FINAL REPORT: MONITORING THE ENDANGERED TIDEWATER GOBY (*EUCYCLOBIUS NEWBERRYI*) USING eDNA IN WATER SAMPLES: ASSAY DEVELOPMENT AND FIELD SURVEY DESIGN

Andrew P. Kinziger (1) and Molly C. Schmelzle (2)

(1) Associate Professor, Department of Fisheries Biology, Humboldt State University, One Harpst Street, Arcata CA 95521

(2) Graduate Student, Department of Fisheries Biology, Humboldt State University, One Harpst Street, Arcata CA 95521

Produced Under Contract Agreement Between US Geological Survey and the Humboldt State University Sponsored Programs Foundation. Full Contract Title: Monitoring the endangered tidewater goby (Eucyclogobius newberryi) using water samples: laboratory tests. Cooperative Agreement number 1434-HQ-97-01547, Research Work Order No. 86

December 2013

SYNOPSIS

The recovery plan for the endangered tidewater goby calls for the development of standardized survey methods for monitoring the presence/absence of isolated populations throughout the geographic distribution of the species. The objective of this proposal was to assess the potential of using environmental DNA (eDNA) in water samples as a tool for determining the presence/absence of the endangered tidewater goby in lagoon and bay habitats in northern California. eDNA is a noninvasive technique that has been successfully applied to a broad range of taxonomic groups, including fishes, and is sometimes less expensive than traditional field monitoring approaches. Studies have shown environmental DNA can closely track presence/absence even when species occur at low levels of abundance and can be useful for monitoring species that cannot be detected using conventional field approaches. However, insufficient testing has been conducted to determine the suitability of eDNA approaches for monitoring tidewater goby. In this report we deliver: (1) laboratory assays that can be used for determining presence or absence of tidewater goby eDNA in water samples, and (2) a field survey design for instituting a monitoring program for tracking presence/absence within isolated habitat units in northern California that was designed to specifically account for imperfect detection. Thus we are now poised to employ eDNA approaches to monitoring tidewater goby presence/absence at field sites in Del Norte, Humboldt, and Mendocino counties.

LABORATORY PROTOCOLS

The following provides summaries of (i) water sample collection, filtration, and DNA extraction methods, (ii) quantitative PCR (qPCR) tests for detecting tidewater goby, and (iii) results from preliminary tests of the eDNA presence/absence qPCR assay. Detailed protocols are provided in the appendices.

Water Sample Collection, Filtration, and DNA Extraction

We collected 2 L surface water samples in pre-sterilized Nalgene bottles. The water was pumped through a disposable filter funnel with 47 mm diameter cellulose nitrate filter paper with a 0.45 µm pore size (Goldberg et al. 2011; Pilliod et al. 2013). Water was pumped using a peristaltic pump in the field or sometimes at the laboratory. Filters sometimes clogged due to high water turbidity and thus the amount of water filtered varied in our tests from 0.5 to 2 L. Filtration occurred within 12 hours of water collection. Following filtration, filter paper was placed into a 1.5 mL tube filled with 95% ethanol until DNA extraction or it was placed directly into a lysis solution. DNA was extracted from the filter paper using QIAGEN DNeasy Blood and Tissue Kit following manufacturer recommendations except that 270 µl ATL buffer and 30 µl proteinase K were used to accommodate larger volumes required by extraction from filters. We also processed negative controls of distilled water to evaluate the potential of contamination.

Due to the high sensitivity of eDNA qPCR assays sterilization and decontamination procedures were carefully followed (see appendix).

qPCR Assay for Tidewater Goby Detection

We developed seven PCR-based assays for detection of tidewater goby in northern California including three that relied on traditional PCR and four that are based on TaqMan quantitative PCR (qPCR) techniques (see appendix). We conducted preliminary tests of these assays and selected a TaqMan qPCR assay (named NC10) for tidewater goby presence/absence detection for several reasons. First, TaqMan MGB qPCR assays are more target-specific than traditional PCR thereby reducing chances for false-positives resulting from co-amplification of non-target species. Second, qPCR is much more sensitive than traditional PCR; TaqMan MGB qPCR has the power to detect target species DNA at concentrations as low as 0.5 target copies/µl (Wilcox et al. 2013). Third, qPCR approaches have been successfully used to estimate abundance in several studies thereby opening the possibility of using qPCR for estimating tidewater goby abundance in future studies (e.g., Takahara et al. 2012).

Our NC10 assay targeted 119 base pairs of the mitochondrial *cytochrome b* gene (*cyt b*). We used mitochondrial DNA (mtDNA) because it is substantially more abundant in cells than nuclear DNA and therefore improves detection power when DNA is present at low concentrations and/or degraded. Further mtDNA is highly variable among species known to co-occur with tidewater goby in our study area and therefore provides the ability to develop a species specific assay. Because *cyt b* sequence data for tidewater goby were limited to one individual originating from a southern California location we generated sequence data for two individuals, one individual originating from Big Lagoon in Humboldt County and a second individual from a Mendocino County population, using the primers L15314 and H15958 with sequencing performed at the University of Washington High Throughput Genomics Center (http://www.htseq.org/; see appendix). (We initially attempted to use mitochondrial control region sequence data previously generated for tidewater goby (Dawson et al. 2001; McCraney et al. 2010) but abandoned this approach when we learned that this gene was too highly variable among populations to develop a single assay applicable across our study area.)

We designed our qPCR assay (NC10) using Primer3Plus (Untergasser et al. 2007) using specialized settings for qPCR and tidewater goby *cyt b* reference sequence data (described above). The selected assay was purchased from Applied Biosystems and contained a primer set and a FAM-labeled minor groove binding, non-fluorescent quencher (MGB-NFQ) probe. Design for NC10 forward primer was NC10F (5'-CCTCAATTCTCGTTCTACTAGTTGT-3'), the reverse primer was NC10R (5'-GAGAATAAGTACGTCTGCTACTAGG-3'), and the probe was NC10P (5'-ACGTGCACTGACCTTCCGGCCTTTCTCC-3'). We ran 25 µl reaction volumes with 2 µl of template, 10 µl Master Mix (Life Technologies Taqman Environmental Master Mix 2.0 (Catalog Number: 4396838)), 1 µl of each primer (10 µM), 1 µl of probe (2.5 µM), and 10 µl diH_2O following cycling conditions 50° C 5 min., 95° C 10 min., 55 X (95° C

0:30 sec., 61° C 1 min., cycle) on an Applied Biosystems 7300 Real-time PCR Instrument. qPCR reactions were set up inside of an enclosure which was irradiated with UV for at least 1 h prior to each use along with all consumables and pipettes. Reagents were also aliquoted into small quantities prior to experiments such that each reagent tube was only opened a single time in a PCR product-free environment.

To ensure that our tidewater NC10 assay was highly species-specific we designed our primers and probe so that they were conserved within tidewater goby but exhibited a large number of binding-site mismatches in comparison to arrow goby (*Clevelandia ios*). Arrow goby is the putative sister taxon to tidewater goby (Dawson et al. 2001) and can co-occur with tidewater goby in our northern California study area. Our NC10 assay had 20 binding site mismatches in comparison to arrow goby (Humboldt County, California), including 8 in the forward primer, 7 in the probe, and 5 in the reverse primer. Test of our qPCR TaqMan assay indicated very high specificity for tidewater goby. None of the nine co-occurring species tested positive: arrow goby, steelhead (*Oncorhynchus mykiss*), threespine stickleback (*Gasterosteus aculeatus*), prickly sculpin (*Cottus asper*), bay pipefish (*Syngnathus leptorhynchus*), topsmelt (*Atherinops affinis*), steelhead (*Oncorhynchus mykiss*), northern anchovy (*Engraulis mordax*), and bay goby (*Lepidigobius lepidus*). Thus, NC10 is robust to problems associated with false-positives resulting from amplification co-occurring species.

Preliminary Results of tidewater goby eDNA assay

We collected water samples from 14 locations for testing of our NC10 qPCR for tidewater goby eDNA presence/absence detection. Ten locations were sites where tidewater goby are known to occur: (1) HSU tidewater goby hatchery tanks, (2) Ten Mile River, Mendocino County, (3) Virgin River, Mendocino County, (4) Pudding Creek, Mendocino County, (5) Davis Lake, Mendocino County, (6) Elk River (Martin Slough), Humboldt County, (7) McDaniel Slough, Humboldt County, (8) Stone Lagoon, Humboldt County, (9) Big Lagoon, Humboldt County, and (10) Elk River (Berta Bridge), Humboldt County. Five locations are sites where tidewater goby were NOT known to occur: (1) Klopp Lake, Humboldt County, (2) Big River, Mendocino County, (3) Freshwater Lagoon, Humboldt County, (4) Little River, Humboldt County, and (5) Mad River, Humboldt County. Water samples were collected from each location, the number of water samples collected varied by location and ranged from 1 to 3. Each water sample was tested for tidewater goby eDNA by running at least one qPCR test but in some cases up to four replicate qPCR tests were run for a given water sample.

Each water sample was tested for presence/absence of tidewater goby eDNA using the NC10 assay. All qPCR tests included a positive control (DNA extracted from tidewater goby collected from Big Lagoon), a negative water sample control (tap water or water from a source that did not contain tidewater goby DNA), and a negative PCR control (substituting nuclease free water for template) to test for contamination. In all qPCR tests the positive controls tested positive for tidewater goby DNA while all negative controls did not detect tidewater goby.

The results from the preliminary tests of NC10 qPCR assay to detect tidewater goby eDNA in water samples are reported in Table 1. Two major findings are evident. First, tidewater goby were never detected at sites where the species is not known to occur (with a single exception, see below). Thus, the method is robust to issues associated with false positives. Second, tidewater goby were generally detected at sites that they were known to occur but in several instances the NC10 assayed failed to detect tidewater goby eDNA even though the species was present at the site. Imperfect detection of tidewater goby using our NC10 assay are partially due to use of different types of water filtration methods. We didn't test filtration methods extensively so definitive finding was possible. Another interesting finding is that our eDNA technique detected tidewater goby from Big River, Mendocino County, a site where tidewater goby have not been previously detected. This site has not been exhaustively surveyed using traditional field survey techniques, contains habitat suitable for tidewater goby, and thus future field surveys are needed.

Table 1. Results from preliminary test to detect tidewater goby eDNA in water samples using NC10 qPCR assay. Each water sample was tested one to three times using the NC10 qPCR assay. Positive detections are indicated by + and negative detection by –.

		Replicate PCRs			
Water sample	Filtration Method	1	2	3	Notes
HSU Hatchery 1	2 L @ 0.45µm	+	+		about 5 fish in 30 gallons
HSU Hatchery 2	2 L @ 0.45µm	+			about 12 fish in 100 gallons
Big Lagoon	1L @ 0.45µm	-			twg present at site
Big Lagoon	1L @ 0.45µm	-			twg present at site
Stone Lagoon	1L @ 0.45µm	+	+		twg present at site
Stone Lagoon	1L @ 0.45µm	+	-		twg present at site
McDaniel Slough	0.36L @ 0.45µm	-	-		twg present at site
McDaniel Slough	.62L @ 3.µm	-	-	-	twg present at site
McDaniel Slough	0.64L @ 8µm 0.45µm	-			twg present at site
McDaniel Slough	1.4L @ 3µm then 0.45µm	-	-		twg present at site
McDaniel Slough	1.4L @ 3µm then 3µm	-	+		twg present at site
McDaniel Slough	1.6L @ 0.45µm	-	-		twg present at site
Elk River (Martin Slough)	ca. 0.5L @ 0.45µm	+	+	+	twg present at site
Elk River (Martin Slough)	ca. 1.25L @ 3µm then 0.45µm	+	+	+	twg present at site
Elk River (Martin Slough)	ca. 1L @ 3µm	+	+		twg present at site
Elk River (Martin Slough)	ca. 1L @ 12µm then 0.45µm	+	+		twg present at site
Elk River (Martin Slough)	2L @ 8µm then 8µm then 0.45µm	+	+		twg present at site
Elk River (Berta Bridge)	1.6L @ 8µm then 8µm then 8µm then 0.45µm	+			twg present at site
Elk River (Berta Bridge)	0.4L @ 12µm then 0.45µm	-			twg present at site
Ten Mile River at HWY 101	ca. 1.2L @ 0.45 µm	-			Field survey: 0 twg per 11 hauls
Ten Mile River upstream	ca. 1.2L @ 0.45 µm	+	+		Field survey: 2 twg per 8 hauls
Virgin Creek	1L @ 0.45µm	+	+		Field survey: 52 twg per 9 hauls
Virgin Creek	1L @ 0.45µm	-			Field survey: 52 twg per 9 hauls
Pudding Creek	1L @ 0.45µm	+	+		Fiield survey: 61 twg per 57 hauls
Pudding Creek	1L @ 0.45µm	+	+		Fiield survey: 61 twg per 57 hauls

Davis Lake	ca. 0.6L @ 0.45µm	+	-	-	Field survey: 96 twg per 1 haul
Davis Lake	ca. 1L @ 3.0µm	+	+	-	Field survey: 96 twg per 1 haul
Mad River	1L @ 0.45µm	-			No twg documented from this site
Mad River	1L @ 0.45µm	-			No twg documented from this site
Freshwater Lagoon	1L @ 0.45µm	-			No twg documented from this site
Freshwater Lagoon	1L @ 0.45µm	-			No twg documented from this site
Litter River	1L @ 0.45µm	-			No twg documented from this site
Klopp Lake	1L @ 0.45µm	-			No twg documented from this site
Big River	2L @ 0.45µm	+			No twg documented from this site

FIELD SURVEY DESIGN

Environmental DNA is a powerful approach for species detection but like field survey methods it is possible that species will not be detected even though it is present using eDNA. Imperfect detection can lead to underestimation of the geographic distribution of tidewater goby and/or false-assumptions regarding extirpation and colonization dynamics of local populations. Occupancy modeling survey designs provide a solution for imperfect detection because they directly account for imperfect detection by calculation of detection probabilities and simultaneously generate estimates of the proportion of sites occupied using presence/absence data. We developed an occupancy modeling approach for monitoring tidewater goby presence/absence using eDNA by modifying the single-season-multi-method model of Nichols et al. (2008). A similar approach has also been described by Schmidt et al. (2013).

Under an occupancy modeling framework the geographic distribution of tidewater goby in the northcoast region would be monitored by collection of *t* water samples within each of *i* study sites (e.g., lagoons or bays) distributed across the landscape. Each water sample would then be tested for tidewater goby eDNA by running *s* replicate qPCR assays on each individual water sample. Presence/absence histories would then be generated for each sampling location (*i*). A history of 010 000 000 indicates that at site *i* a total of $t = 3$ distinct water samples were collected and $s = 3$ qPCR tests were conducted on each water sample. eDNA from tidewater goby was detected in the second qPCR test from water sample one and went undetected in all qPCR tests in the second and third water samples. These data are then used to estimate occupancy (Ψ = probability that sample unit *i* is occupied) while simultaneously accounting for imperfect detection resulting from (1) failure to detect the species during field collection of water samples (e.g., by *t* replicate water samples) and (2) failure to detect the species during laboratory analysis (e.g., by *s* replicate qPCR tests). Occupancy modeling approaches account for uncertainty associated with imperfect detection by estimating detection probabilities from replicate water samples (θ) and from the replicate qPCR tests (p_s^t). Detection probability for the water samples is defined as θ_t the probability that a species is present in water sample *t* given that sample unit *i* is occupied. Detection probability for the qPCR tests is defined as p_s^t the probability of detection in water sample *t* by qPCR test *s* given that sample unit *i* is occupied and that the species is present in the water sample. The water sample detection probabilities are useful for accounting for heterogeneity of tidewater goby eDNA within lagoon habitats. Tidewater goby eDNA is unlikely to be uniformly mixed within lagoon and bay habitats due to microhabitat preferences of the species, variation in abundance in space or time, depth, water flow, sunlight and other factors. In contrast, qPCR detection probabilities provide an indication of the robustness of the lab tests; or the probability that the lab assay or test will detect DNA of the target species given it is present in a given water sample. It is possible that errors in laboratory procedures or low concentration of tidewater goby eDNA may result in non-detection by qPCR.

To evaluate the performance of occupancy modeling approach for monitoring tidewater goby presence/absence we conducted a simulation analysis using the software GENPRES (Bailey et

al. 2007). GENPRES provides estimates of the precision (standard error) that might be expected given user specified values for Ψ, θ, and p_s^t. The simulations are useful for determining how to allocate effort in order to achieve a desired level of precision. Our simulations were designed to determine how increasing the number of replicate water samples collected from each field site would influence our estimates of precision. For this test we set the number of survey sites (*i*) to 40 and the occupancy probability of those sites (ψ) to 0.75. These values seemed reasonable because tidewater goby were previously detected at 30 isolated sites in Del Norte, Humboldt and Mendocino counties but we expect to survey sites where tidewater are likely to occur but have never been detected in future survey efforts (ie, 30 occupied sites/forty survey locations = 0.75 occupancy). Detection probabilities for replicate water samples (θ) and for the replicate qPCR tests (p_s^t) were set to 0.50 and constant. We ran three scenarios: (i) low - 3 water samples per site, (ii) high - 9 water samples per site, and (iii) intermediate - 20 sites with 3 water samples per site and 20 sites with 6 water samples per site.

The results of the simulations demonstrated that improvements in the precision (standard error) of the estimates of occupancy probability (ψ) would be realized by increasing the number of water samples per site (Table 2). However, the trade-off is that project costs substantially increase with the number of water samples collected per location.. For example, if three water samples are processed per site a total 9 qPCR tests would need to be run (3 water samples X 3 replicate qPCR tests per water sample = 9 total qPCR tests). In contrast, if nine water samples are processed per site a total 27 qPCR tests would need to be run (9 water samples X 3 replicate qPCR tests per water sample = 27 total qPCR tests). Thus, while a substantial improvement in precision is afforded by collection of nine water samples per site the associated cost of doing so would triple.

Three features of the occupancy modeling design that directly influence precision warrant mention. First, increasing the number of sampling sites will improve the precision of the estimator. We ran an additional simulation identical to scenario (i) above except that the number of survey sites was increased from 40 to 100. This analysis resulted in a substantial improvement in the precision of the occupancy probability estimate (ψ = 0.7500 (SE 0.0875), p_s^t = 0.5000 (SE 0.0768), and θ =0.5000 (SE 0.0667)). Second, although we set detection probabilities for replicate water samples (θ) and for the replicate qPCR tests (p_s^t) to 0.50 we suspect that these estimates are too low especially for p_s^t. If detection probabilities for the replicate qPCR tests (p_s^t), were say 0.80, which some literature suggests (e.g., Schmidt et al. 2013), then precision would be improved over the values reported. Lastly, because there are a finite number of locations for tidewater goby to occupy in our study region we could apply a finite population correction to our occupancy estimates. The finite population correction for single-season-multi-method can be calculated as

$$SE_{finite} = \psi_{SE}\, x\, (1 - (1 - \mathrm{p})^{l})$$

Where *l* is the number of levels and for the design outlined above it is three: sample unit, water sample, and the PCR test. The finite population correction is important because it will improve the precision (ie, reduce SE and 95% Confidence Intervals) of the occupancy estimates (Table 2).

Table 2. Summary of GENPRES simulations used to determine how the number of water samples per site influences precision. Ψ = Pr (sample unit occupied); θ_t = Pr(species present in immediate water sample at location *t* | sample unit occupied); p_s^t = Pr(detection in water sample *t* by qPCR test *s* | sample unit occupied and species present in immediate water sample).

Scenario	# sites (*i*)	water samples per site (*t*)	PCR tests per water sample (*s*)	*p* (SE)	θ *(SE)*	Ψ*(SE)*	$\Psi(SE_{finite})$
Low	40	3	3	0.5000 (0.0833)	0.5000 (0.0884)	0.7500 (0.1135)	0.7500 (0.0993)
High	40	9	3	0.4716-0.5490 (0.0458-0.0493)	0.9633 (0.0641)	0.8333 (0.0680)	0.8333 (0.0595)
Intermediate	40	20 sites w/ *t*=6 and 20 sites w/ *t*=3	3	0.5000 (0.1054)	0.5000 (0.1215)	0.7500 (0.1384)	0.7500 (0.1211)

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Appendix 1

ENVIRONMENTAL DNA STERILIZATION/DECONTAMINATION METHODS FOR NON-DISPOSABLE FIELD AND LAB SUPPLIES

Environmental DNA analysis is highly susceptible to contamination. Therefore, decontamination and sterilization of eDNA supplies is of extreme import. High quantities and concentrations up to 50% are used during field collection and post-filtration steps. Bleach is corrosive and an irritant. It should be handled with extreme care. Ensure that lids to bleach solutions are secure, always wear gloves, and if necessary don a face mask if fumes are overwhelming. An MSDS is available at: http://msds-data.com/clorox/.

Sampling Containers (2 L Nalgene bottles), one sample container per field sample

- Rinse with RO water
- Fill the sampling container with 20% bleach solution. Shake vigorously for approximately 15 seconds. Let soak for several hours.
- Rinse thoroughly with RO water. Be sure to clean cap with both bleach and RO water.
- Place in UV hood for at least 24 hours.
- Store in a dry cool place away from potential contamination sources.

Sampling Containers (2 L Nalgene bottles), one sample container, > 1 field sample

- There are currently a total of 12 2 L Nalgene sampling bottles. If the number of water samples per site exceeds the number of sampling containers, filtration will have to be conducted in the field. The containers must be cleaned and washed vigorously with 50% bleach solution.**
- Rinse with RO water.
- Fill the sampling container, not all the way but significantly with 50% bleach solution. Shake vigorously and make sure all inner surfaces are decontaminated.
- Pour out bleach in a container solely for used bleach solution. Rinse thoroughly with RO water and new sample site water. Be sure to clean cap with both bleach and RO water.

Whatman Filter funnels

- There are two designated 15 quart cleaning containers for filter funnels. One container for bleach solution and one container for RO rinse and soak.
- After filtration, place the used filter funnels into an approximate 20% beach solution in the designated container, tap water ok.
- Soak for several hours.
- Transfer filter funnels into the 15 quart container filled with RO water. Soak for several hours or overnight.
- Air dry filter funnels.
- Package no more than three filter funnels into Autoclave bag. Seal.
- Autoclave in the Biology building, 3rd floor. Run at 87.3, 0.1 pressure for a 30 minute sterilization. Use the SMDRY cycle, approximately 1 hour run time.

- Keep the filter funnels packaged in a dry and cool place until ready to use either in the field or in the lab. Filters will have to be placed into the funnel at the time of the next water sample filtration.

Glass jar vacuum reservoirs

- If the glass jars are used for pre-filtering experiments they must be cleaned and decontaminated thoroughly before the next sampling event.
- Be careful as the glass jar may become slippery and wet when filled with liquid.
- Rinse with RO water.
- Wash thoroughly with 20% bleach water
- Rinse again with RO water.
- Autoclave, Use the SMDRY cycle approximately 1 hour run time.
- Store in dry and cool place.

Glass jar lids and #8 rubber stoppers

- Wash with 20% bleach and rinse with RO water
- Place in UV hood for 24 hours

Forceps / Scalpel

- During filtration and in between each use, wash with 50% bleach solution and rinse thoroughly with DI water. Shake dry.
- It is important to decontaminate the forceps and scalpel to ensure that remnant DNA is not transferred to other sample filters.
- After filtration wash with 50% bleach solution, rinse with RO water, and place into UV hood for 24 hours.

Silicon tubing

- Wash with 20% bleach and rinse with RO water.
- Place in UV hood for 24 hours

Sampling jars for DI water (Orange lids)

- If no contamination is evident rinse thoroughly rinse with RO water and place in UV hood for 24 hours.

**It is recommended to bring approximately 2 bottles of 250 ml of 50% bleach. 50% bleach will completely remove DNA, a lesser concentration will not do so effectively. Also bring 2 x 250 ml of DI water to rinse the forceps. Both need to be changed every few days. The bleach because the sodium hypochlorite evaporates every time the bottle is opened and the DI because it

will start to smell like bleach indicating that is no longer pure (Pilliod, 2013 email correspondence).

Appendix 2

ENVIRONMENTAL DNA WATER SAMPLING FIELD GEAR LIST, LAB FILTRATION WITHIN 12 HOURS OF COLLECTION

I. Gear List

1. Write in the Rain Field notebook, pencils
2. 2-L Sampling bottles all pre-labeled plus 1 -2 extra for impromptu sampling (ensure bottles are bleached, washed, and UV treated)
3. Cooler and ice for 2-L sample bottles
4. Trip blank and field blank, fill blank sampling bottles with deionized water prior to departure
5. Refractometer
6. YSI for water quality measurements
7. Turbidity meter if available
8. GPS unit

II. Field Sampling

1. If filtration is not scheduled to occur in the field, place samples on ice immediately, filter as soon as possible. It is recommended to filter samples within 24 hours. Store and preserve as indicated by individualized research and lab needs: in lysis solution, in 95% ETOH, or store at -20°C until the extraction process can be initiated.
    a. Drop filter directly into lysis solution if the extraction process will occur within 24 hours of filtering.*
    b. Drop filter into 95% ETOH if filtering takes place in the field and extraction isn't scheduled immediately.
    c. Drop filter either in 95% ETOH or place into a sterile microfuge tube and store at -20°C if filtering takes place in the lab and extraction isn't scheduled immediately.
2. It is recommended to have several negative (blanks) samples for each field sampling event to test for false positives associated with handling, transport, and equipment use (Goldberg et al., 2013).

a. Site negative-if filtering takes place in the field, each site sampled needs a corresponding negative. Pour DI water into a 2 L sampling container at each site. It should be filtered in the same manner as the environmental water samples. Filter approximately 500 ml to 1000 ml for each site.

b. Trip negative- if filtering takes place back in the lab, at the start of the day transfer 500 ml of DI water into a glass sampling container and filter with all of the collected samples.

c. Equipment negative-to test for contamination of sampling equipment transfer 500 ml – 1L of DI water into a 2 L plastic sampling container and filter.

3. At the sampling site, record:

    a. GPS point

    b. Vegetation

    c. Substrate

    d. Water quality (DO, temperature, salinity, NTUs)

    e. Tidal influences and/ or water levels

4. **Taking a Sample**: Submerge the pre-labeled sterile 2-L sampling container approximately 10 cm below the surface of the water until completely filled. Swirl and rinse with sample water. Take sample.

5. Steps to avoid cross-contamination (Pilliod, 2012):

    a. Don a new pair of gloves for each filtration.

    b. Don't touch the inside of the filter funnel

    c. Only use disinfected and sterile sampling bottles, hoses, and forceps

    d. Store used gloves and disposable supplies sealed in a separate Ziploc bags and away from clean and sterile gloves and supplies.

    e. If re-using sample bottles and glass jar reservoirs the bottles must be sterilized with a 50% bleach solution. Rinse with DI or distilled water at least three times. Rinse with sample water 3 times before collecting new water sample. See above more information.

* Lysis solution (Qiagen DNeasy Blood and Tissue Kit) – **If halving** the final filter paper use manufacture's protocol of 180 µl ATL buffer & 20 µl proteinase K. Increase the lysis solution

by 50% from the standard manufacture's protocol to account for the size of filter paper. It is recommended to use 270 µl ATL buffer & 30 µl proteinase K **if using the whole filter paper** during the extraction process. Other researchers have doubled the amount, 360 µl of ATL & 40 µl of proteinase K. We have found that using the former amount, 270 µl ATL & 30 µl of proteinase K works as well and uses less Qiagen product. Increased amounts are used because the standard amount is not enough solution for the entirety of the filter paper to be adequately lysed.

Extra Considerations for field sampling

Water in a lentic and / or estuarine system may have high turbidity. This makes water filtration for eDNA research challenging and sometimes a timely process requiring patience and careful attention, especially if pre-filtering is necessary. If there is an intention to filter in the field allot for extra time in cases where a water system is extremely turbid. If it will take too much time keep the sample on ice in a cooler and filter within 12-24 hours if possible and use the amount of water sampled as a covariate of the sampling process.

Appendix 3

ENVIRONMENTAL DNA FILTRATION

Pre-filtering steps

1. Take a water sample
    a. Pre-label 2-L sampling containers
    b. Pre-label corresponding 1.5 ml sterile microfuge tubes for filter storage in either lysis solution and/ or preservation, 95% ETOH or -20°C.
2. Find a clean and level area away from hazards to conduct water filtration
3. Set up the Cole Palmer air Cadet-Electric pump and the vacuum glass containers.
4. Connect the pump to the air nozzle on the glass jar lid with approximately 12-18 inches of sterile Masterflex silicon tubing (size L/S 15).
    a. Make sure the pump has the appropriate attachment for the tubing.
    b. Ensure the tubing and glass jars have been cleaned and sterilized prior to use
5. If filtration is scheduled for the field, an electric source is needed. Plug the pump into an inverter which should be plugged into a vehicle's AC power. Make sure both work properly before heading into the field.
6. Place the #8 rubber stopper with a drilled out ½ inch hole securely into the vacuum glass jar lid, hole provided.
    a. If using the glass jar system properly label each jar with a corresponding sampling container, especially if pre-filtering samples.
    b. Glass jars will facilitate pre-filtering and reduce contamination
7. Securely fit a new or sterilized Whatman Disposable filter funnel with the 47mm Cellulose Nitrate, 0.45 um pore diameter filter into the rubber stopper**. 0.45 micron nitrocellulose filters have a very high binding capacity for nucleic acids and proteins.** To avoid cross contamination where single-use gloves for each filtration.
    a. Don't touch the inside of the filter when removing it from the package.
    b. If using an autoclaved filter take extreme care when placing in new filters from the filter storage package. Use sterilized and dedicated forceps for handling clean filters.

Filtering steps

1. Start the pump to begin filtration.*
2. Slowly pour the sample water into the filter funnel. Swirl the last 300 – 500 ml of remaining sample to recapture any DNA on walls of container.
    a. Make sure there is a vacuum.
    b. If water enters the silicone tubing, stop the pump immediately.
    c. Watch / listen for holes in the filter
3. Upon completion of a water sample (all possible water has passed through the filter into the glass collection reservoir). **Remove the filter paper.**
    a. Wear sterile gloves
    b. Use sterile and disinfected forceps (50% bleach wash and DI water rinse)**
    c. Carefully remove the filter funnel (top) from the bottom by pulling the tab, slowly twist up and off.
    d. Take forceps and fold the filter three times or fold in half and roll.
    e. Place filter into properly labeled microfuge tube (Lysis solution, 95% ETOH, or empty tube for -20°C storage).
    f. Place the tube with filter securely inside into a field sampling case.
    g. Remove the filter funnel from the rubber stopper and place in a Ziploc bag separate from clean filter funnels.
4. Sterilize and disinfect the forceps with the 50% bleach and DI water system.

Important Considerations for field sampling

•Water in a lentic and /or estuarine system may have high turbidity and may be tannic. This makes water filtration for eDNA research challenging and sometimes a long process that requires patience and careful attention. Pre-filtering may be necessary. If filtration is to occur in the field allot for extra time in cases where a water system is extremely turbid. If it will take too much time think about keeping the samples on ice in a cooler. Filter within 12- 24 hours.

•Between each sample filtration clean forceps with a 50% bleach solution and rinse with DI water. Repeat three times. Rinse the forceps thoroughly to make sure there is no residual bleach on the forceps. Dry before touching the filter paper.

•It is recommended to bring approximately 2 bottles of 250 ml of 50% bleach. 50% bleach will completely remove DNA, a lesser concentration will not do so effectively. Also bring 2 x 250 ml of DI water to rinse the forceps. Both need to be changed every few days. The bleach because the sodium hypochlorite evaporates every time the bottle is opened and the DI because it will start to smell like bleach indicating that is no longer pure (Pilliod, 2013 email correspondence).

Appendix 4

ENVIRONMENTAL DNA SPIN-COLUMN EXTRACTION STEPS

Qiagen D'Neasy Blood and Tissue Kit Modified Spin-Column Protocol

1. *Lysis solution (Qiagen DNeasy Blood and Tissue Kit) – **If halving** the final filter paper use manufacture's protocol of 180 µl ATL buffer & 20 µl proteinase K. Increase the lysis solution by 50% from the standard manufacture's protocol to account for the size of filter paper. It is recommended to use 270 µl ATL buffer & 30 µl proteinase K **if using the whole filter paper** during the extraction process. Other researchers have doubled the amount, 360 µl of ATL & 40 µl of proteinase K. We have found that using the former amount, 270 µl ATL & 30 µl of proteinase K works as well and uses less Qiagen product. Increased amounts are used because the standard amount is not enough solution for the entirety of the filter paper to be adequately lysed.

2. With sterilized forceps carefully roll and fold the filter, place it into a standard sterilized 1.5 ml tube with lysis solution (see step 1).

    a. The lysis solution may be added to a microfuge tube prior to adding the filter paper (field) or it may be added after the filter is placed into a tube (lab).

    b. Vortex periodically and if necessary centrifuge to ensure the filter paper is submerged completely in the lysis solution.

3. Incubate lysis solution with filter paper at 56°C for 3 – 8 hours. Overnight incubation is also fine.

4. Vortex and centrifuge prior to commencing extraction process.

5. If the lysis solution was increased by 50% to account for the filter size then the next step in the extraction process also needs to be increased by 50% from the manufacture's protocol. Add 300 µl of AL buffer and 300 µl of absolute ethanol. The increase in buffer solution and ethanol at this step ensures concentrations are consistent.

6. Before transferring the solution to the spin-column, centrifuge for 5 minutes at 13,000 RPM. This will force the filter to the bottom of the tube and the solution containing the DNA to the top (email correspondence with Tim Gingera).

7. The filter paper is a surrogate for a tissue sample. This adds a unique step to the extraction process.

    a. There is more buffer being used

    b. The filter needs to be removed from solution.

8. It is necessary to have sterilization solutions at the extraction table for the forceps that are used to remove the filter paper. Set-up a wash with either a 20% bleach solution or 95 % ETOH and a RO water rinse. Make sure to wash and rinse extremely well to ensure that residual DNA doesn't transfer to subsequent samples.

9. Remove and discard the filter and sterilize the forceps before moving onto the next sample.

10. If the original lysis solution amounts were increased not all of it will fit into the spin-column at once. Pipette approximately half of the solution into the spin column and centrifuge according to the manufacturer's protocol. Add the remainder of the solution into the spin-column, centrifuge according to the manufacture both times (8,000 rpm for 1 minute).

    a. 600 µl is the max amount of solution that can be pipetted into the spin-column at once. This is standard for 180 ATL, 20 µl proteinase K, 200 µl of AL buffer, and 200 µl of ethanol.

    b. 270 µl ATL, 30 µl proteinase K, 300 µl AL buffer, and 300 µl of ethanol totals 900 µl of solution. It is easy to transfer a little more than half the total amount each transfer.

    c. 360 µATL, 40 µl proteinase K, 400 µl AL buffer, and 400 µl of ethanol totals total 1200 µl of solution. Transfer 600 µl at a time.

    d. Transfer to correctly labeled spin-column, centrifuge, transfer the remainder to the same spin-column, centrifuge.

11. From this step onward follow the manufacturer's protocol.

12. After the last step is complete, the elution step, transfer the solution with DNA into a new and sterile microfuge tube, pre-label. **DO NOT KEEP IN THE SPIN COLUMN**.

    a. The final elution is ready for PCR.

    b. Store in a dedicated and safe freezer.

Important Extraction Notes

•If there is a chance of contamination during the extraction process, place the buffer solutions, uncapped, in a UV hood for 20-30 minutes before using. **Do not place the proteinase K in the UV hood.**

•Maintain a clean and sterile work place.

•The ATL buffer may form precipitate or turn into a gel like substance upon storage, incubate at 56°C until the solution is clear and ready to use.

•Some researchers recommend halving the filter paper before placing into lysis solution. This method can be used in case something goes wrong with the PCR reaction. In addition, there is less risk of contamination since the filter is smaller and more manageable during the lysis and extraction process. Be sure to double the DNA value to account for the division when using qPCR.

•Wipe down extraction table before and after every use with RNase. Wipe off pipettes, centrifuge, and tip waste jar.

•Only open tubes with your left hand and only pipette/hold stuff with your right (reverse if you're left handed).

•Be careful to never to move your arm or hand over open tubes, work from right to left.

•Only have one sample or reagent tube open at a time to avoid cross contamination

•Like all steps in the eDNA process use aerosol resistant pipette tips and pipettes specific to the extraction process.

•Aliquot reagents so that your stock doesn't become contaminated.