. Other potential species such as mollusks or amphibians (e.g. Thomsen *et al.,* 2012; Goldberg *et al.*, 2011; Ficetola *et al.,* 2007) cannot be captured via electrical current and could therefore benefit from eDNA via qPCR in large drainages or where traditional sampling is difficult due to terrain. However, future researchers must invest heavily in gathering data on the hydrology of the system in which they are working in as the hydrology heavily dictates the ability of eDNA to correctly identify targets, with false negative rates being quite high, as indicated in this study.

For future researchers, it is highly recommended that qPCR be employed for eDNA. PCR does not seem to be as sensitive in many ways to target specificities, and seems to be more susceptible to environmental factors. Also, in a well mixed system such as the tidal

James River, PCR has a limited ability to indicate the source of the DNA present. In larger systems (e.g. > 3rd order), or in tidal systems, hydrology is critical to success in correctly identifying positives for eDNA. Future studies would do well to study hydrology before ever conducting a field eDNA test. The ability of qPCR to detect biomass is quite advantageous but, in order to discern these biomass levels, samples will likely need to be taken densely both temporally and spatially. This approach however has the potential for future gradient predictive models of presence based on seasonal eDNA data.

Limitations of this study mostly relate to model assumptions and hydrology. In order to estimate Atlantic Sturgeon biomass, average values for male and female mass were calculated to generate masses per site in fall of 2013. Acoustic tag telemetry, and average masses of individuals caught during fall of 2013 likely underestimate the biomass present. Hydrology is not well understood in the study area in as far as how the water fluctuates during its time flowing toward the ocean. The study area is freshwater tidal, so water fluctuates both upstream and downstream making the sources of eDNA difficult to discern once captured. The current literature indicates that eDNA is able to correctly identify targets in streams of all sizes and with target species of many kinds. While there has been a lot of development in the laboratory approaches to this problem, much less effort has been allocated to the influences that hydrology may have on detection rates. Indeed, eDNA data thus far has been taken at face value with little mention of the influence of hydrology (e.g. Thomsen *et al.*, 2012). The purpose of this method is to ascertain life history data about species that would otherwise be quite difficult. Capturing DNA from ocean water is a good first step but does not describe the behavior of a particular species. Thomsen notes this through potential travel time versus degradation time but makes no effort to indicate the effect in that particular study. They note that DNA could be degraded in as little as 10 hours but also indicate that the DNA could travel 40 - 600 km in that

time, limiting the behavioral information that can be gained unless samples are quantified in relative terms in the context of hydrology. Indeed, Roussel (2015) claims that the implementation of eDNA as a management program is based on just a few studies where biomass is known and no limit of detection is noted. Roussel also further supports that lack of effort to report effect of hydrology on correct capture rates. On the contrary, eDNA seems to be capable of correctly identifying cryptic species well in unidirectional small streams (e.g. Goldberg *et al.*, 2011; Ficetola *et al.*, 2008) due in large part to less complexity in hydrology and higher concentrations given the smaller volumes of water. Given the complexity of the James River in the study area present here, careful consideration of hydrology will be necessary for the future development eDNA tools for Atlantic Sturgeon that begins to describe behavioral preference for spawning habitat.

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Table 1. Location data for sites in study area. Two liter samples were taken at each of these sites through spawning event of 2013 (August-November). 79 two liter samples were kept for further analysis for proof-of-concept.

Site	Latitude	Longitude
B150	37.379332	-77.35093
B138	37.380072	-77.322787
Presquile	37.338773	-77.270136
Mouth of App	37.324441	-77.277002
App 1.5	37.313535	-77.294588
B107	37.30903	-77.241441
Rice Center	37.325225	-77.205656

Table 2. Coordinates for Positive Control test sites with Dr. Matt Balazik. These sites were selected as environmental positive control sites to indicate efficacy of eDNA methods at sites were found to have Atlantic Sturgeon present the time via radio tag or side-scanning sonar.

Site	Latitude	Longitude
Ab G25	37.15854	-76.64241
Below G25	37.14205	-76.69955
Bottom Sample 3	37.14872	-76.63891
Bottom Sample 4	37.15056	-76.63841

Table 3. Polymerase Chain Reaction *CO1* (FishF2_t1/FishR2_t1) primer sequences and qPCR (*CoxII)* primer sequences. Primer sequences for *CO1* were received from Dr. Ivanova via Barcode of Life. *CoxII* primers were developed based on basic BLAST for *CoxII* and subsequent retrieval of sequence and design via Primer BLAST/Primer 3.

Primer	Sequence
FishF2_t1	TGTAAAACGACGGCCAGTCGACTAATCATAAAGATATCGGCAC
FishR2_t1	CAGGAAACAGCTATGACACTTCAGGGTGACCGAAGAATCAGAA
qAoxCoxIIF	CCTAGTTTCCGCAGAAGATGT
qAoxCoxIIR	CCTGGTCGTGAGGTGATAAAG

Table 4. Tank numbers and biomass estimates. Biomass calculated based on average mass of Atlantic Sturgeon of 47 g times 200 individuals divided by total volume of tanks. Tanks are in open system so mass is thought of two be in one tank.

Tank #	Total Volume (L)	Number of Individuals (mean mass = 47 g)	Biomass (g/L)
8, 12, 14	2914.77	200	3.23

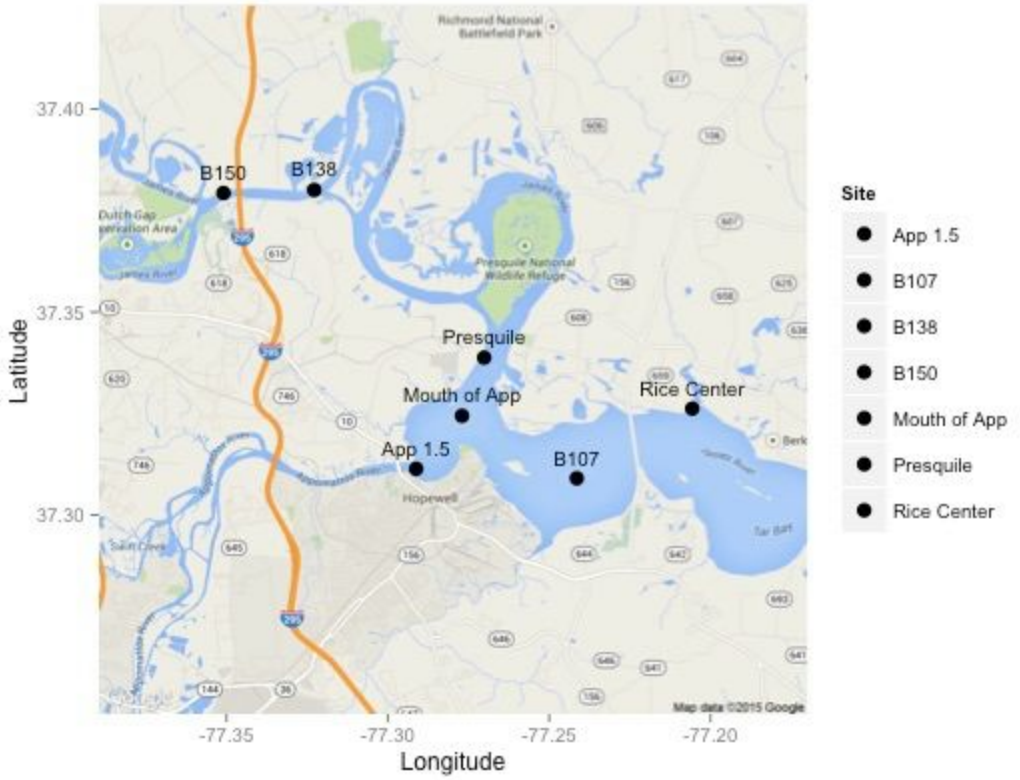

Figure 1. Map of study area on James River near Richmond, VA. Two liter samples were taken at each of these sites through spawning event of 2013 (August-November). 79 two liter samples were kept for further analysis for proof-of-concept.

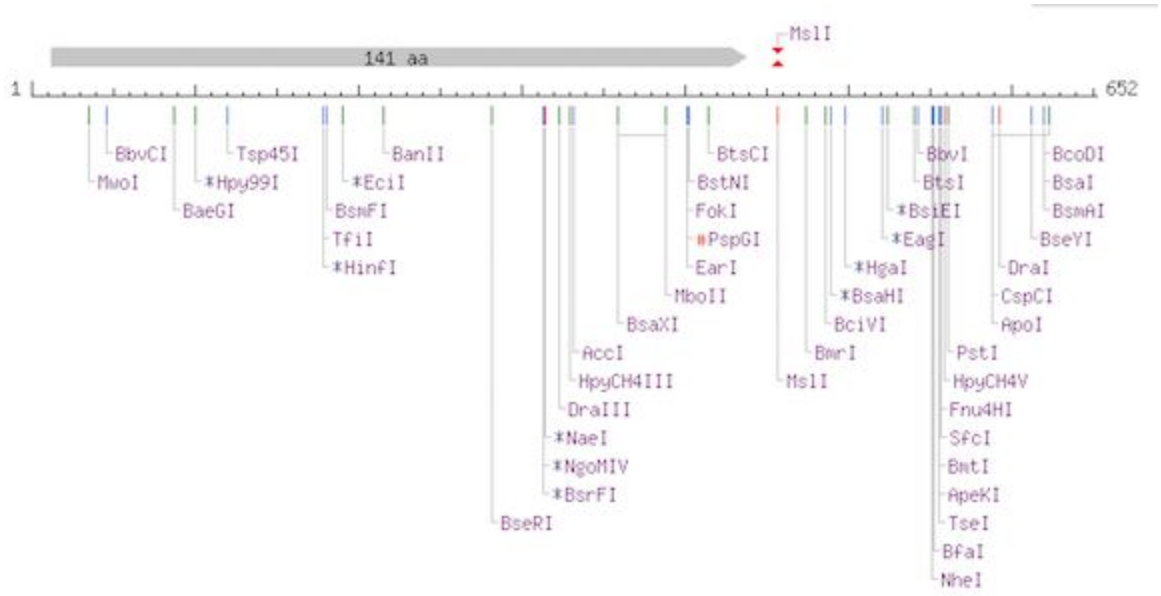

Figure 2. Example of NEBcutter software output by New England Biolabs. This image shows example output for Atlantic Sturgeon *CO1* sequence. Enzyme cuts sites are denoted by lines to purple letters, where red represents blunt ends and other colors represent types of sticky ends.

Lane	Taxa
1	Black Drum
2	Red Drum
3	Blue Catfish
4	Blueback Herring
5	Gizzard Shad
6	Menhayden
7	Pumpkinseed Sunfish
8	Satinfin Shiner
9	Redear Sunfish
10	Bluegill Sunfish
11	White Catfish
12	American Eel
13	Flathead Catfish
14	Threadfin Shad
15	Longnose Gar
16	Bowfin
17	Atlantic Sturgeon

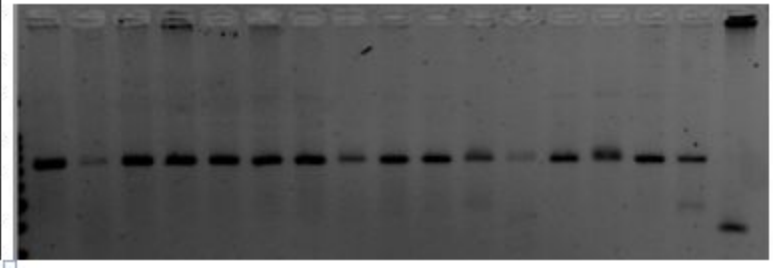

Figure 3. Table describes lanes in gel to the right. Gel represents *DraIII* digest of positive (Atlantic Sturgeon) and negative control (all others) fin clips as a quick/high throughput means of discriminating between taxa.

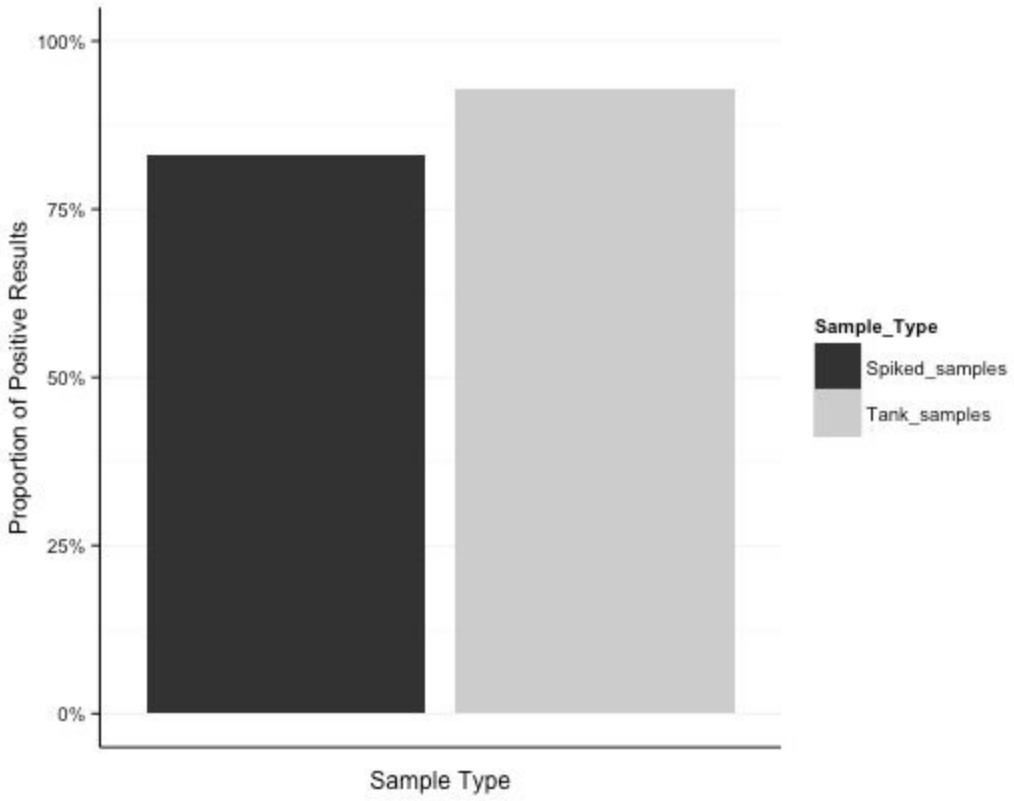

Figure 4. Two Liter tank sample (n = 24) and Spiked sample (n = 6) positive results proportions. Samples contained 91 and 83% positive results, respectively. These samples are post restriction digest with *DraIII* and prior to sequencing.

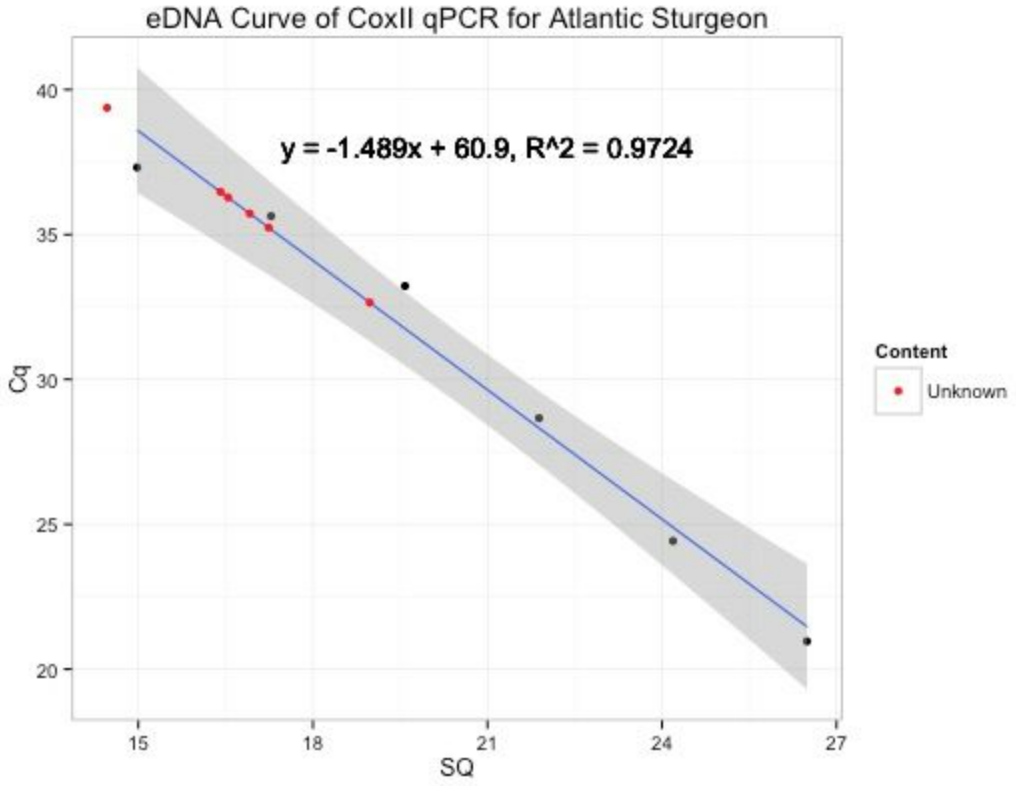

Figure 5. qPCR standard curve. 1:10 dilution of an Atlantic Sturgeon fin clip at 10 ng/µl. Standards are in blue and a 1:10 dilution of the tank sample containing all individuals (3.23 g/L) are in red. Model fit indicated on the graph. These data indicate a limit of detection of 32.3 ug/L.

Appendix A - Tables

Table 1. Table of estimated Kilograms of biomass of Atlantic Sturgeon present on given eDNA sampling days based on radio tag telemetry and gill net catchment during Fall of 2013.

Date	B107	App 1.5	Presquile	B138	B150
6-Aug	0	3780	3780	3780	0
13-Aug	3780	3780	3780	3780	0
20-Aug	11340	11340	3780	3780	3780
27-Aug	15120	11340	3780	15120	18900
3-Sep	3780	15120	15120	11340	26460
10-Sep	18900	34020	7560	3780	22680
17-Sep	15120	26460	22680	18900	3780
24-Sep	30240	18900	7560	7560	41580
1-Oct	22680	41580	71820	60480	22680
8-Oct	0	30240	11340	15120	30240
15-Oct	11340	34020	30240	30240	11340
22-Oct	0	3780	3780	0	0

Table 2. Chesapeake Bay Program's estimated volume of water in segment of river for study area, JMSTF

Segment	Volume (m^3)
Upper James River (JMSTF)	286,187,500
Middle James River (JMSOH)	431,500,000
Lower James River (JMSMH)	977,000,000
Mouth of the James River (JMSPH)	434,000,000

Appendix B- Figures

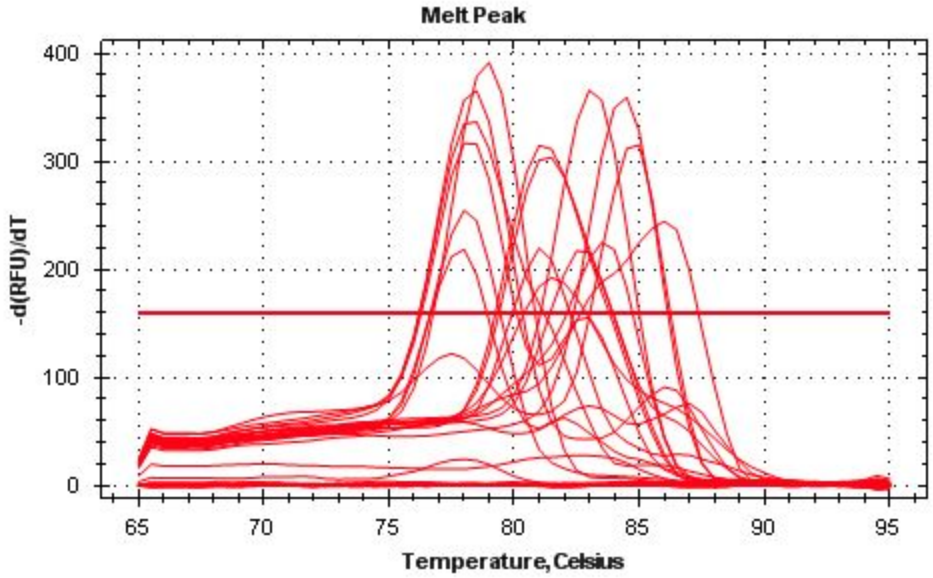

Figure 1. Melt Curve of *CoxII* primers containing all 17 taxa. Atlantic Sturgeon amplicons can be seen at its predicted melting temperature of ~ 78°C. Other fin clips can be seen as other melting curves around this temperature.

Appendix C. R scripts

A. Barplot_samps - generates barplots for positive tanks samples as a proportion in barplot.

```
samps <- read.csv(file = "Samples.csv", sep = ",", header = T)
require(ggplot2)
p <- ggplot(samps, aes(x = Sample.Type, y = Number)) +
  geom_bar() + scale_fill_grey() +
  theme_bw() +
  xlab("Sample Type") + ylab("Number of Samples") +
  theme(axis.text.x = element_text(size = 16))
p
library(scales)
results <- read.csv(file = "edna_results.csv", sep = ",", header = T)
r <- ggplot(results, aes(x = Sample_Type, y = prop_positive, fill = Sample_Type)) +
            geom_bar() + scale_x_discrete(breaks=NULL)  +
            scale_y_continuous(limits = c(0, 1), labels = percent) +
            scale_fill_grey() + theme_bw() + theme(axis.line = element_line(colour = "black"),
                                        panel.border = element_blank()) +
            xlab("Sample Type") + ylab("Proportion of Positive Results")

r

theme(axis.ticks = element_blank(), axis.text.x = element_blank())
scale_x_discrete(breaks=NULL)
```

B. Site_map - Generates map of study area based on Latitude/Longitude using google tile data imported by ggmap.

```
sites <- read.csv(file = "sites.csv", sep = ",", header = TRUE)
xy.dat <- sites[c("Longitude", "Latitude")]
sites <- SpatialPointsDataFrame(coords = xy.dat, sites)
# get sites
require(ggmap)
require(gstudio)
map <- ggmap(population_map(sites, map.type = "roadmap", zoom = 12), stratum = Site)
```

C. qpcr_plotting - This Script takes imported qPCR data and helps to plot in a better looking manner for publication. Need Unknowns and Standards, and plot against each other in an Lm, then plot with equation/R^2 on graph.

```
qpcr <- read.csv("MASSTEST_21315_summary.csv", sep = ",", header = T)
# standard curve data
qpcr$SQ <- log(qpcr$SQ)
# log transform as in bio-rad software
d <- qpcr[ qpcr$Content=="Std",]
# grab only standards
p <- ggplot(d, aes(x = SQ, y = Cq)) + geom_point() + stat_smooth(method = lm)
# run lm on standards only
p + geom_point( aes(x=SQ,y=Cq),data=qpcr[ qpcr$Content=="Unknown",],color="red") +
  labs(title = "Standard Curve of CoxII qPCR for Atlantic Sturgeon")
# add unknowns in different color and title

# set up equation and R^2 for plotting on graph
m <- lm(d$Cq ~ d$SQ)
a <- signif(coef(m)[1], digits = 4)
b <- signif(coef(m)[2], digits = 4)
R2 <- 0.9724
textlab <- paste("y = ",b,"x + ",a , ", R^2 = ", R2, sep="")
print(textlab)

# add equation and R^2 to graph
p <- p + geom_point( aes(x=SQ,y=Cq, fill = Content),data=qpcr[
qpcr$Content=="Unknown",],color="red") +
  geom_text(aes(x = 21, y = 38, label = textlab), color="black", size=5, parse = FALSE) +
theme_bw() +
  labs(title = "eDNA Curve of CoxII qPCR for Atlantic Sturgeon")
```