Assessing the Status of Native Freshwater Mussels (Unionidae) in Los Angeles & Ventura Counties

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California Regional Water Quality Control Board

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October 2018

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Executive Summary

Freshwater mussels have been found to be highly sensitive to ambient ammonia levels (Augspurger et al. 2003). The Environmental Protection Agency (EPA) revised acute and chronic ammonia recommendations to protect mollusk populations in 2013 (USEPA 2013). While North America is a hotspot of freshwater mussel diversity, these aquatic invertebrates have suffered significant declines in recent times and few populations are thought to remain in southern California. This study conducted surveys to evaluate the status of three mussel species, California floater (*Anodonta californiensis*), Western pearlshell mussel (*Margaritifera falcata*) and Western ridged mussel (*Gonidea angulata*) throughout the Los Angeles Region of WQCB (most of Los Angeles and Ventura counties). These mussels were historically present in southern California (Howard et al. 2015) but their populations have been reduced, and potentially extirpated due to habitat destruction, alterations to water systems, and pollution.

We visited over 80 sites throughout Los Angeles and Ventura counties to search for native mussel populations. Many of the sites were located within watersheds where mussels were historically present. Traditional visual surveys were performed in addition to the more recently developed molecular survey technique known as environmental DNA (eDNA) analysis. eDNA analysis involves using molecular methods to detect species-specific DNA sequences from water samples. Water quality analyses were also performed at selected sites to analyze ammonia and other nutrient levels.

The visual surveys did not locate any live mussels; however, shells of the *Anodonta* mussel clade (*A. californiensis/nuttaliana*), which includes the California floater, were found at one site, Malibou Lake. This population was locally abundant in Malibou Lake in 2016 but the mussels appear to have been lost in a winter storm that buried the mussels in sediment prior to the 2017 surveys (M. Hart personal communication 2017). eDNA surveys detected one native species, *M. falcata*, in Castaic Lagoon. This is likely due to mussel tissue, or possibly live larvae, being deposited into Castaic Lagoon via the California State Water Project, as this species has not been noted in the region and is not known to inhabit reservoirs. Our results indicate that native freshwater mussels are likely extirpated in the study area. However, current river and creek restoration plans, such as those underway for the Los Angeles & San Gabriel Rivers Watershed Council, offers a potential strategy for reintroduction of native mussels to some of their historical ranges in the region.

Introduction

In 2013, the U.S. Environmental Protection Agency (EPA) revised standards for ambient ammonia levels in freshwater systems (USEPA 2013). Ammonia occurs naturally in aquatic systems but additional pollution from sources such as wastewater treatment plants, agricultural runoff, animal waste, and other industrial/urban waste, can increase ammonia to levels that are toxic for aquatic life (Driscoll et al. 2003). The EPA recommends maximum ammonia thresholds based on the aquatic species with the lowest tolerance. Previous research indicated that salmonid species were the aquatic fauna most vulnerable to elevated ammonia levels. However, a more recent study indicates that freshwater mussels of the family Unionidae, which were not included in previous research, are more sensitive to ammonia than salmonids (Augspurger et al. 2003). This led to the revision of ammonia standards to an acute ambient total ammonia nitrogen (TAN) level maximum of 17 mg/L and a 30-day chronic average of no more than 1.9 mg/L, to prevent the loss of native mollusk species. Mollusk populations are currently in decline globally (Lydeard et al. 2004), with noted declines and extirpations in California (Coney 1993, Howard et al. 2015, Blevins et al. 2017).

The purpose of this study was to investigate the status of freshwater mussels within the Los Angeles Region (coastal Los Angeles and Ventura Counties), the area under jurisdiction of the Los Angeles Regional Water Quality Control Board (Regional Water Board) and perform water quality analyses to characterize current ammonia levels in relevant waterways. To meet these objectives we followed a multi-step process that included analyzing historic ranges of native mussel taxa, performing visual surveys, analyzing DNA from water samples to detect current presence of mussels, and analyzing standard water quality and nutrient concentrations.

An in-depth literature review was conducted to establish the historical distribution of the targeted native mussel species in southern California. This involved accessing archival records from natural history museums along with historic reports of occurrence and status (Appendix A). We selected sites for our surveys and associated water quality measures based on our literature review and GPS analysis of Los Angeles and Ventura County watersheds. We aimed to select sites containing habitat suitable for mussels.

Our findings from this project can be used to inform management agencies regarding the presence of mussel populations in the study area. Water quality results can be used to estimate nutrient tolerance where mussel populations are present and may indicate areas that are unsuitable for mussel populations due to high levels of ammonia or other nutrients. The study also recommends locations where habitat and water conditions may be suitable for the re-introduction of native freshwater mussels.

Historically, three mussel species occurred in southern California: the California floater (*Anodonta californiensis*), Western pearlshell mussel (*Margaritifera falcata*) and Western ridged mussel (*Gonidea angulata*), of which only *A. californiensis* and *G. angulata* are recorded from the Los Angeles Region (Appendix A). There are few records of native mussels in these counties, and most existing records date back to the early 20th century. Since then southern California has experienced extensive development and extensive alteration of regional rivers that historically supported mussels (e.g. channelization of the Los Angeles & San Gabriel Rivers). Furthermore, all three native mussels have a parasitic larval stage that is reliant on a fish host. Declining populations of native fishes in the region may have thus contributed to the decline of native mussels (Jepsen et al. 2012, Tremblay et al. 2016). We anticipated that native freshwater mussel populations had experienced severe declines in the Los Angeles Region, potentially to the point of regional extirpation, but no comprehensive studies have been conducted to determine if residual or relict populations remain. A complete summary of the historic distribution, habitat requirements, etc. of these native mussels was compiled in the initial stage of this project and is included as Appendix A.

Prior to this study, visual surveys for native mussels described in Howard et al. (2015) did not locate any current native mussel populations in their historic locations in southern California. The only recent sighting was in Malibou Lake, a private artificial lake/reservoir in the Santa Monica Mountains. According to residents, a population of mussels that resemble *A. californiensis* has existed in the lake for decades and are relatively abundant (M. Hart, personal communication, 2017). There are no historical records of *Anodonta* in the Santa Monica Mountains, and it is likely that this population was introduced through fish stocking.

When attempting to detect rare species in a large geographical area, it is important to consider how to maximize capability of detection using state-of-the-art survey methods. Conventional visual surveys are widely used to assess mussel presence, but lack the ability to detect sparse populations. Because of this, we applied recently developed eDNA survey strategies to assess presence of native mussels in the study area. Traditional survey techniques, which require visual encounters through snorkeling and clear bottom buckets, are useful in that they can confirm the species' presence by direct observation and provide means for population size estimation. However, these techniques are limited in the area that can be surveyed due to high manpower and time required. Traditional survey techniques are further limited because the equipment must be properly cleaned between sites to ensure that no invasive species or pathogens are transported to new water systems (CDFW 2013).

To increase the number of sites and area surveyed for native mussels, we sampled for mussel eDNA at each site. eDNA sampling collects the genetic material aquatic animals shed into the water (tissue, blood, mucus, etc.) and, through DNA extraction and amplification via quantitative polymerase chain reaction (qPCR), detects unique DNA sequences specific to the target species (Ficetola et al. 2008).

This survey technique has been shown to be highly effective at detecting aquatic species (Jerde et al. 2011, Smart et al. 2015). Recently eDNA has been applied to monitor for mollusk species, both to detect invasions of non-native species such as the quagga and zebra mussel (Egan et al. 2015, Peñarrubia et al. 2016, Cowart et al. 2017) and to detect rare native mollusks populations (Stoeckle et al. 2015, Dyer and Roderique 2017, Dysthe et al. 2018, Currier et al. 2018, Preece et al. 2018). Surveys comparing traditional and eDNA methods found that eDNA was at least as effective, if not more effective, at detecting mussel presence (Currier et al. 2018) and could delineate the extent of local populations (Dysthe et al. 2018).

DNA is detectable in water for several days to weeks after an organism sheds tissues containing DNA (Thomsen et al. 2012a, Cowart et al. 2017), so positive results indicate current presence. In addition, the DNA travels downstream where it can still be detected (Deiner et al. 2014, Wilcox et al. 2016), effectively expanding the assessment area. By including eDNA surveys, we can cover larger areas upstream of the immediate sampling site. However, this technique does not provide information on the number of individuals present in the population but instead serves as a presence/absence indicator. To determine the population size, it would be necessary to conduct follow-up visual survey in the areas that produced a positive result.

Methods

Study Area

Locations throughout the Los Angeles Region, encompassing coastal drainages of Los Angeles and Ventura counties, were selected as sampling locations for native mussel presence surveys (Map 1). Sites represent all major watersheds, including both the mainstems and major tributaries. Within Los Angeles County, the Los Angeles River watershed, San Gabriel River watershed, and various streams in the Santa Monica Mountains were surveyed. In Ventura County, the Ventura River, Santa Clara River, Calleguas Creek watersheds were surveyed. Efforts were made to include sites with historical records of the native mussels. A list and map of the sample locations is provided in Appendix B. Visits to each site involved collecting water samples for eDNA and water quality analyses followed by traditional visual surveys (which could disturb substrates and alter suspended sediments that would interfere with chemical assays if performed first).

Visual Survey

Visual surveys involved walking the stream bank in search for shells or live specimens. Clear bottom bucket and snorkeling surveys were performed at selected sites. Native mussel species are known to inhabit a range of microhabitat types so five stream sections were included in the visual surveys to cover a range of habitat

types. At sites with low water levels, only bank walks were performed. The surveyors walked slowly along the stream bank, scanning the water and bank for specimens. In-water surveys involved using a clear-bottom bucket in semi-deep areas (approximately 1-2 feet deep) and snorkeling in deep areas (> 2 feet deep), if allowed by water clarity at time of sampling. The surveyors scanned the bottom of the stream systematically to visually confirm the presence of any specimens. Any mussel or fish species found during the survey was recorded, including nonnative mollusks. All gear used was dried or frozen between sites, following California Fish and Wildlife protocols (CADFW 2013), to prevent the introduction of invasive species, such as New Zealand mud snails, to following sites. Visual surveys were completed May through August of 2017.

eDNA Sample Collection

Water samples for eDNA analysis were collected at each site by filtering the water to capture cells the mussels shed into the water. In the initial sampling, water samples were collected for eDNA analysis from the site and either filtered on site or transported on ice to the University of California, Santa Barbara and filtered within 24 hours of collection. Three 500mL water samples were filtered through separate 1.0 µm polycarbonate track-etched (PCTE) filters. For each site, one negative control of 500mL of deionized (DI) water was filtered immediately after the three field samples to serve as a check against contamination between sites. A detailed description of the field collection protocol is presented in Appendix C.

Follow-up sampling was conducted in 2018 at a sub-set of seven sites to confirm prior results, especially if any of the replicated water samples showed a 'positive' result, and particularly to apply a newer eDNA sampling technique using an enclosed filter cartridge (Sterivex™, GP polyethesulfone, 0.22 µm pore size). This method has been shown to increase DNA extracted from water samples (Spens et al. 2017, Cruaud et al. 2017). As with the initial 2017 sampling, three 500mL water samples and a negative control of DI water were filtered for each site. All samples were filtered in the field using single-use equipment to reduce the likelihood of cross-contamination between sites from re-used items. For a detailed description of the sample collection and DNA extraction process used in the initial samples in 2017 and follow-up samples in 2018, see Appendices C, D, E & F.

Mussel Marker Validation

The three primer and probe sets, hereafter referred to as markers, were designed at UC Davis for a similar mussel survey in the Central Valley of California (Preece et al., in review) . These markers were effective in detecting the targeted native mussel species in eDNA samples in field surveys performed in Central Valley. Two of the markers are species-specific to either *G. angulata* or *M. falcata*, while the third detected several species of closely related *Anodonta* mussels, of which only

one species, *A. californiensis/nutalliana*, is known to have occurred in southern California (Howard et al. 2015). We used these markers and performed additional validations to ensure they would effectively detect only the targeted mussel species using our protocols. To confirm the effectiveness of the markers, they were tested using both mussel tissue and field eDNA samples collected from sites with known mussel populations.

Each marker was tested for specificity using DNA from seven different species. The seven species included: the single target species; the two other native mussels; two non-native bivalve species found in southern California, the Asian clam (*Corbicula fluminea*) and quagga mussel (*Dreissena bugensis*); and two amphibians species found in the study area, the introduced American bullfrog (*Lithobates catesbeianus*) and the California newt (*Taricha torosa*). Samples were tested in triplicate using high concentrations of DNA in serial dilutions following DNA quantification using a Nanodrop machine. The DNA used in these assays was extracted and kept in the post-PCR laboratory area where field samples were not processed, in order to avoid possible contamination.

We sequenced the amplicon from the three native mussel species to serve as the known sequences that could be used to compare to any positives from the field samples (protocol details in Appendix G). The amplicon was Sanger sequenced by Eton Biosciences (San Diego, California) and compared to DNA sequences available on the National Center for Biotechnology Information (NCBI) database using the BLAST search to confirm the targeted mussel species.

Water samples were collected from streams in Oregon and northern California with known mussel populations to test detection in field samples. Two sites in Oregon with known *M. falcata* and *Anodonta spp*. populations and one site in Napa County, California with known *G. angulata* were sampled using the initial eDNA field collection protocol.

eDNA Sample Assays

The DNA was extracted from the filters using the phenol chloroform isoamyl alcohol (PCI) extraction protocol modified from Deiner et al. (2015). For a detailed description of the extraction protocol, see Appendix D. Each site was tested in duplicate for the targeted three native mussel species. The three replicates and single negative control eDNA field samples from each field site were run in duplicate in qPCR, so that each site tested six field sample replicates and two negative controls. The first qPCR run included an internal positive control (Taqman Exogenous Internal Positive Control) to check for inhibitors, such as tannins or other contaminants, which can disrupt the qPCR reaction. Samples that displayed inhibition were treated with a OneStep PCR Inhibitor Removal Kit (Zymo). The mussel primer and probe concentrations, as well as the qPCR cycles, were the same

as those used in the maker validation assays (Appendix G). Each plate included DNA quantitative standards that served as positive controls for the targeted mussel species. The standards included three replicates of three concentrations of the targeted mussel DNA in a 1in 10 dilution series, beginning at 0.05 ng. The standards were loaded on the plate in a separate room from where the eDNA field samples were processed and no equipment is shared between rooms. Each qPCR plate also included a minimum of three negative template controls and three DNA extract controls.

A portion of the positive samples was sequenced to confirm the mussel DNA's presence, following the protocol used to sequence the mussel tissue extract. The samples were checked in duplicate for amplification through gel electrophoresis on a 1% agarose gel stained with GelGreen (Biotium). Samples that displayed amplification bands were purified using the Qiagen MinElute PCR Purification Kit, sequenced by Eton Biosciences (San Diego, California), compared to the known DNA sequences, and the NCBI database.

Water Quality Analysis

Twenty-five sites throughout the study area were analyzed for phosphate (PO_4), nitrite (NO_2-N), nitrate (NO_3-N), and total ammonia as nitrogen (NH_3 & NH_4). Sites were selected for sampling based on the habitat suitability from initial mussel surveys. We collected 50mL samples, filtered them through 0.22µm filter, and stored at -4°C until analysis. Samples were analyzed at the UCSB Marine Science Institute nutrient analysis laboratory using flow injection analysis on a QuikChem 8500 (Lachat Instruments).

Results

Eighty-three field sites throughout the Los Angeles Water Quality region were visited in 2017 and 2018. Nine of these sites were dry at the time of visits and therefore not surveyed further, as freshwater mussels require perennial availability of water for survival. The remaining 74 sites were visually surveyed for mussels, and eDNA water samples were collected for DNA detection of native mussels. A map and list of the sites is provided in Appendix B.

Visual Surveys

Live native mussels were not found at any of the survey sites, but non-native mollusk species were observed at several locations (Table 1). Shells of the *Anodonta* mussel clade (*A. californiensis/nuttaliana*), which includes the California floater, were found at one site, Malibou Lake, during the snorkeling survey. Malibou Lake is

an artificial private lake located in the Santa Monica Mountains and stocked with non-native fish species for recreational angling. The specimens found at this location were large for *Anodonta* (Image 1), with the largest specimen 130mm in length. Dr. Arthur Bogan, mollusk curator at North Carolina Museum of Natural Science, confirmed the specimens were *Anodonta californiensis/nuttaliana*. The local residents report that the mussels had been present in the lake for decades but none had been found alive since a large rainstorm in the winter of 2016, which deposited a large amount of sediments in the lake and buried the specimens we recovered. Approximately 20 cm of silt covered the shells of the specimens that we did find during surveys.

Species	**Site**
Anodonta mussel (*Anodonta californiensis/nuttaliana*)*	Malibou Lake
Asian clam (*Corbicula fluminea*)	Malibu Creek site 1
Asian clam (*Corbicula fluminea*)	Malibu Creek site 2
Asian clam (*Corbicula fluminea*)	Conejo Creek site 1
Quaga mussel (*Driessena bugensis*)	Piru Creek site 1
Asian clam (*Corbicula fluminea*)	Piru Creek site 2

Table 1.
Field sites where mussels and clams were found during visual surveys. *Shells only, no live specimens observed.

Image 1.
A. californiensis/nuttaliana, shell collected from Malibou Lake. Ruler displays centimeters for scale.

Mussel Marker Validation

The *M. falcata* and *G. angulata* primer sets were specific to the targeted *M. falcata* or *G. angulata* tissue extracts, respectively. The *Anodonta spp*. primer set amplified the targeted *Anodonta spp*. tissue extract well but also cross-amplified *M. falcata* and *G. angulata* when present in high concentrations (50ng DNA). However, when the non-target DNA was diluted to 0.05ng of *M. falcata* DNA or 5ng of *G. angulata* DNA, it was no longer detected. This indicates a low likelihood of cross-amplification in eDNA samples due to low DNA concentrations. None of the mussel primer sets amplified the non-native mussel, *C. fluminea,* or amphibian DNA.

The DNA extracts from the three mussel species were sequenced to establish known control sequences that can be used to compare to any field positive sample sequences (Table 2). Each sequence was unique and matched only the targeted species sequences available on the NCBI database.

California Floater (*Anodonta spp.*) (117 base pairs, including primers): GGAGAGTGGTGTTGGTACTGGTTGGACGGTATATCCACCTTTATCTGGAAATGTTGCTCATTC TGGGGCTTCTGTGGATTTGGCCATTTTCTCTTTACATCTTGCTGGTGCYTCTTC
Western Ridged (*Gonidea angulata*) (98 base pairs, including primers): GGTTTTGATTACTTGTACCGGCTCTTTTTTTATTATTAAGGTCTTCATTAGTAGAGAGTGGTG TTGGGACTGGTTGGACAGTGTATCCGCCGTTGTCT
Western Pearlshell (*Margaritifera falcata*) (84 base pairs, including primers): TAATATGCGCTCCCCTGGCGTAGTTGCTGAACGAATCCATTKTTCGTGTGAGCCGTCACTGTG ACGGCTATTTTGTTAGTGGCG

Table 2. DNA sequences of the targeted native mussel species.

The markers successfully detected the targeted DNA presence at low concentrations when sampled within detection assay limits. The *Anodonta spp.* marker set detected the targeted DNA in all three replicates to $2.5x10^{-4}$ ng and one replicate at $5x10^{-5}$ ng; *M. falcata* marker detected all three replicates at $5x10^{-6}$ ng and nothing lower; and *G. angulata* marker detected all three replicates at $2.5x10^{-6}$ and two at $5x10^{-6}$ ng.

eDNA Field Samples

The eDNA samples collected from the positive control site in Oregon and northern California with known mussel populations tested positive for the targeted mussel species. The amplicons were successfully sequenced to confirm the targeted mussel DNA matched the known sequence for each mussel species (Table 3). The *Anodonta spp.* primer set did not amplify the *M. falcata* DNA that was present in the Johnson Creek samples. This indicates that the *Anodonta spp.* primer set is unlikely to cross-amplify the other mussel species, likely because the non-target DNA concentrations are too low and the non-target amplification is not efficient enough to cause a false positive.

	Western pearlshell (*Margaritifera falcata*)	Oregon/California floater (*Anodonta spp.*)	Western ridged (*Gonidea angulata*)
Johnson Creek, OR	**+**	-	-
Crystal Spring Creek, OR	**+**	**+**	-
Napa River, CA	-	**+**	**+**

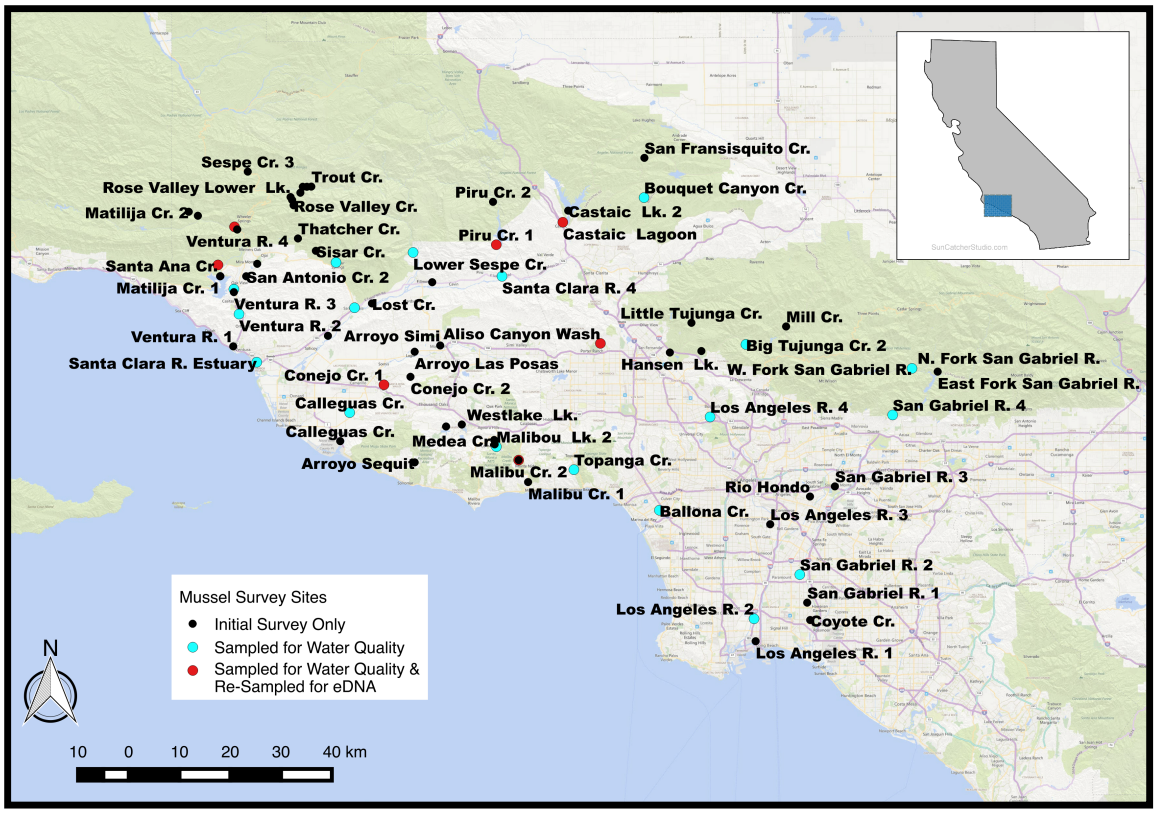
Table 3. Environmental DNA detection of the native mussel species from the Oregon field sites using qPCR.

In the initial eDNA surveys, less than 2% of the eDNA samples collected in the study area were positive for a mussel species. Out of these positives, 23% were field negative controls, suggesting some level of false positives. None of the extract controls (n=20; 120 qPCR runs) or negative template controls included in each plate (n=16; 96 qPCR runs) were positive. Three sites had multiple positives for a given species from the six qPCR replicates. These three sites were sequenced and in only

two sites did sequences match mussel DNA: Piru Creek for *G. angulata* and Santa Ana Creek for both *G. angulata* and *M. falcata*.

Positives occurred as frequently in the field negative controls as the stream samples, suggesting contamination rather than true positives. The positives from the 2017 samples could have resulted from field equipment contaminated with mussel DNA after being used at positive control sites, due to the re-use of the filter funnels. In response, we changed the field collection protocol for the follow-up Year 2 sampling (Appendix E) to use enclosed filter cartridges and single-use collection material to reduce the likelihood of contamination from field equipment.

Seven field sites (Map 1) were selected for eDNA re-sampling. The sites were selected because they were positive for at least one of the native mussels, and visits to the sites showed the potential for mussel habitat. The water samples were collected in the field using the enclosed filter cartridges and single-use collection materials. The same volume of water and number of replicates were collected at each site, as in the initial protocol. The DNA was extracted from the filters using a modified version of the original protocol to adapt it to the enclosed cartridge. See Appendix E and F for a complete description of the sample collection and DNA extraction protocols using the enclosed filter cartridges. The re-sampled sites yielded similar or greater DNA concentrations when compared to the initial sampling event in all but one site (Table 4), suggesting that the enclosed cartridge filter method is as or more efficiency for capturing eDNA.



Map 1. Map of Field Sites. The map displays only the sites that had water during the initial visual and eDNA mussel surveys. The colors indicate which of these sites were also sampled for water quality (blue) and which were sampled for water quality and additional eDNA sampling (red).

Sample Site	Initial Sampling (ng DNA/uL ± SD)	Re-Sampling (ng DNA/uL ± SD)
Castaic Lagoon	90.6 ± 12.4	134.5 ± 48.7
Malibu Creek site 2	18.5 ± 8.5	23.4 ± 6.2
Aliso Canyon Wash	6.7 ± 1.8	135.3 ±59.3
Conejo Creek site 1	175.9 ± 97.9	130.4 ± 15.2
North Fork Matilija site 2	6.2 ± 2.4	187.1 ± 29.8
Piru Creek site 1	57.2 ± 13.0	11.8 ± 3.1
Santa Ana Creek	5.8 ± 3.5	22.8 ± 4.9

Table 4. Yields from DNA extractions
The concentration of DNA from the seven sites selected for eDNA re-sampling. Two DNA concentrations are listed, one from the initial filter funnel protocol, the second from the more recent sampling event that used the enclosed filter cartridge protocol.

Of the seven field sites re-sampled for eDNA, only one site, Castaic Lagoon, was positive for a native mussel species, the western pearlshell (*Margaritifera falcata*). While only one of the six replicates from Castaic Lake was positive from this sampling event, which could indicate a false positive, the initial eDNA samples produced three positive replicates, the most positives from any field site.

Additional eDNA samples were collected from Malibou Lake to determine if the DNA from mussels was present in the top layer of sediment in the lake. DNA can settle in higher concentration in sediment, potentially making it easier to detect a rare species (Buxton et al. 2018). Six benthic samples were collected from submerged areas around the edge of the lake and tested for the California floater mussel clade (*Anodonta*). The samples were processed using a commercial kit designed to extract DNA from soils (Qiagen DNeasy PowerMax Soil Kit) and tested in duplicate. The samples did not yield any positives for the California floater, validating that live mussels are no longer present in this water body despite shells from prior occupation.

Water Quality Analysis

Twenty-five field sites were tested for water nutrients, phosphate (PO_4), nitrite (NO_2-N), nitrate (NO_3-N), and total ammonia nitrogen (TAN) (NH_3 & NH_4), regularly used to evaluate water quality (Table 5). None of the sites exceeded the stricter total ammonia nitrogen acute (17 mg/L) recommendation in the 2013 update. While only a single water sample was tested at each site, all ammonia levels were below the recommended chronic 30-day average (1.9 mg/L). .

Site Name	Phosphate (mg/L)	Nitrite (mg/L)	Nitrate (mg/L)	TAN (mg/L)
Alison Canyon Wash	0.07	0.00	0.29	0.02
Ballona Creek	0.35	0.04	0.61	0.00
Bouquet Canyon Creek	0.08	0.00	0.00	0.00
Big Tujunga Creek, site 1	0.04	0.00	0.01	0.00
Calleguas Creek	8.04	0.03	6.30	0.02
Conejo Creek, site 1	5.15	0.07	6.65	0.09
Los Angeles River, site 2	0.76	0.14	3.52	0.14
Los Angeles River, site 4	2.91	0.10	4.86	0.77
Castaic Lagoon	0.01	0.00	0.01	0.00
Malibu Creek, site 2	0.33	0.00	0.01	0.01
Malibou Lake	0.27	0.00	0.01	0.01
North Fork Matilija, site 2	0.05	0.00	0.07	0.01
Piru Creek site 1	0.03	0.00	0.07	0.01
San Antonio Creek, site 1	0.04	0.00	0.10	0.00
Santa Clara River, site 1	0.14	0.04	10.56	0.01
Santa Clara River, site 3	0.05	0.03	1.44	0.01
Santa Clara River, site 4	1.64	0.00	2.02	0.00
Lower Sespe Creek	0.05	0.00	0.01	0.00
San Gabriel River, site 2	0.20	0.05	1.95	1.09
San Gabriel River, site 4	0.00	0.00	0.01	0.00
West Fork San Gabriel River	0.02	0.00	0.32	0.01

Santa Paula Creek	0.04	0.00	0.07	0.04
Santa Ana Creek	0.05	0.00	0.02	0.00
Topanga Creek	0.11	0.00	0.01	0.00
Ventura River, site 2	0.12	0.02	2.27	0.02

Table 5. Water analysis
Concentrations of phosphate (PO_4), nitrite (NO_2-N), nitrate (NO_3-N), and total ammonia nitrogen (TAN) (NH_3 & NH_4).

Interpretation of Data

The findings suggest that native mussels are likely to have been extirpated from the Los Angeles Region. Of the more than 83 field sites visited, visual surveys failed to detect any native freshwater mussels and eDNA detected western pearlshell (*M. falcata*) in Castaic Lagoon only. However, Castaic Lagoon is fed from Castaic Lake, which is part of the California State Water Project (SWP). The water in the SWP is sourced from northern California where native mussels are more abundant. DNA sloughed off of the animal can persist for several weeks (Thomsen et al. 2012b, Cowart et al. 2017), making it possible that mussel tissue, or possibly live larvae, traveled through the SWP and were deposited into Castaic Lagoon where they were detected using eDNA techniques.

Malibou Lake, located in the Santa Monica Mountains, is reported to have supported a population of *Anodonta californiensis/nuttaliana* for decades, according to local residents, but the failure to find any live specimens or DNA on repeated visits suggests that the population has been recently extirpated. Local residents and maintenance staff who complete work in and around the lake have not observed a live specimen since 2016 when a winter storm deposited large volumes of silt into the lake (M. Hart, personal communication, 2017). Neither visual nor repeated eDNA surveys beginning in 2017 found evidence of live specimens; however, several shells were recovered from the lake bottom, under approximately 20 cm of mud. It is possible that the 2016 storms smothered the adult mussels. Subsequent fish die-offs observed in early 2017 may have killed many of the mussel's fish hosts, eliminating or reducing mussel larvae.

Although we did not find any mussels on our surveys, we recommend continued monitoring of Malibou Lake for *Anodonta spp.* in coming years. It is possible that some mussel glochidia (larvae) that were attached to fish survived in the lake and may repopulate this water body. If specimens are found, it would be useful to conduct a complete genetic analysis to evaluate where the mussels are from.

The total ammonia-nitrogen levels in the sited samples are not above the recommended levels provided in the 2013 EPA guidelines (US Environmental Protection Agency 2013). Other nutrients, including phosphate and nitrate were high in some locations (Table 5), such as Conejo Creek, where agriculture borders

the stream and fertilizer runoff is likely increasing the nutrient levels. The low nutrient levels found at most sites indicate that water pollution is not, at least at the levels measured on a single visit, responsible for the loss of native mussels. Historic water quality levels and seasonal water quality variation was beyond the scope of this project, but under current conditions, the water quality appears suitable to support mussel populations. Non-native mussels, quagga mussels and Asian clams, were found in abundance in multiple locations, further indicating that water quality may be suitable to mollusks. However, further study would be necessary to evaluate differences in tolerance between the invasive and native mussels. In addition, research has suggested that the larval stages of freshwater mussels are more sensitive to ammonia than adults and population may have difficulty recruiting in high ammonia environments (Strayer and Malcom 2012)

Factors other than pollution levels may be responsible for the loss of freshwater mussels in the region. Native mussels have complex life cycles with an obligate parasitic glochidia (larval) stage that relies upon a fish host. The loss of native fish in southern California (Moyle and Williams 1990, Dagit et al. 2017) can make it more difficult for mussel glochidia to find a host and reach adulthood. Some mussel species have been documented to successfully use non-native fish hosts (Trdan and Hoeh 1982, Spring Rivers 2007) but little work has been performed evaluate the success of *A. californiensis*, *G. angulata*, and *M. falcata* in using common non-indigenous species, such as bluegills (*Lepomis macrochirus*), in southern California areas.

Urban development has also likely contributed to the loss of native mussel populations in southern California. For example, historically *A. californiensis* were found in the Los Angeles River and *G. angulata* were found in Ballona Creeks (LACM, Ingram 1948), both of which have been channelized in concrete. Other historical records describe abundant water in areas that are now much drier. One historic *G. angulata* record refers to wetlands in what is now the downtown Los Angeles area (LACM, Ingram 1948) and has long since been drained. Further alterations of river flows that disrupt natural flows or remove refuges, can result in loss of these long-lived species (Vannote and Minshall 1982, Haag and Williams 2013). Some populations of native mussels are still extant in southern California in water systems such as the Kern River, but few populations have been found in recent surveys (Howard et al. 2015). Malibou Lake serves as an example of the mussel's ability to persist in southern California for extended periods of time, even in artificial locations, and indicates how quickly small populations can be lost because of rare environmental events, such as sediment-producing storms.

Small isolated populations in southern California are at risk of extinction from natural stochastic events and without larger source populations, have no ability to reestablish once lost. To reduce the risk of the complete loss of mussels in southern California, it will be necessary to reintroduce them to their historic stream systems. Introducing mussels into isolated areas only serves to slightly increase the population size and does not overcome the problem of stochastic risk. Alternatively,

reintroductions into larger river stems can better serve the system by developing into an established and more resilient source population (Haag and Williams 2013). Recent advancements in techniques to produce large numbers of mussels in captive environments (e.g. Owen et al. 2010, Lima et al. 2012, Ma et al. 2017), make it possible to produce the number of young mussels for reintroduction projects.

Habitat evaluation will be necessary prior to reintroduction efforts. Successful reintroduction depends upon four main habitat factors: (1) adequate perennial water, (2) the availability of suitable fish hosts, and (3) the requisite type of substrate (4) water quality. While the increasing frequency of drought conditions in the region limits the number of sites that will satisfy the first condition, current restoration efforts underway in the region may help to achieve the other factors. The Los Angeles River is currently undergoing several restoration efforts (City of Los Angeles 2007) that could contribute to increasing habitat suitability. Plans include restoring natural substrate composition, increasing groundwater recharge, reduce non-point source pollution, and slowing the flow of the river, which would provide suitable habitat for mussels and refuge areas for fish species. Coordinating mussel reintroduction efforts with current ongoing river and creek restoration plans offers a potential strategy for targeted and successful reintroduction of the mussel to several key areas in the region. Similarly, in the Santa Clara watershed of Ventura County, the State Coastal Conservancy, The Nature Conservancy and the Santa Clara River Conservancy are cooperating in an effort to promote restoration of native flora and fauna in this ecosystem; similar efforts are underway in the Ventura River watershed as well. Unlike the geographically similar Los Angeles/San Gabriel system, the Santa Clara retains major elements of its natural hydrological functioning so this system provides an even greater opportunity to promote the ecological and hydrologic conditions that could support a re-introduced population of native freshwater mussels. While no historic evidence support their previous presence, the lack of sampling in an earlier era could be responsible for absence of such records and along with the presence of suitable habitat, it is quite likely that this system, as well as the Ventura system, could in the future provide opportunity to expand the range of severely depleted freshwater mussels in the Los Angeles region and more broadly, for California.

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Appendices

Appendix A. Ecology & Historic Distribution of Native Freshwater Mussels in Southern California

Native Freshwater Mussels of California[1]

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Ammonia Criteria and Freshwater Mussels

In 2013 the U.S. Environmental Protection Agency (EPA) released an update to the national ambient water quality criteria recommendations for ammonia (EPA 2013a). Ammonia, a common freshwater pollutant, can be toxic to aquatic life, but especially so to freshwater mussels. Wastewater treatment facilities are a direct source of ammonia to freshwater habitats; other sources include agricultural runoff and animal and industrial wastes. Recent studies have determined that freshwater mussels are much more sensitive to ammonia than fish and most other aquatic invertebrates, and EPA's revised criteria are more stringent than earlier standards. Because states can calculate site-specific criteria for ammonia that are dependent on the presence of freshwater mussels at a site or within a waterbody, state agencies may choose to assess the distribution of freshwater mussels to establish ammonia criteria (EPA 2013a, b). Because knowledge regarding the mussel species present in California, including their life history and distribution, is important for understanding the potential for water quality criteria to influence mussels and vice versa, and the following sections provide a summary of freshwater mussels in general, as well as California's native species.

Introduction to Freshwater Mussels

Freshwater mussels (hereafter "mussels") are a type of bivalve mollusk found in perennial freshwater habitats. The outer shell of a mussel is comprised of two valves ("bivalve") connected by a hinge ligament and adductor muscles. The animal inside the shell has a muscular foot used to anchor the mussel when burrowed into river, stream, or lake banks and beds. Mussels filter water through gills, collecting food such as algae and plankton and depositing nutrients that are later taken up by other benthic species. Their unique association with host fish (see "Life History" below) is one main difference between mussels in the order Unionoida (which includes North American species of mussel) and bivalves invasive to North America in the order Veneroida (e.g., zebra and quagga mussels). Globally, mussels in the class Unionoida number nearly 890 species, and North America is a "hotspot"

[1] *This material is reproduced in part from Fact Sheets written by Emilie Blevins for the USFS Pacific Southwest Region, with funding from FS Agreement Number 2016-CS-11052007-087.*

for freshwater mussel diversity, with more than 300 species documented. This totals more than a third of all species globally (Graf and Cummings 2015).

Species can vary in appearance, life history, and distribution, but as a whole, mussels play a similar and important role in aquatic habitat and provide valuable ecosystem services. As filter feeders, mussels filter large quantities of water, siphoning out bacteria, phytoplankton and zooplankton, fungal spores, and algae, although the specific dietary needs of mussels are relatively unknown (Haag 2012). Howard and Cuffey (2006a) found that a bed of western pearlshell (~12,000 individuals) in a reach of the South Fork Eel River, CA, could remove as much as 4 kg (dry mass) of suspended material each day, making organic material available to other aquatic organisms through biodeposition (Vaughn 2010). Kreeger (2011) found that a bed of mussels (~1,000 individuals of any of the western North American species) could remove about 2 kg (dry mass) of suspended solids each year from rivers in an Oregon watershed. Where mussels occur in beds, much of the water column may be filtered as it flows over, especially during lower flows and at higher densities, improving water quality and clarity (Vaughn et al 2004). Mussels improve and provide habitat for other aquatic invertebrates and are food for aquatic and terrestrial wildlife (Howard and Cuffey 2006a; Vaughn 2010; Limm and Power 2011; Scordino et al. 2016). Freshwater mussels also have significant cultural importance to many Native Americans, especially in the Pacific Northwest where they served as a traditional food resource (CTUIR 2015; Norgaard et al. 2013).

The western U.S. (including watersheds west of the Continental Divide) is home to many fewer species than in the eastern U.S. Among western species are those in the families Unionidae (genera: *Anodonta*, *Gonidea*) and Margaritiferidae (genus: *Margaritifera*). The number of western mussel species has been the subject of considerable debate, and historically numerous species were described from each genus on the basis of variation in shell morphology. Taxonomy has stabilized for two of the three western genera, with the species *Margaritifera falcata* and *Gonidea angulata* each being the only western North American representatives from their genus. In fact, *Gonidea angulata* is the only extant member of its genus globally.

The number of species of western *Anodonta*, in comparison, is still debated, although recent taxonomists (Turgeon et al. 1998; Graf and Cummings 2007) have typically recognized 6 species (Table 1). Genetic and morphological analyses by Chong et al. (2008) and Mock et al. (2010) indicate that the members of Clade 1 (*A. californiensis* and *A. nuttalliana*) likely belong to a single species, properly identified as the winged floater (*Anodonta nuttalliana*). However, genetic sampling was not as complete for members of Clade 2, and further research is necessary to understand the number of species in that clade. The woebegone floater (*Anodonta dejecta*) has an uncertain status stemming from a complicated taxonomic history. However, recent analyses of genetic samples of southwestern *Anodonta* (the historic range of *A. dejecta*) have not recovered a species of *Anodonta* distinct from Clade 1 (Mock et al. 2010; Culver et al. 2012), which also ranges in the southwestern U.S.

Table 1. Western *Anodonta* recognized in recent checklists and their phylogenetic relationships (clades) based on Chong et al. (2008).

Common Name	Scientific Name	Clade
California floater	*Anodonta californiensis*	1
winged floater	*Anodonta nuttalliana*	1
western floater	*Anodonta kennerlyi*	2
Oregon floater	*Anodonta oregonensis*	2
Yukon floater	*Anodonta beringiana*	3
woebegone floater	*Anodonta dejecta*	undetermined

Conservation Status

Freshwater mussels are among the most imperiled animals on earth (Lydeard et al. 2004). In North America, nearly 30 species are thought to have become extinct over the last 100 years, and 65% of remaining species are imperiled (Haag and Williams 2014). Western North American mussels are generally widespread compared to eastern species and still often occur in multiple watersheds of western states. However, recently completed extinction risk assessments for *M. falcata*, *G. angulata*, and *Anodonta* clades 1 and 2 indicate that western mussels have also declined in distribution (Table 2; Blevins et al. 2016a-d).

Mussels are extremely sensitive to habitat alteration and destruction, and changes in the natural flow regime (timing, volume, and rate), as well as the general availability of water, have been pervasive in western U.S. waters since widespread settlement in the 1800s. Future impacts as a result of increased demand, as well as reduced reliability and availability under climate change, are anticipated (Richter et al. 2016). General threats to mussels in North America include abiotic (impoundments, shoreline or channel modification, restoration activities, dredging and mining, reduced water quality, sedimentation and scouring, and water withdrawal and diversion) and biotic (livestock grazing in riparian areas, the introduction of nonnative plant, fish and invertebrate species, and loss or decline of host fish) impacts (reviewed in Jepsen et al. 2012).

Table 2. Conservation status of western mussels according to the IUCN Red List and NatureServe.

Species	IUCN Red List Status	NatureServe California Status
Margaritifera falcata	Near Threatened	S1S2
Gonidea angulata	Vulnerable	S1S2
Anodonta clade 1	Vulnerable	*A. nuttalliana:* SNR*; A. californiensis:* S2?
Anodonta clade 2	Least Concern	*A. oregonensis*: S2?; *A. kennerlyi*: SNR

Life History

Mussels are generally inconspicuous members of the aquatic community whose presence may only be noticed when shells are washed on banks or found in piles (predator middens) onshore. In contrast, live mussels are typically partially buried in the sediment and are relatively sessile as adults. Mussels can be found in small numbers or in dense aggregations ("mussel beds") in streams, rivers, lakes, and ponds (Nedeau et al. 2009).

Adult mussels filter oxygen and food from water, and during breeding, male mussels also release sperm that is filtered by females (Figure 1). Within specialized chambers of the gill known as marsupia, embryos develop into larvae termed glochidia. Glochidia are released,

often as a mucus mass called a conglutinate. Glochidia, which are generally obligatory parasites of fish, require a period of encystment to complete development. Glochidia attach to host fish gills, fins, head, and flanks (D'Eliscu 1972; Moles 1983; Martel and Lauzon-Guay 2005; Spring Rivers 2007; O'Brien et al. 2013; Maine et al. 2016). Host fish vary by mussel species (see next sections), but following metamorphosis, juvenile mussels excyst from their fish host and settle into the bottom substrate where they grow and mature. Juvenile mussels may be difficult to observe, as they are quite small compared to adults and may be more deeply buried (Howard and Cuffey 2006b). The timing of reproduction, age at maturity, and maximum lifespan of mussels all vary by species.

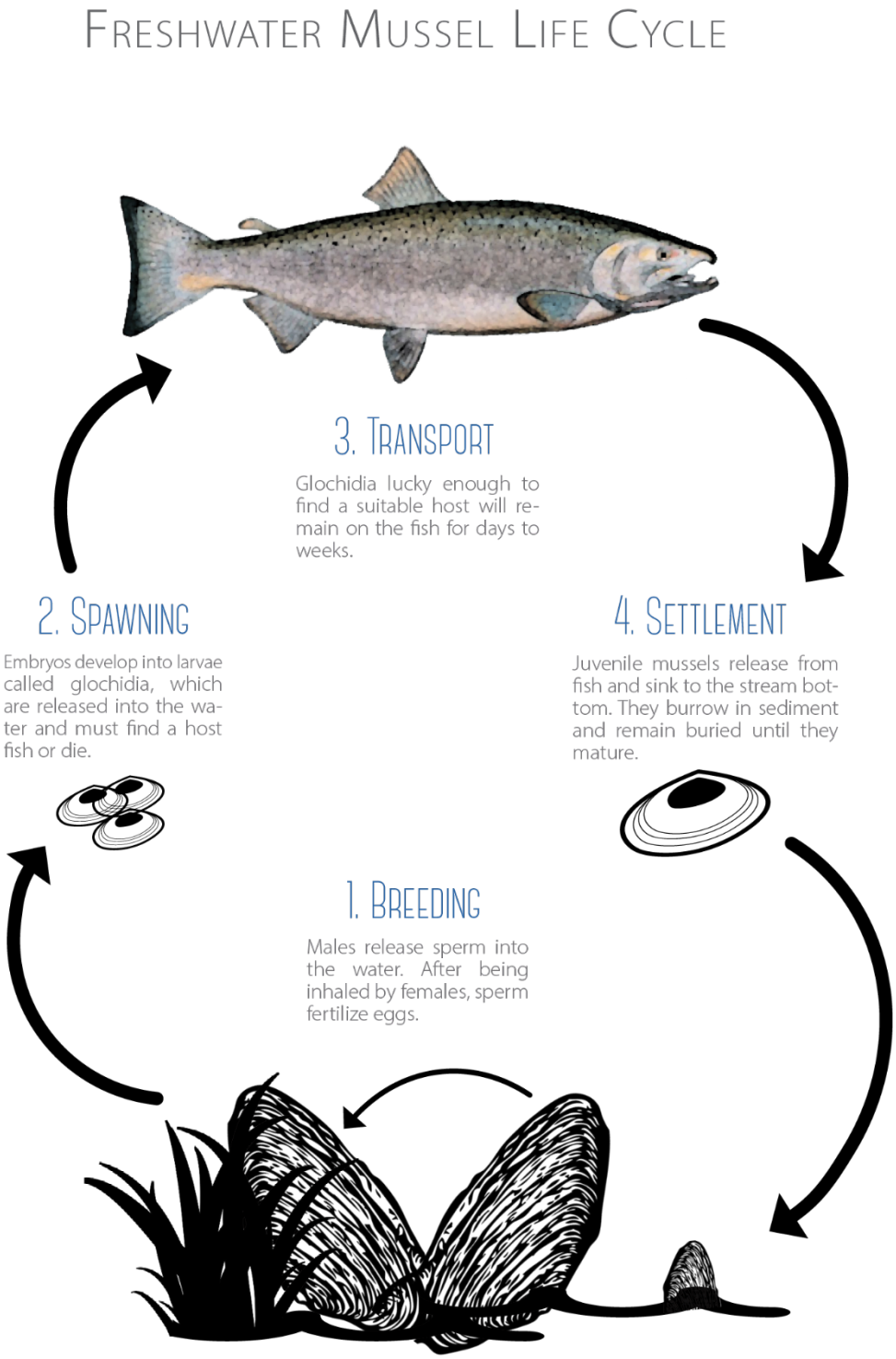


Figure 1. Freshwater mussel life cycle. Diagram created by Michele Blackburn, Xerces Society. Image Credits: Salmon, Wikimedia Commons; small mussel icon, Daniel Gamage, The Noun Project; mussel block print, Patrick Norton, Crystal Springs Partnership; grass, bryn mackenzie, The Noun Project.

Mussels native to California

Margaritifera falcata

Margaritifera falcata is known by the common name "western pearlshell." The species is characterized by an elongate outer shell up to five inches in length, outwardly light brown to black in coloration, and with a curved dorsal margin and slightly arcuate ventral margin (Figure 2; Nedeau et al. 2009). The nacre (the mother-of-pearl interior of a shell) can vary in color, ranging from "peach blossom" to "salmon red and dull purple…often a beautiful, rich purple in fresh, unfaded specimens" (Henderson 1929). One triangular-shaped pseudocardinal tooth is present on the right valve, while two are present on the left; one lateral tooth is present on each valve, though it may be difficult to distinguish (Nedeau et al. 2009). Underwater, papillae appear as fringes along the incurrent opening and mussels are oriented upright and partially buried.

M. falcata, like other members of the family Margaritiferidae have relatively long lifespans; maximum age estimates meet or exceed 100 years, though more commonly older individuals are <100 years in age (Vannote and Minshall 1982; Stock 1996; Toy 1998; Starkey 2015; Allard et al. 2015). Age at sexual maturity is also tied to growth rate; Toy (1998) reports sexual maturity between 9 and 12 years of age and >36 mm in size. Based on Toy's estimates, sexual maturity would occur closer to the age of 45 years in Merrill Creek, OR, where the growth rate is relatively slow (Allard et al. 2015).

Figure 2. Western pearlshell, *Margaritifera falcata*, in typical habitat. Photo credit: Roger Tabor, USFWS. Photo used under Creative Commons Public License (CCPL Attribution-NonCommercial 2.0).

The species occurs in perennial rivers, streams and creeks, inhabiting areas characterized by boulders and gravel substrate, and including sand, silt and clay (summarized by Roscoe and Redelings 1964). Where ranges and habitat overlap, the western pearlshell may co-occur with other species of mussels. Stability of habitat, including low velocity, low gradient, low shear stress, and protected substrate is typical, and the species generally inhabits areas with stones or boulders or occupies eddies or pools protected from scouring flows; it may also be found in riffles, runs, pools or glides (Howard and Cuffey 2003; Vannote and Minshall 1982; Stone et al. 2004; Hegeman 2012; Davis et al. 2013; May and Pryor 2015). In the Upper Truckee River, CA, Howard (2013) reported the species from a variety of habitats within a single reach, including undercut bank, thalweg, run, and riprap and cobble with sand and gravel substrate. In a study comparing mussel and spawning salmon habitat in the Trinity River, CA, May and Pryor (2016) found that mussels were typically found in deeper, finer grained substrate, and occurred closer to vegetated streambanks and in lower velocity areas.

M. falcata specializes on salmonid host fishes, including natives like Chinook (*Oncorhynchus tshawytscha*), coho (*Oncorhynchus kisutch*), cutthroat trout (*Oncorhynchus clarkii*), and rainbow/steelhead trout (*Oncorhynchus mykiss*) (Murphy 1942; Karna and Millemann 1978). Other potential host fish are reviewed in Blevins et al. (submitted). The species can occur in dense beds containing thousands of individuals; for example, Murphy (1942) reported a bed of 20,000 mussels in the Truckee River, CA. May and Pryor (2016) estimated approximately 141,000 western pearlshell in a 1.25 km reach of the Trinity River, CA, and a recent restoration project in the Upper Truckee River, CA, revealed 26,000 western pearlshell inhabiting a 2,255 m (7,400 ft.) reach of the river (LTBMU, unpublished data). However, the species may occur singly or in much smaller aggregations; Howard and Cuffey (2003) found typical aggregation sizes between 50 and 100 individuals in the South Fork Eel River, CA.

A recent assessment of rangewide extinction risk based on trends in occurrence suggests that the species has declined by as much as 17% across its range (Blevins et al. 2016a). In California, Western Mollusk Sciences (2008), Howard (2010), and Howard et al. (2015) report population extirpations from 65% of resurveyed historical sites, as well as a large decline (a bed containing 20,000 individuals in a 2 km stretch of river in the 1940s but only ~150 in the 2000s) in the lower Truckee River. Howard (2010) observed the species at four sites (three of which were historic) in the Northern California ecoregion, although the species was not documented during surveys at three additional historic sites. Howard (2010) notes that this ecoregion likely contains the best freshwater mussel habitat in the state and is the least hydrologically-altered. The species was also observed at 20 sites (seven of which were historic) in the Sacramento-San Joaquin ecoregion, though the species was not documented during surveys at four additional historic sites.

Gonidea angulata

Gonidea angulata is known by the common name "western ridged mussel." The species is characterized by an outer shell up to five inches in length, outwardly yellowish-brown to black and thick, obovate to trapezoidal in shape (Figure 3; Nedeau et al. 2009). A prominent ridge is visible along the outer shell running from the beak to the posterior margin, although its prominence is variable with some specimens nearly lacking the ridge or having two ridges (Sowerby 1869; Hemphill 1891; Dall 1908). One pseudocardinal tooth is typically present on each valve, but a lateral tooth is absent (Nedeau et al. 2009). Underwater, papillae appear bifurcated along the incurrent opening and mussels may be almost completely buried.

G. angulata may live 20 to 30 years (Mageroy 2015), though published observations may underestimate maximum age. Vannote and Minshall (1982) studied population characteristics of the species in the Salmon River, ID, and among habitat types the authors reported age ranges of 10 to 22 years (large, block-boulder-controlled reaches), 12 to 18 years (cobble/boulder-shielded runs), and 12 to 24 years (sand and gravel bars). Individuals are thought to reach sexual maturity around the age of seven years based on growth rate observations, though mussel growth rate can vary significantly among populations (Mageroy 2015).

Figure 3. Western ridged mussel, *Gonidea angulata*, buried in sediment. A caddisfly also rests on the shell. Photo credit: Roger Tabor, USFWS. Used with permission.

The species is reported from a variety of habitats, including perennial rivers, lakes and reservoirs. Where ranges and habitat overlap, the western ridged mussel may co-occur with other species of mussels. In both rivers and lakes, the species is often found in well-oxygenated substrate, areas of constant flow, and waters generally <3 m deep (COSEWIC 2010). Spring Rivers (2007) report finding *G. angulata* "tightly wedged between boulders or in cracks of bedrock or diatomaceous earth… In locations where sand and silt were abundant, they were deeply burrowed and tightly anchored into the substrate with only their siphons exposed;" mussels were observed in similar habitat in B.C. (Stanton et al. 2012). O'Brien et al. (2004) found the species "in the riffle sections…tightly anchored between cobble and boulders such that their shells had formed the shape of the rock they grew up against." However, in Idaho, *G. angulata* was more abundant in areas with sand and gravel bars, and less abundant in boulder dominated reaches (Vannote and Minshall 1982). Hemphill (1891) observed that the species was burrowed in "beds of compact gravel and sand" and "seemed to prefer the steep sides of banks that led into deep water as burrowing places, rather than the flat bars, although some were found in the latter places." Lake habitat substrate where the species has been found ranges from "large cobble, gravel and sandy openings, muddy sediments with sparse vegetation, cobble and gravel over sand, and areas where sediment became turbid when disturbed" (Fisheries and Oceans Canada 2011).

G. angulata use a number of different fish, including natives like hardhead (*Mylopharodon conocephalus*), pit sculpin (*Cottus pitensis*), and tule perch (*Hysterocarpus traski*) (Spring Rivers 2007). Other potential host fish include species of dace and sculpin, as well as other species (reviewed in Blevins et al. (submitted)). The species can occur in small numbers or in dense aggregations. In northern California, Howard (2010) reports that when found, *G. angulata* were often "sparsely dispersed and not found in dense beds," though she notes that at three sites on the Klamath National Forest (Klamath River) and one site on the Modoc National Forest (Pit River, 50 m-long pool), *G. angulata* numbered in the thousands and were "densely packed near channel banks."

A recent assessment of rangewide extinction risk based on trends in occurrence suggests that the species has declined by as much as 43% across its range (Blevins et al. 2016b). In California, Taylor (1981) and Coney (1993) considered *G. angulata* to be extirpated from the southern portion of the state and most of the Central Valley, an observation also confirmed by Howard et al. (2015). Howard (2010) observed the species at seven sites (six of which were historic) in the Northern California ecoregion, noting that it likely contains the best freshwater mussel habitat in the state and is the least hydrologically-altered. The species was also observed at eight sites (five of which were historic) in the Sacramento-San Joaquin ecoregion, though Howard et al. (2015) notes the species is extant at only 55% of resurveyed historical sites in the state.

Anodonta species

Mussels in California belonging to the genus *Anodonta* include those in clade 1 and clade 2 (*Anodonta californiensis, Anodonta nuttalliana, Anodonta oregonensis*, and *Anodonta kennerlyi*), although *A. kennerlyi* has rarely been reported from the state (Xerces/CTUIR 2017a), and Anodonta clade 2 (*Anodonta oregonensis* and *Anodonta kennerlyi*) probably did not occur in southern California based on a review of museum specimens (Xerces/CTUIR 2017b). *Anodonta* species are variable in shell morphology and require genetic analyses to confirm clade membership, although research by Hegeman et al. (unpublished data) has demonstrated that the length to height ratio may be useful for determining clade; their work has shown that shells with a ratio <1.805 typically belong to the *A. nuttalliana* clade, while shells with a ration >1.805 typically belong to the *A. oregonensis/kennerlyi* clade. In general, *Anodonta* shells are characterized as thin and fragile, usually four to five inches in length, and outwardly yellow, green, brown, or black. The shape varies greatly from elliptical to ovate. A "wing" may be present adjacent to the hinge posteriorly and can range greatly in height. *Anodonta* have no hinge teeth (Figure 4; Nedeau et al. 2009). Underwater, papillae appear singly along the incurrent opening (Figure 5).

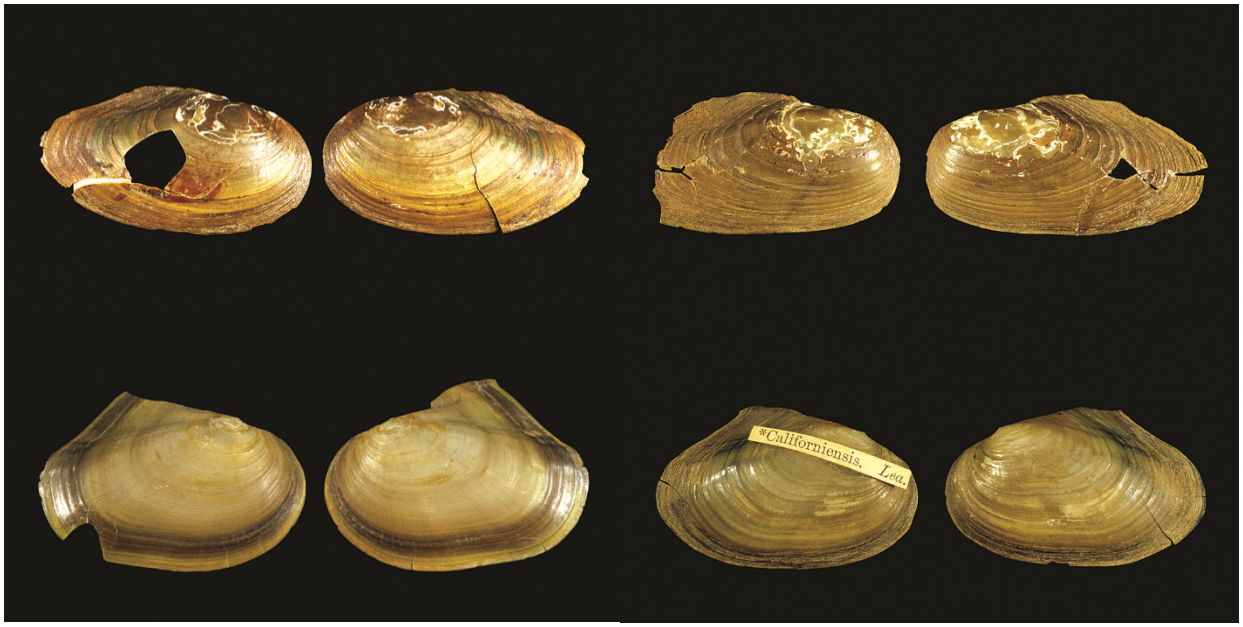


Figure 4. Exterior view of floater mussel shells from original type specimens (from left): Top: *A. oregonensis, A. kennerlyi.* Bottom: *A. nuttalliana, A. californiensis*. Photo credit: Sheila Nadimi, CTUIR. Used with permission.

Anodonta and closely related species appear to grow quickly, maturing in 4 to 5 years, with a maximum life span of ~15 years (Heard 1975; Dudgeon and Morton 1983), though *A. beringiana* (clade 3) has been reported as old as 20 years in age (Kendall et al. 2010) and growth rate of western mussels can vary among aggregations, even within the same waterbody (Clarke 2010; Allard et al. 2015; Mageroy 2015). Heard (1975) reported breeding mussels ranging in age from 5 to 11 years.

Western *Anodonta* inhabit a broad range of habitat types, including lakes, reservoirs, and ponds, as well as perennial streams and rivers (Taylor 1981; Chong et al. 2008; Nedeau et al. 2009). Where ranges and habitat overlap, multiple species of floaters may co-occur, and

they may also co-occur with western pearlshell or western ridged mussels. In riverine habitat, *Anodonta* occurs in glides, riffles, pools, and runs, often in areas with fine sediment, including shallow muddy and sandy habitats (Frest and Johannes 1995; Nedeau et al. 2009; Hegeman 2012). Howard and Cuffey (2003) noted the species occurred almost exclusively in pool habitat in the South Fork Eel River and was found only in the most downstream 25% of the study reach. In lake habitat, the species can occur in large numbers; following draining, thousands of individuals were observed in Stow Lake, CA (Ingram and Kenyon 1947). Spring Rivers (2007) observed *Anodonta* in habitat ranging from nearshore areas consisting of fine substrates as well as near or within flow refugia created by emergent vegetation and submerged logs.

Figure 5. Floater mussels, *Anodonta* sp., in sandy habitat. Photo credit: Roger Tabor, USFWS. Used with permission.

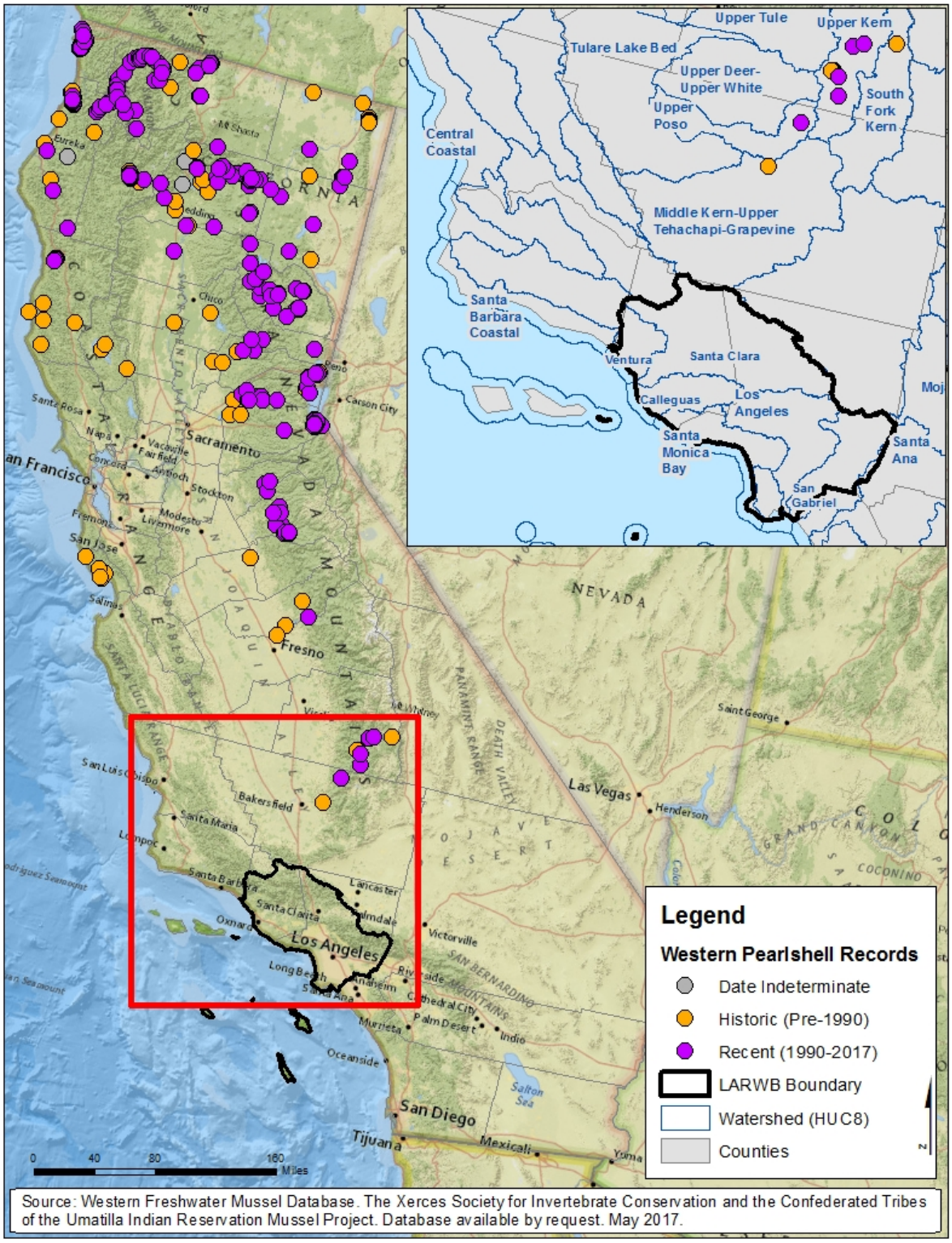
Anodonta use a number of host fish, including natives like hardhead (*Mylopharodon conocephalus*), pit sculpin (*Cottus pitensis*), prickly sculpin (*Cottus asper*), redside shiner (*Richardsonius balteatus*), Sacramento pikeminnow (*Ptychocheilus grandis*), speckled dace (*Rhinichthys osculus*), torrent sculpin (*Cottus rhotheus*), tule perch (*Hysterocarpus traski*), and threespine stickleback (*Gasterosteus aculeatus*) (Martel and Lauzon-Guay 2005; Spring Rivers 2007; O'Brien et al. 2013; Maine et al. 2016; Barnhart, unpublished data). Other potential host fish are reviewed in Blevins et al. (submitted). The species can occur in small numbers or in dense aggregations. For example, Davis et al. (2013) counted more than 1,500 *Anodonta* from surveys of 82 sites in the Klamath River and tributaries, though they observed 95% of those mussels at just one site (furthest upriver site surveyed). Similarly, though Howard and Cuffey (2003) observed ~8,000 individuals in a reach of the South Fork Eel River, with most aggregations consisting of 50 to 100 individuals, two aggregations observed in a 100 m-long pool were comprised of ~6,300 individuals.

A recent assessment of rangewide extinction risk based on occurrence records for members of *Anodonta* clade 1 (*A. nuttalliana* and *A. californiensis*) suggests a decline of as much as 33% across the range (Blevins et al. 2016c). In comparison, occurrence records for *Anodonta* clade 2 (*A. oregonensis* and *A. kennerlyi*, demonstrated a decline of as much as 26% across the range (Blevins et al. 2016d). Despite numerous historical records for *Anodonta* clade 2 in southern California (Gregg 1947; Xerces/CTUIR 2017a, b), the species is thought to be generally extirpated from the region (Taylor 1981; Coney 1993; Howard et al. 2015). However, it is notable that a population of floaters, presumably from this clade, was recently reported from a lake in the Santa Monica Mountains, and surveys should target additional waterbodies in the Santa Monica mountains. It is possible that floater populations exist elsewhere in the region, or mussels may have been more recently re-introduced by fish with attached glochidia.

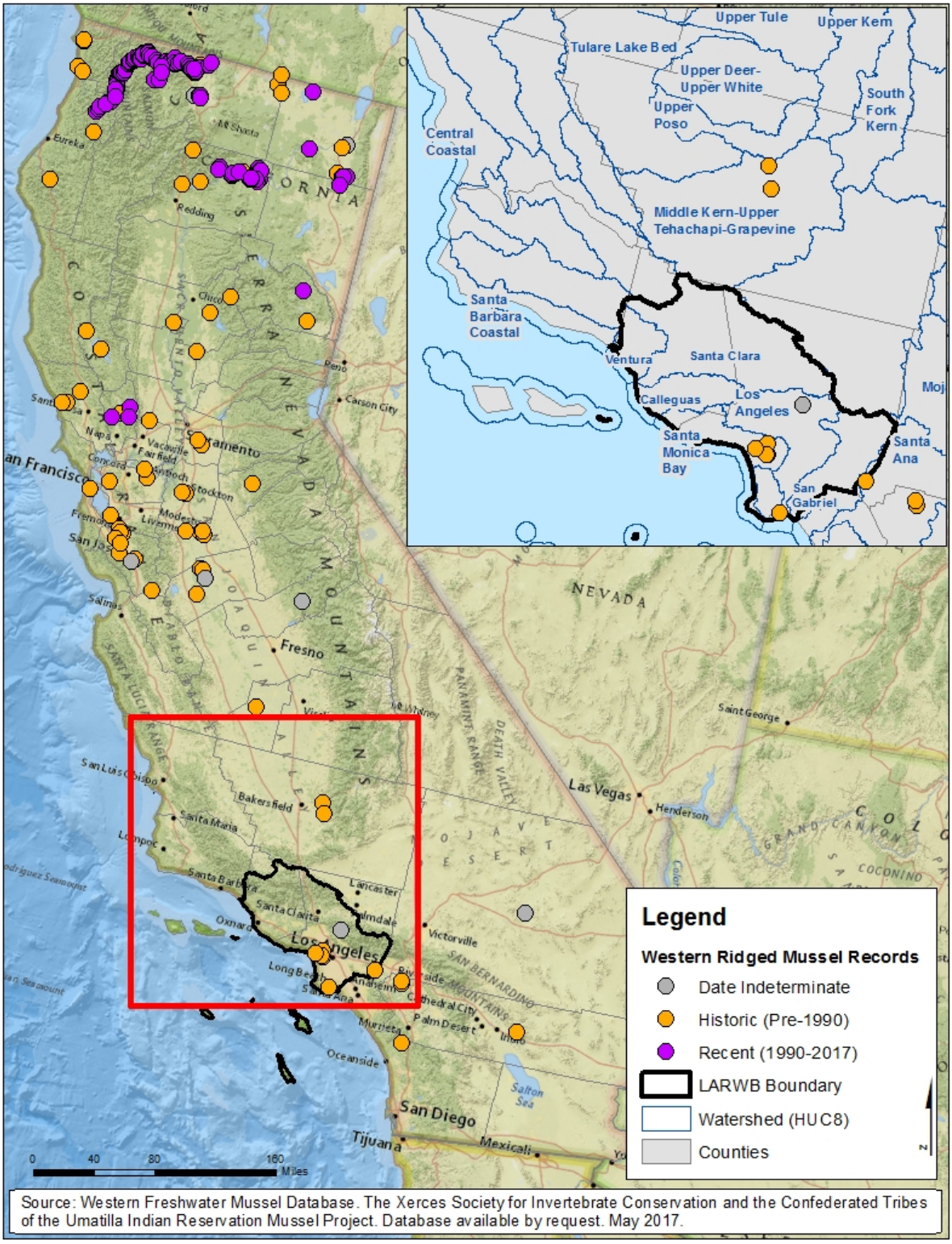
In northern California Howard et al. (2015) have also observed declines in total population size and number of populations of *Anodonta* generally; *Anodonta* was historically the most commonly reported genus of freshwater mussel in California, yet the genus was recently documented at only 33% of sites from which it was once reported in the state (Howard et al. 2015). Collection of genetic samples for species' identification would clarify whether only one or both clades remains widespread in California.

Distribution of Mussels in California and Region 4

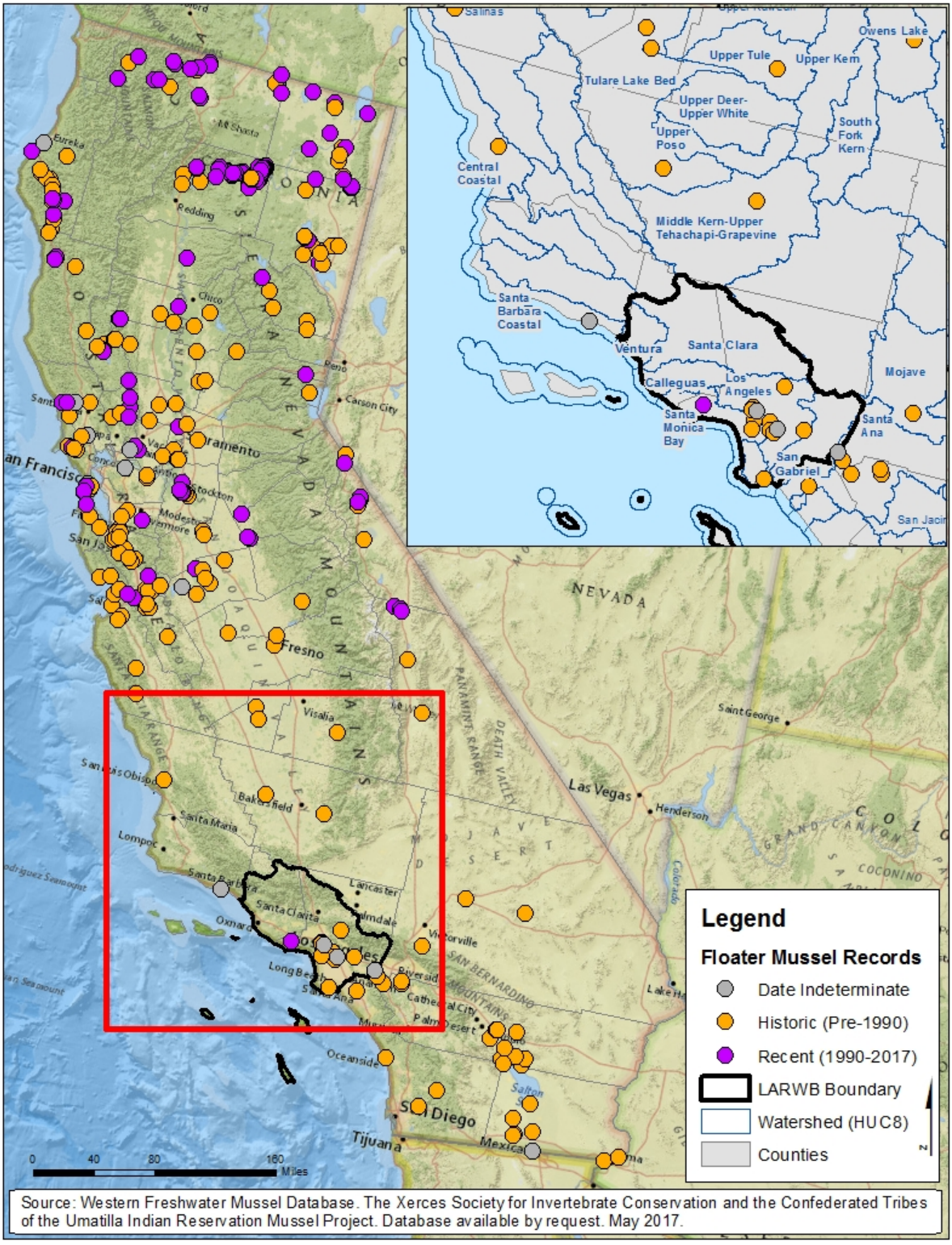
The following maps depict historic and recent observations of mussels from each genus in California and Region 4.



Map 1. Western pearlshell records in California and the LA Regional Water Board vicinity. Note that "date indeterminate" records are museum records that most likely predate 1990 but for which a date is not provided.



Map 2. Western ridged mussel records in California and the LA Regional Water Board vicinity. Note that "date indeterminate" records are museum records that most likely predate 1990 but for which a date is not provided.



Map 3. Floater mussel records in California and the LA Regional Water Board vicinity. Note that "date indeterminate" records are museum records that most likely predate 1990 but for which a date is not provided.

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Persons Contacted

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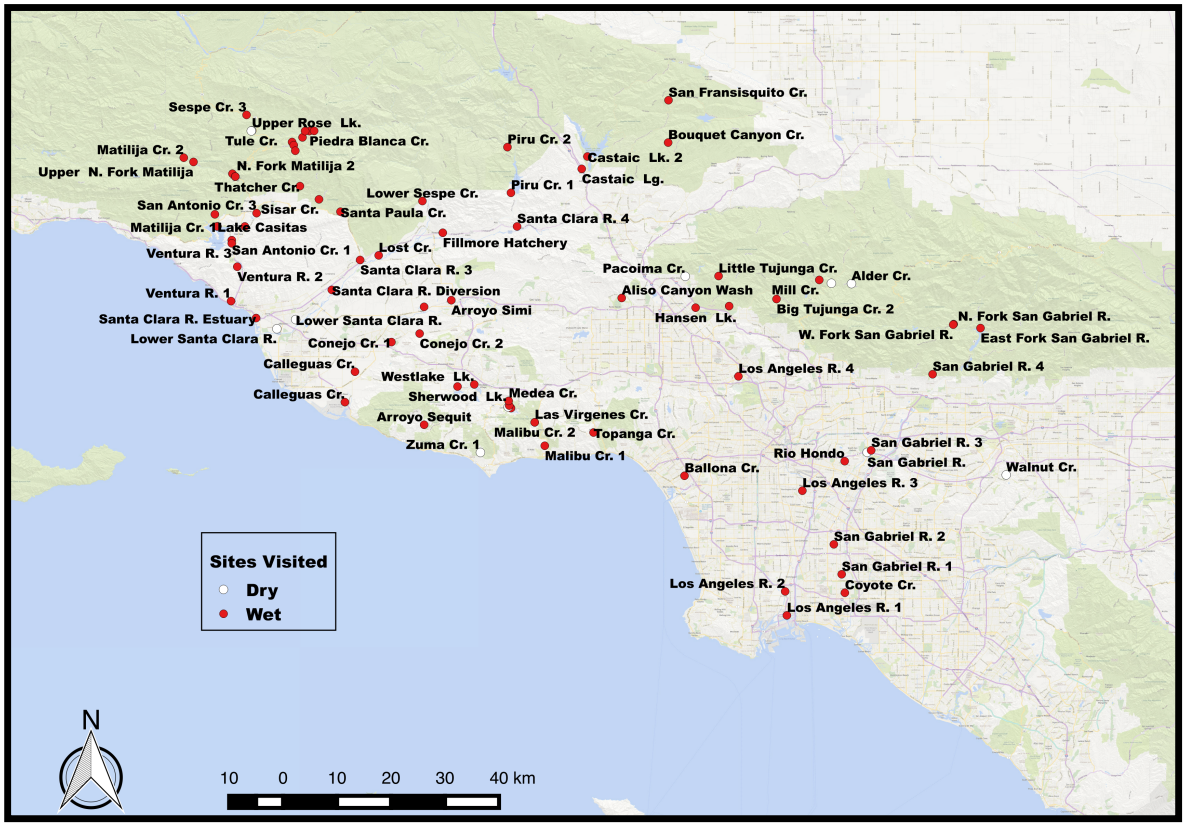
Appendix B. Mussel Field Survey Sites

Map (Map B1) and list (Table B1) of the sites visited for the project.



Map B1. Map of Survey Sites in Los Angeles and Ventura Counties.
Eighty-three sites in Los Angeles and Ventura Counties were visited for native mussels surveys. Colors indicate dry or wet sites. Dry sites were excluded from further surveys while visual and eDNA surveys were performed at sites with water.

County	Watershed	Site Name	Latitude	Longitude
Los Angeles	Los Angeles River	Alder Creek*	34.30945	-118.07343
Los Angeles	Los Angeles River	Aliso Canyon Wash	34.28592	-118.53091
Los Angeles	Los Angeles River	Big Tujunga Creek, site 1	34.27256	-118.31699
Los Angeles	Los Angeles River	Big Tujunga Creek, site 2	34.28416	-118.22263
Los Angeles	Los Angeles River	Camp Colby*	34.31003	-118.11339
Los Angeles	Los Angeles River	Hansen Lake	34.26996	-118.38374
Los Angeles	Los Angeles River	Little Tujunga Creek	34.32195	-118.33804
Los Angeles	Los Angeles River	Los Angeles River, site 1	33.76281	-118.20205
Los Angeles	Los Angeles River	Los Angeles River, site 2	33.80244	-118.20545
Los Angeles	Los Angeles River	Los Angeles River, site 3	33.96863	-118.17118

Los Angeles	Los Angeles River	Los Angeles River, site 4	34.15706	-118.29854
Los Angeles	Los Angeles River	Mill Creek	34.31556	-118.13754
Los Angeles	Los Angeles River	Pacoima Creek*	34.32153	-118.40433
Los Angeles	Los Angeles River	Rio Hondo	34.01737	-118.08693
Los Angeles	San Gabriel River	Coyote Creek	33.80018	-118.08658
Los Angeles	San Gabriel River	East Fork San Gabriel River	34.23647	-117.81647
Los Angeles	San Gabriel River	North Fork San Gabriel River	34.24290	-117.86997
Los Angeles	San Gabriel River	San Gabriel River, site 1	33.83073	-118.09287
Los Angeles	San Gabriel River	San Gabriel River, site 2	33.88020	-118.10850
Los Angeles	San Gabriel River	San Gabriel River, site 3	34.03518	-118.03419
Los Angeles	San Gabriel River	San Gabriel River, site 4	34.16052	-117.91184
Los Angeles	San Gabriel River	San Gabriel River, site 5*	34.03191	-118.04218
Los Angeles	San Gabriel River	Walnut Creek*	33.99471	-117.76533
Los Angeles	San Gabriel River	W. Fork San Gabriel River	34.24210	-117.87086
Los Angeles	Santa Clara River	Bouquet Canyon Creek	34.54085	-118.43846
Los Angeles	Santa Clara River	Castaic Lagoon	34.49777	-118.61051
Los Angeles	Santa Clara River	Castaic Lake	34.51805	-118.59917
Los Angeles	Santa Clara River	San Fransisquito Creek	34.61017	-118.43774
Los Angeles	Santa Monica Mtn.	Arroyo Sequit	34.07735	-118.92431
Los Angeles	Santa Monica Mtn.	Ballona Creek	33.99328	-118.40587
Los Angeles	Santa Monica Mtn.	Las Virgenes Creek	34.08133	-118.70417
Los Angeles	Santa Monica Mtn.	Malibou Lake, site 1	34.10897	-118.75488
Los Angeles	Santa Monica Mtn.	Malibu Creek, site 1	34.04278	-118.68411
Los Angeles	Santa Monica Mtn.	Malibu Creek, site 2	34.08132	-118.70412
Los Angeles	Santa Monica Mtn.	Medea Creek	34.11665	-118.75595
Los Angeles	Santa Monica Mtn.	Sherwood Lake	34.14030	-118.85780
Los Angeles	Santa Monica Mtn.	Topanga Creek	34.06457	-118.58706
Los Angeles	Santa Monica Mtn.	Westlake Lake	34.14377	-118.82446
Los Angeles	Santa Monica Mtn.	Zuma Creek*	34.03170	-118.81216
Ventura	Calleguas Creek	Arroyo Las Posas	34.27134	-118.92435
Ventura	Calleguas Creek	Arroyo Simi	34.28226	-118.87021
Ventura	Calleguas Creek	Calleguas Creek, site 1	34.11459	-119.08205
Ventura	Calleguas Creek	Calleguas Creek, site 2	34.16468	-119.06201
Ventura	Calleguas Creek	Conejo Creek, site 1	34.21342	-118.98938
Ventura	Calleguas Creek	Conejo Creek, site 2	34.22740	-118.93324
Ventura	Santa Clara River	Fillmore Hatchery	34.39299	-118.88715
Ventura	Santa Clara River	Lion Creek	34.54932	-119.16647
Ventura	Santa Clara River	Lost Creek	34.35588	-119.01483

Ventura	Santa Clara River	Piedra Blanca Creek	34.55974	-119.15270
Ventura	Santa Clara River	Piru Creek, site 1	34.45853	-118.75143
Ventura	Santa Clara River	Piru Creek, site 2	34.53342	-118.75838
Ventura	Santa Clara River	Rose Valley Creek	34.52743	-119.18085
Ventura	Santa Clara River	Rose Valley, Lower Lake	34.54174	-119.18724
Ventura	Santa Clara River	Rose Valley, Upper Lake	34.53655	-119.18391
Ventura	Santa Clara River	Santa Clara River Diversion	34.29919	-119.10807
Ventura	Santa Clara River	Santa Clara River Estuary	34.25296	-119.25884
Ventura	Santa Clara River	Santa Clara River, site 3	34.34833	-119.05174
Ventura	Santa Clara River	Santa Clara River, site 4	34.40335	-118.73926
Ventura	Santa Clara River	Santa Clara River, site 5*	34.23528	-119.21755
Ventura	Santa Clara River	Santa Clara River, site 6*	34.25047	-119.18014
Ventura	Santa Clara River	Santa Paula Creek	34.42755	-119.09093
Ventura	Santa Clara River	Sespe Creek, site 1	34.55970	-119.16130
Ventura	Santa Clara River	Sespe Creek, site 2	34.44501	-118.92773
Ventura	Santa Clara River	Sespe Creek, site 3	34.58616	-119.27791
Ventura	Santa Clara River	Sisar Creek	34.44785	-119.13367
Ventura	Santa Clara River	Trout Creek	34.56002	-119.14330
Ventura	Santa Clara River	Tule Creek*	34.55993	-119.26839
Ventura	Ventura River	Lake Casitas	34.40355	-119.33590
Ventura	Ventura River	Matilija Creek, site 1	34.40339	-119.33653
Ventura	Ventura River	Matilija Creek, site 2	34.51610	-119.40292
Ventura	Ventura River	North Fork Matilija, site 1	34.48526	-119.30040
Ventura	Ventura River	North Fork Matilija, site 2	34.48978	-119.30604
Ventura	Ventura River	San Antonio Creek, site 1	34.38079	-119.30728
Ventura	Ventura River	San Antonio Creek, site 2	34.40339	-119.28123
Ventura	Ventura River	San Antonio Creek, site 3	34.42517	-119.25789
Ventura	Ventura River	Santa Ana Creek	34.42323	-119.34095
Ventura	Ventura River	Thatcher Creek	34.46947	-119.17159
Ventura	Ventura River	Upper North Fork Matilija	34.50912	-119.38354
Ventura	Ventura River	Ventura River, site 1	34.28076	-119.30870
Ventura	Ventura River	Ventura River, site 2	34.33728	-119.29639
Ventura	Ventura River	Ventura River, site 3	34.37562	-119.30730
Ventura	Ventura River	Ventura River, site 4	34.48514	-119.30025
Ventura	Ventura River	Ventura River, site 5	34.48514	-119.30025
Clackamas	Willamette River	Tualatin River^	45.34991	-122.67694
Multnomah	Willamette River	Crystal Springs Creek^	45.47475	-122.64167
Multnomah	Willamette River	N. Fork Johnston Creek^	45.46500	-122.39472
Napa	Napa River	Napa River^	38.41859	-122.35298

Table B1. List of Survey Sites.
The following sites were visited for visual and eDNA surveys for freshwater mussels. Eighty-three of the sites are in Los Angeles and Ventura Counties; four sites are outside those counties and were used as positive control sites where mussel populations are present.
* Survey was not performed because site was dry.
^ Positive control mussel site outside of Los Angeles and Ventura Counties.

Appendix C. Open Filter eDNA Field Collection Protocol

Supplies:

Lab Equipment Laminar flow hood UV hood Bleach Bleach buckets Longmire solution Ziplock bags Bags for each site that includes: 8 x 1.5mL labeled vials with 500 uL Longmire solution 4 x 1µm PCTE filters (47mm diameter) 4 x funnel with filter Forceps and scissors set 2 x pair of gloves Solo cup Bottle of water	General Field Items: Peristaltic pump Tubing Flask with rubber stopper GPS device Indelible pen Vial box Vial rack Bag for used items Extra/Spare Items: 1.5 mL vials with 500 uL Longmire solution Forceps & scissors Labeling tape Gloves Tubing Bottled water

Protocol:

Laboratory Preparation:

1. All filter funnel pieces (the base that holds the filter and the funnel) must be submerged in 20% bleach for at least 1 hour, rinsed thoroughly with clean water, dried, and placed under the UV for 30 minutes.
2. One 1µm PCTE filter (47mm diameter) is placed in each filter and the filter base and funnel pieces are sealed using parafilm while under the laminar flow hood.
3. 700mL of Longmire solution is added to each 1.5mL centrifuge vial and then sterilized for 30 minutes under the UV while open. Vials are closed under the laminar flow hood and labeled with site ID.
4. Bags are assembled for each site under the laminar flow hood. Each bag contains: four assembled filter funnels, eight sample vials, one set of new forceps and scissors, two pairs of gloves, a plastic solo cup, and a bottle of water.

eDNA Sample Collection

1. Connect pump to vacuum flask with rubber stopper.
2. Put on gloves and take materials out of site bag. Make sure all vials are labeled with site and date. Place vials in rack and open all eight.
3. Attach filter funnel to the vacuum flask. Use Solo cup to collect water from a running portion of the stream and filter 500mL through the filters. Do not touch the water with anything other than the gloved hand in order to avoid site cross contamination. Repeat this for remaining 2 sample filters. If 250ml cannot be filtered due to clogging, record the amount of water filtered through each filter.
4. Put on clean gloves and filter 250 mL of bottled water through remaining filter as a field control.
5. Cut the control filter in half and one half into the 2 control vials and close them.
6. Cut the 3 sample filters in half and put them into the 6 sample vials. Do not touch the outside of the vial with anything that has touched the water.
7. Remove gloves, close remaining vials.
8. Ensure vials are labeled with site and date. Place all vials into the sample box.
9. All used items should be placed into a large bag for later cleaning or disposal.
10. Upon return from the field, half the samples will be stored -20°C until extraction. The other half of the samples will be stored at -80°C in case of future need.

Appendix D. DNA Extraction from Open Filters

Extraction

- **Work performed in a room where tissue samples and PCR products have not been handled.**
- **Create 2 negative control extract with each batch of samples.**

Part 1:

1. Thaw up to 34 samples (filter in Longmire solution) in fridge or on ice. And create 2 negative control vials of only Longmire solution.
2. Add 20 µL of Proteinase K (4mg/mL) to each vial. Mix vials by inverting them several times.
3. Incubate the vials overnight at 56°C on rotator.
4. Label and UV three 1.5 mL tubes for each sample for use in later steps.
    - 1.5 mL snap cap set for CI (these vials will be discarded so don't bother labeling with detail)
    - 1.5 mL snap cap set for final solution to use for lab work – label with site and extraction date
    - 1.5 mL screw cap set for half of final solution to store at -20C for reserve – label with site and extraction date

Part 2:

- **Perform this part under fume hood.**
- **All waste with PCI and CI must be disposed of properly.**
- **100% ethanol should be kept in freezer until use.**
- **If samples sit for a while after centrifuging and layers aren't' sharp, they can be re-centrifuged before collecting the top layer.**

1. Add 500 µL PCI (phenol chloroform isoamyl alcohol) to double to the total volume of liquid to each vial (1 sample : 1 PCI). PCI stratifies into two layers; use the bottom layer (organic phase).
2. Shake manually for 5 minutes.
3. Centrifuge for 5 minutes at 10,000 rpm.
4. Label new 1.5 mL vials for each sample. Add enough CI each vial to make a 1:1 ratio of sample:CI.
5. Collect the top layer from the sample/PCI vial and put it into the labeled 1.5 µL vial with the CI. Dump the bottom layer into the waste container.
6. Shake manually the sample/CI vial for 5 minutes.
7. Centrifuge for 5 minutes at 10,000 rpm.
8. Collect the top supernatant layer and place that in a new 1.5 µL vial (if you accidently took some of the bottom layer of Phenol, you can repeat steps 6-9). Discard the bottom layer.
9. Add 50 µL of 5M NaCl to each vial (~10% of the sample volume).
10. Add 1,000 µL of **ice cold** (-4°C) 100% EtOH to each vial (~200% of the sample volume).
11. Invert samples a few times to mix them.
12. Freeze overnight at -20°C.

Part 3:

- **Centrifuge must be performed in the cold room at 4°C.**
- **Move centrifuge into cold room the night before use and when you move it back don't use it for several hours. All parts need to be at ambient temperature.**
- **Always load vials into the centrifuge with the hinge part out so the pellet will always be at the bottom hinge side of the vial, even if not visible.**

1. Centrifuge samples for 30 minutes at 14,000 rpm at 4°C.

2. Decant off EtOH, being careful not to lose the pellet (might not be visible).

3. Add 900 µL of 70% EtOH. Pipette up and down enough times to break up and wash pellet.

4. Centrifuge for 30 minutes at 14,000 rpm at 4°C.

5. Pipette off EtOH, being careful not to take the pellet (but remove as much EtOH as possible).

6. Let the vial air dry with lid open in laminar flow hood to dry. **ALL** EtOH must be gone!

7. Re-suspend DNA in 200 µL of 0.25X Tris-EDTA buffer solution (TE). If samples were split earlier use 100 µL of 0.25X Tris-EDTA for each.

8. Place in hot plate at 55°C for 10 minutes to re-dissolve DNA, remove from incubator, gently vortex and store at -20°C (or at -4°C, if processed in next couple of weeks). Recombine any split samples after both are re-suspended.

9. Put 100 µL into a separate screw top vial to be kept on reserve in the -20°C.

Appendix E. Enclosed Filter eDNA Field Collection Protocol

Supplies:

Bags for each site include: 4 x Sterivex filter cartridges (0.22 pore size; Luer lock ends) 2 x pair of gloves 2 x solo cups bottled water (>250 mL) 1 x 60mL syringe (Luer lock tip) 1 x 10mL syringe (Luer lock tip) of Longmire solution 8 x Luer lock filter caps	General supplies/spares Indelible pen GPS device Trash bags Spares of: Gloves 60mL syringes 10mL syringes Sterivex filter cartridges Filter caps Bottled water Ziploc bags

Protocol:

Laboratory preparation

All supplies are to be kept in a clean area of the lab separate from the processing areas of any field samples.

1. Prepare enough bags for all the field sites, plus one extra, for each site you intend to visit.
2. Fill the 10mL syringe with at least 8mL of Longmire solution and cap it.
3. Keep all other syringes, filters, and caps in their sterile individual bags until used in the field.

eDNA Sample Collection

Avoid getting the sample water over everything. Even with gloved hands, the less you touch the water and splash water around, the better. Do not touch the water or anything that has been exposed to the water without gloves on. Do not step in the water.

1. Once at the site, put the first pair of gloves on and take out all the filters, solo cups, bottled water, and 60mL syringe. Put the Ziploc bag of the remaining items away from the water so it doesn't get splashed.
2. Pour the bottled water into a solo cup and using the 60mL syringe, filter 250mL of water through the filter, which will be the control filter. Set this filter aside away from the rest of the work.
3. Using the solo cups, collect the sample water and filter 500mL of water through each of the three remaining filters. Water should be collected from the flowing portion of the water or from multiple sites around still water. Do not collect sediment; if the water is not deep enough to collect a full cup of water, use the second cup to fill the first.

4. Push air through the filter to remove the remaining water in the filter capsule. Set the filters aside.
5. Using clean gloves, take the **control** filter and cap the bottom end. Using the 10mL syringe of Longmire solution and inject 1mL of solution into the control filter. Remove the syringe and cap the end.
6. Cap the ends of the three sample filters and inject 1mL of Longmire solution into each filter. Cap the input end.
7. Place the 4 filters into the Ziploc bag. Label the bag of filters with the site location information and place on ice for transportation to the lab. Put all the single-use materials in the trash bag.
8. At the lab, wipe off each filter with 10% bleach twice and then wipe it with 70% ethanol twice to remove all DNA from the outside of the filters. Label the filters with the site information and be sure to label which filter is the negative control filter. Store the filter cartridges at -4°C until extraction.

Appendix F. DNA Extraction from Enclosed Filters

Extraction

- **Work performed in a room where tissue samples and PCR products have not been handled.**
- **Create 2 negative control extract with each batch of samples.**

Part 1:

5. Thaw up to samples (filter in Longmire solution) in fridge or on ice. And create 2 negative control vials of only Longmire solution (500 µL).
6. Add 40 µL of Proteinase K (4mg/mL) to each vial. Mix vials by inverting them several times.
7. Incubate the vials overnight at 56°C on rotator.
8. Label and UV four 2 mL tubes for each sample for use in later steps.
    - 2mL snap cap set for PCI (these vials will be discarded so don't bother labeling with detail)
    - 2mL snap cap set for CI (these vials will be discarded so don't bother labeling with detail)
    - 2mL snap cap set for final solution to use for lab work – label with site and extraction date
    - 2mL screw cap set for half of final solution to store at -20C for reserve – label with site and extraction date

Part 2:

- **Perform this part under fume hood.**
- **All waste with PCI and CI must be disposed of properly.**
- **100% ethanol should be kept in freezer until use.**
- **If samples sit for a while after centrifuging and layers aren't' sharp, they can be re-centrifuged before collecting the top layer.**

13. Push all the liquid out of the filter cartridge into a 2mL vial using a new 10mL vial for each sample.
14. Add enough PCI (phenol chloroform isoamyl alcohol) to double to the total volume of liquid to each vial (1 sample : 1 PCI). PCI stratifies into two layers; use the bottom layer (organic phase). If necessary, split the sample into two 2mL vials
15. Shake manually for 5 minutes.
16. Centrifuge for 5 minutes at 10,000 rpm.
17. Label new 2 mL vials for each sample. Add enough CI each vial to make a 1:1 ratio of sample:CI.
18. Collect the top layer from the sample/PCI vial and put it into the labeled 2mL vial with the CI. Dump the bottom layer into the waste container.
19. Shake manually the sample/CI vial for 5 minutes.
20. Centrifuge for 5 minutes at 10,000 rpm.
21. Collect the top supernatant layer and place that in a new 2mL vial (if you accidently took some of the bottom layer of Phenol, you can repeat steps 6-9). Discard the bottom layer.
22. Add 50 µL of 5M NaCl to each vial (~10% of the sample volume).
23. Add 1,000 µL of **ice cold** (-4°C) 100% EtOH to each vial (~200% of the sample volume).
24. Invert samples a few times to mix them.
25. Freeze overnight at -20°C.

Part 3:

- **Centrifuge must be performed in the cold room at 4°C.**
- **Move centrifuge into cold room the night before use and when you move it back don't use it for several hours. All parts need to be at ambient temperature.**
- **Always load vials into the centrifuge with the hinge part out so the pellet will always be at the bottom hinge side of the vial, even if not visible.**

10. Centrifuge samples for 30 minutes at 14,000 rpm at 4°C.

11. Decant off EtOH, being careful not to lose the pellet (might not be visible).

12. Add 900 μL of 70% EtOH. Pipette up and down enough times to break up and wash pellet.

13. Centrifuge for 30 minutes at 14,000 rpm at 4°C.

14. Pipette off EtOH, being careful not to take the pellet (but remove as much EtOH as possible).

15. Let the vial air dry with lid open in laminar flow hood to dry. **ALL** EtOH must be gone!

16. Re-suspend DNA in 200 μL of 0.25X Tris-EDTA buffer solution (TE). If samples were split earlier use 100 μL of 0.25X Tris-EDTA for each.

17. Place in hot plate at 55°C for 10 minutes to re-dissolve DNA, remove from incubator, gently vortex and store at -20°C (or at -4°C, if processed in next couple of weeks). Recombine any split samples after both are re-suspended.

18. Put 100 μL into a separate screw top vial to be kept on reserve in the -20°C.

Appendix G. PCR Descriptions

Quantitative PCR

Quantitative PCR was performed to detect the presence of native freshwater mussels from eDNA samples.

qPCR markers for the three targeted native mussel species.

Developed by University of California, Davis.

California floater (*Anodonta californiensis/nuttalliana/oregonensis*):
ANCA forward primer: GGAGAGTGGTGTTGGTACTGGTT
ANCA reverse primer 1: GAAGAAGCACCAGCAAGATGTAAAG
ANCA reverse primer 1: AAGAGGCACCAGCAAGATGTAAAG
ANCA reverse probe: GTGGATTTGGCCATTTT

Western Ridged (*Gonidea angulata*):
GOAN forward primer: GGTTTTGATTACTTGTACCGGCTC
GOAN reverse primer: AGACAACGGCGGATACACTGT
GOAN probe: GGTCTTCATTAGTAGAGAGTG

Western Pearlshell (*Margaritifera falcata*):
MAFA forward primer: TAATATGCGCTCCCCTGGC
MAFA reverse primer: CGCCACTAACAAAATAGCCGTC
MAFA probe: TTCGTGTGAGCCGTCAC

qPCR Setup

1. All samples are run in duplicate for a total of six replicates per site (3 samples/site x 2 qPCR runs). The negative controls are also run in triplicate. Each run will include a positive control of mussel tissue extract and a negative control of water.
2. All samples must be checked for inhibitors with the IPC (Internal Positive Control) in the first run of qPCR
    a. All samples from a site that shows inhibition must be processed through the StepOne™ PCR Inhibitor Removal Kit (Zymo Research)
    b. Samples treated with for inhibitors are then run again with IPC to check on inhibitor removal
3. The targeted mussel's DNA is used to standards at three concentrations with three replicates for a total of nine.
4. At least three negative template controls (NTC) are included in each plate. NTC include the master mix and 5 µL of DNA free water.

qPCR Master Mix:

Reactions were 25 µL total.
5 µL sample template (eDNA extract)
1x TaqMan Environmental Master Mix 2.0

400nm final concentration for each primer
80nm final concentration for the probe

qPCR Cycling Conditions
50°C for 2 minutes
95°C for 10 minutes
50 cycles of:
95°C for 15 seconds
60°C for 1 minute

Standard PCR
Standard PCR was used when samples were needed for genetic sequencing. This was performed to create known DNA sequences for each of the three native mussels species and also to confirm positives from eDNA samples.

Standard PCR for DNA Sequencing
Reactions were 25 µL total.
5 µL sample template for eDNA extract or 5-10ng DNA of mussel tissue extract
1x TaqMan Environmental Master Mix 2.0
400nm final concentration for each primer

Standard PCR Cycling Conditions
Samples were run on a standard PCR thermocycler using the following cycles: 50°C for 2 minutes and 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute, with a concluding step of 72°C for 5 minutes.