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UNITED STATES DISTRICT COURT

NORTHERN DISTRICT OF CALIFORNIA

SAN FRANCISCO DIVISION

FRIENDS OF GUALALA RIVER, et al.,

Plaintiffs,

vs.

GUALALA REDWOOD TIMBER, LLC,

Defendant.

Case No. 20-cv-06453-JD

DECLARATION OF DR. CHRISTOPHER A. FRISSELL IN SUPPORT OF PLAINTIFFS' MOTION FOR PRELIMINARY INJUNCTION

Date:	June 24, 2021
Time:	10:00 a.m.
Courtroom:	11
Judge:	Hon. James Donato
Action Filed:	September 15, 2020
Trial Date:	None

I, Dr. Christopher A. Frissell, declare as follows:

1. If called as a witness, I could and would truthfully testify to the following based on my personal knowledge.

2. Attached hereto as Exhibit A is true and correct copy of my expert report concerning the impact of the project at issue in this litigation on Northern California steelhead (*Oncorhynchus mykiss*) and Central California Coast coho salmon (*Oncorhynchus kisutch*) prepared at the request of Plaintiffs Friends of Gualala River and Center for Biological Diversity in this action.

3. The content of Exhibit A, inclusive of its exhibits, are true and correct.

I declare under the penalty of perjury under the laws of the United States of America and the State of California that the foregoing is true and correct.

Executed on May 18, 2021 in Polson, MT.

Christopher A. Frissell

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EXHIBIT A

REPORT OF DR. CHRIS FRISSELL REGARDING IMPACTS OF THE PROJECT ON COHO SALMON AND NORTHERN CALIFORNIA STEELHEAD

I. EXECUTIVE SUMMARY

I have been asked to evaluate the likelihood that the proposed logging and associated activities (collectively, the "Project") of certain floodplain areas of the Gualala River, in northern Sonoma County, California, as identified and described in the Dogwood Timber Harvest Plan, Approved March 30, 2018, (the "Dogwood THP Area") is reasonably certain to result in the "take" of coho salmon (*Oncorhynchus kisutch*) ("Coho") and Northern California steelhead (*Oncorhynchus mykiss*) ("Steelhead").

I have been informed by counsel for Plaintiffs in the action titled *Friends of Gualala River, et al. v. Gualala Redwood Timber, LLC*, No. 20-6453-SBA (the "Action") that the standard by which I am to evaluate whether a take of Coho and Steelhead is reasonably certain to occur is whether any Coho or Steelhead is reasonably certain to be: harassed; harmed; killed; or injured. I have been further informed that "harm" under this standard includes significant habitat modification or degradation where it actually kills or injures individual Coho or Steelhead by significantly impairing essential behavioral patterns, including breeding, feeding, or sheltering. I have also been informed that "harass" means an intentional or negligent act or omission which creates the likelihood of injury to wildlife by annoying it to such an extent as to significantly disrupt normal behavioral patterns which include, but are not limited to, breeding, feeding, or sheltering. I have applied this standard, inclusive of these definitions, to the analysis contained in this report.

Based on that analysis my conclusions are as follows: Take of steelhead and adverse modification of habitat of listed steelhead and coho salmon by the Dogwood timber sale is highly likely from the following sources:

1. Logging removal of large trees curtailing the natural recruitment of wood to channels and floodplains, further depleting natural wood loading.

2. Tractor logging and yarding directly damaging floodplain channels and wetlands that are the key wintering habitats of steelhead and coho salmon, and triggering or re-initiating erosion on slopes adjacent to the floodplain.
3. Haul roads delivering chronic fine mineral sediment to river, tributary, and floodplain habitats.
4. Logging on floodplains producing summer stream temperature increases in streams where high temperature already limits salmonid survival and productivity.
5. Logging and road practices that increase erosion and delivery of sediment also increasing nutrient loading to a nutrient-overloaded river, increasing harm from algae blooms, eutrophication, and resulting oxygen depletion in the river and estuary .

II. QUALIFICATONS

A. Education

I am a fisheries ecologist and watershed scientist with expertise in land management impacts on habitat and populations of fishes and amphibians, and the development and evaluation of conservation and restoration strategies for freshwater ecosystems and species. My education is as follows: I hold a Bachelors degree in Zoology from the University of Montana, and M.S. and Ph.D. degrees in Fisheries Science from Oregon State University, where the focus of my graduate thesis research was the cumulative effects of land use and watershed disturbance on freshwater ecosystems and salmon populations.

B. Work Experience and Publications

A list of publications that I have authored in the last ten years is included in my curriculum vitae, attached as Exhibit 1 to this report. I have more than 30 years of experience as a research scientist in the field of aquatic ecology, fishery and conservation biology, and watershed science, having held research and teaching faculty positions at Oregon State

University, The University of Montana and Salish Kootenai College. I have published more than 40 scientific and technical publications on the subjects of aquatic ecology, fishery and conservation biology, forestry management effects on salmonid habitat, and watershed science, in professional journals, symposia, and books, and book chapters. In addition, I am author of more than 30 research reports for various institutions and agencies. I have served as peer reviewer or reviewing editor for more than a dozen professional journals and government research publications. I have served on 15 professional and government panels that provided technical guidance about stream and river protection, or applied research to inform resource protection, to state and federal fish, wildlife, environmental, and forest management agencies in three states. For example, I served on the Temperature Standards Review Subcommittee of the Triennial Water Quality Standards Review for the Oregon Department of Environmental Quality during 1992-95. In recent years I have served as editor and coordinator of two recent scientific peer review panels for state agency research projects intended to characterize stream responses to forest management in Pacific coast states. While on the faculty as a researcher at Oregon State University, I was funded to lead a 6-year research project on salmon habitat protection in Oregon coastal rivers. In 1992, I completed my doctoral dissertation on the cumulative effects of forestry, agricultural, and exurban land use on salmon habitat in Oregon South Coast rivers. That research focused on the full spectrum of threats to physical habitat of salmon in coastal watersheds, including water temperature, sediment conditions, landslides and road erosion, large wood, and channel dynamics. The rivers I where conducted my dissertation research have much in common with rivers of California's north coast with regard to geology, climate, hydrologic and erosional processes, estuarine conditions, salmon and steelhead ecology, land use, and vegetation, aside from the prominence of coast redwoods.

For ten years I was a full-time Research Assistant and Research Associate Professor at the University of Montana's Flathead Lake Biological Station, where I continued to conduct research and supervise graduate students on ecology of salmonid fishes and freshwater habitat conservation. That research included regional assessments of salmon conservation value and

watershed and stream ecological integrity, to inform priority areas for salmon conservation and recovery efforts across large areas of the Pacific Northwest. For 11 years I held the positions (alternately) of Senior Staff Scientist or Conservation and Science Director with the Pacific Rivers Council, where I worked specifically on the interface of scientific information and land management, with considerable involvement in federal and state policy development for stream protection and salmon and trout recovery, including in California. While in this role, in 2000 I co-authored a book chapter with H. H. Welsh and T. D. Roeloffs and entitled "Aquatic Ecosystems of the Redwood Region," in the book *The Redwood Forest: History, Ecology, and Conservation of the Coast Redwoods*, published by Island Press.

C. Background Documents Reviewed

The primary document that I reviewed to prepare this report is the Dogwood Timber Harvesting Plan, Approved March 30, 2018 (the "Approved Dogwood THP"). This includes: (1) the Plan of Timber Operations contained in Sections II, III, and IV of the Approved Dogwood THP; (2) Gualala Redwood Timber, LLC Timber Harvest Plan Letter of Conformance (July 31, 2019); and (3) Official response to significant environmental points raised during the timber harvesting plan evaluation process concerning revisions to the alternatives and cumulative impacts sections of the plan from the director of the California Department of Forestry and Fire Protection (CAL FIRE). In addition, I reviewed the following:

- CALFIRE's 2014 Anadromous Salmonid Protection Rules: Revised Interpretive Questions and Answers for RFPs and Landowners (ASP);
- Pertinent sections of NMFS (National Marine Fisheries Service) 2012 Final Recovery Plan for Central California Coast Coho Salmon Evolutionarily Significant Unit;
- Pertinent sections of NMFS (National Marine Fisheries Service) 2016, Final

Coastal Multispecies Recovery Plan. National Marine Fisheries Service, West Coast Region, Santa Rosa, California.

- Pertinent sections of NMFS (National Marine Fisheries Service), 2007 Federal Recovery Outline for the Distinct Population Segment of Northern California Steelhead. National Marine Fisheries Service, West Coast Region, Santa Rosa;
- CRWQCB (California Regional Water Quality Control Board). 2001. Technical Support Document for the Gualala River Watershed Water Quality Attainment Action Plan for Sediment
- CRWQCB (California Regional Water Quality Control Board). 2009. Evaluation of Anadromous Salmon Protection Rules Relative to the Water Quality Objective for Temperature, transmittal dated 3 September 2009, Santa Rosa, CA;
- NMFS (National Marine Fisheries Service)2009, NMFS comment letter to California Board of Forestry and Fire Protection on ASP, transmittal date 8 September 2009, Long Beach, CA;
- the Complaint filed in the Action;
- scientific literature and agency documents, as cited at the end of this report in the section entitled *Literature Cited*.

D. <u>Satellite, Geographic Information System ("GIS"), and Field Data Reviewed</u>

- I reviewed Google Earth satellite imagery and GIS overlays of the Dogwood THP Area and surroundings to assess the scale of logging and the associated impact on the Coho and Steelhead and their habitat in the Dogwood THP Area and its environs.
- I reviewed an analysis of canopy closure as measured by LiDAR data and by ground-based canopy photography, as reported in Exhibit 2 (Curt Bradley Report);

- I reviewed the report describing the results of eDNA sampling for steelhead and coho salmon conducted at select locations in the Gualala River basin and estuary in December, 2020 by Sarah O'Neal, included here as Exhibit 3 (Sarah O'Neal Report).

1. Site Visit

On April 23, 2021, I visited the Dogwood THP Area and made particular observations at the locations identified with circles on the map attached hereto as Exhibit 4. In the field I observed the general pattern of trees marked for logging in the Dogwood Timer Sale in relation to their distance to streams, floodplain swales and other habitats likely to be seasonally wet during winter. I also observed the location of existing haul roads, older skid trails, and proposed tractor trails for logging the Dogwood sale, particularly with regard to direct disturbance of streambanks and seasonally wetted floodplain habitats, and with regard to evidence of sediment generation and delivery from road surfaces and ditches to river, stream and floodplain habitats in and adjacent to the Dogwood sale units.

2. Environmental DNA Sampling

As part of my review I assessed environmental DNA ("eDNA") samples collected to determine the presence of Coho and Steelhead in and near the Dogwood THP Area. Exhibit 3 identifies the locations from which eDNA samples were collected that contained Coho DNA. The methodology by which those samples were collected and analyzed is likewise described in Exhibit 3. The eDNA samples revealed that in late 2020 steelhead trout were widespread, with eDNA traces in every wetted habitat sampled within and near the Dogwood THP area. The eDNA sampling also revealed that coho salmon occurred in late 2020 at the confluence of the North and South Forks of the Gualala River. Finally, both steelhead and coho salmon presence was indicated by eDNA detections in the Gualala River estuary in December of 2020, immediately prior to the time the bar opened up with a continuous surface water connection to the sea during a major winter storm.

III. COHO SALMON

A. Background

1. Coho Generally

Coho salmon are widely distributed along the Pacific coast of the USA and Canada, but their distribution has become fragmented as numbers have dwindled, and the distribution of populations within river basins have shrunk. Coho begin life incubating as eggs during winter months, and later hatched alevins, within well-sorted, porous stream gravels where they were spawned by their parents. In spring they emerge and become free-swimming, and for their first summer they occupy quieter-water pools where the feed ansd shelter from predators. Most juvenile Central California coho grow for just over a year in fresh water, when they achieve large enough size to successfully smolt and survive the transition to the marine environment. As stated in the Recovery Plan (NMFS 2012) The suitability of the stream to provide the necessary habitats for coho salmon to survive at each life stage is critical to their persistence in our rivers and streams. This means streams must have: (1) clean loose gravels free of fine sediment; needed for spawning and egg development; (2) adequate pools and natural instream cover for juveniles; (3) connected alcoves and off-channel habitats for juveniles to survive winter flows; (4) clean cool water; and (5) unimpaired passage to and from the ocean. Off-channel habitats refers to overflow channels, small tributaries, swales and other wetlands located on floodplains, which become accessible to coho salmon and essential to their survival during high runoff events. Coho may use estuaries to rear in when summer conditions upstream are stressful and too warm for effective growth, and in winter they use off-channel floodplain habitats for survival, but typically are found in lesser numbers than steelhead.

2. Coho Habitat Requirements

During incubation, eggs and very young coho require streambed gravels remain relatively unclogged by fine silt and sand, such that water flows through intragravel pore spaces and delivers oxygen while removing carbon dioxide and other toxins from the egg pocket. Intrusion of organic matter into egg pockets can also reduce survival because the biological oxygen

demand of microbial breakdown of the organic matter depletes oxygen availability to eggs. Factors that destabilize streambeds, such as pulsed delivery of coarse and fine sediments during floods, and erosion sources that deliver fine sediments to streams during the winter and spring incubation period, can severely reduce the survival of eggs and recently-hatched alevins. Because they are spawn earlier than steelhead, coho eggs and fry are more likely to be vulnerable in the gravel during flood events when streambeds shift, crushing, dislodging and killing eggs and young. But chronic inputs of fine sediment to spawning streams is even more likely to kill coho, by clogging interstices and reducing food supply and feeding efficiency of young-of-the year and yearling fish.

3. Coho Status and Threats

The Gualala River Watershed lies within the Central California Coast Coho Salmon Evolutionary Significant Unit (ESU), which is listed as endangered under the Endangered Species Act *(NMFS 2005)*. Although coho salmon are limited in their distribution in the Gualala River watershed today, NMFS (2012) estimated that the basin supported an annual run size of 4000 coho in the 1960s, dropping to 1000 in the 1980s, and 200 in the 1990s. In recent years coho have been reported by trained underwater fish observers in five survey locations in tributaries to the North Fork Gualala (GRWC 2021). Based on inspection of the training and survey protocols used by the Gualala Watershed Council, and based on my experience with coho salmon distribution and detectability in other watersheds with greatly depleted populations, I'm confident that these observations confirm that coho are still extant in the Gualala Watershed (or alternatively, have recently recolonized there.) Environmental DNA surveys in December of 2020 (Exhibit 3) detected trace amounts of coho salmon DNA at two locations within the Gualala estuary (prior to bar break). In my opinion these most likely reflected small numbers of juvenile coho salmon occupying among the most obviously suitable habitats within the estuary.

Alternatively may represent DNA transported from juvenile coho salmon rearing somewhere upstream, which could be the North Fork, or could be other locations where small populations of coho might occur undetected, given in the absence of comprehensive annual surveys in the basin.

Coho are well-adapted to incrementally explore and colonize suitable habitat within a watershed from extant population sources, over time in stepping stone fashion, as some adult coho stray from their natal areas, especially when spawning density is high in their natal stream. Therefore is to be expected that the coho documented to be present in the North Fork are likely to contrinute to eventual recovery of coho populations in the South Fork and it's tributaries, assuming habitat recovers and stream shade and temperature, gravel, pool, and shelter conditions favorable to coho survival redevelop there. Protecting such source populations for future recovery—and restoring habitats where they may expand their distribution-—is indeed the basic foundation of recovery plans for coastal coho salmon throughout their range.

Coho salmon are vulnerable to alteration of spawning and incubation habitat, principally via erosion and sedimentation, warming of waters that restricts rearing habitat, loss of cover and pool-forming features due to the depletion of large wood following logging, direct mechanical disturbance or sediment delivery from roads and disturbed soils to seasonally wetted floodplain habitats and small tributary streams and overflow channels that provide shelter and food at high flows, and loss of rearing space and oxygen depletion in mainstem pools and in small estuarine lagoons due to sedimentation or eutrophication following algae outbreaks.

IV. NORTHERN CALIFORNIA STEELHEAD

A. Background

1. Steelhead Generally

The Gualala River steelhead (*Oncorhynchus mykiss mykiss*) comprises one collection of populations contributing to the Northern California Distinct Population Segment (DPS) of steelhead. The distribution of this DPS ranges from Redwood Creek south to the Russian River in Sonoma County, CA. However, with few exceptions, steelhead like other salmon home to natal spawning areas, therefore the DPS consists of a multitude of localized spawning populations that are behaviorally adapted to survival and reproduction in the specific natal stream to which they return after their ocean life. The Gualala River population is considered by NMFS to be one of 27 "essential independent populations" distributed across the DPS (NMFS 2016, p. 1).

2. Steelhead Habitat Requirements

Steelhead can be seen to begin their lives as eggs placed in stream gravels in appropriate streambed conditions in streams and rivers by adult steelhead that have returned from the sea to spawn in their natal streams. Eggs and fry require an incubation period of many weeks during late winter and spring, during which a relatively streambed persists, and gravels remain relatively unclogged by fine silt and sand, such that water flows through intragravel pore spaces and delivers oxygen while removing carbon dioxide and other toxins from the egg pocket. Factors that destabilize streambeds, such as pulsed delivery of coarse and fine sediments during floods, and erosion sources that deliver fine sediments to streams during the winter and spring incubation period, can severely reduce the survival of eggs and recently-hatched alevins, as they remain immobile within the stream bed. Incubating eggs and alevins also require relatively cold and stable stream temperatures (5-10 °C) for optimum survival, and if water warms above 11 If water temperature warms above 11 °C during this period, survival drops substantially (Carter 2005).

Once they emerge from the gravel in spring and early summer, steelhead fry feed primarily in tiny invertebrates and other food items suspended in the water column. At this life stage during their first summer they require extensive shallow wetted stream margins, where velocity allows them to maintain position in the water column and shallow water discourages larger fish from preying on them. Fine sediment that fills interstices of the streambed gravels can reduce survival of fry and young-of-year steelhead because those interstices are the primary hiding cover those fish rely on to escape predation from birds, mammals, and reptiles that forage in stream shallows. As they grown during the first summer, steelhead fingerling become more mobile and able to exploit a wider variety of food resources. However, as they grow, their temperature requirements become more narrow, and increasingly warm stream temperatures over an optimum of about 17°C daily maximum sustained over a period of 7 days result in reduced individual growth and lower density and abundance, as juveniles emigrate to seek cooler and more favorable summer habitat elsewhere in the river basin. Above about 20°C steelhead are more frequently displaced by minnows and other fishes adapted to warmer water, above 21-22°C steelhead move to seek cooler water pockets to survive (Nielsen et al. 1994). Unless coldwater refugia are available, juvenile steelhead may be eliminated (displaced or killed by stress, disease, or predators) when stream temperature exceeds 22-24°C for multiple days (Carter 2005).

At such warm temperatures, under conditions of nutrient loading and high algal production as seen in the Gualala River, biological oxygen demand increases to such levels that oxygen depletion occurs at night, further stressing and displacing juvenile steelhead from warmed river habitats even during somewhat cooler early morning temperatures (Higgins 1997). Juvenile steelhead displaced by high water temperatures in the Gualala River and its major tributaries are known to congregate downstream in the Gualala lagoonal estuary while it is bar-bound and cut off from direct surface connection with the ocean (Higgins 1997). Cool water strata persist at depth in the estuary, and invertebrate food resources during bar-bound conditions can be prolific enough to support high densities of juvenile salmonids and other fishes in the estuary (Higgins 1997), as is also typical of other similar small, bar-bound estuary lagoons in this

region of California (Bond 2006, Hayes et al. 2008) and in rivers I have studied in south coastal Oregon.

Juvenile steelhead in California typically spend between 1 and 3 years in fresh water before they reach sufficient size to transform into smolts and enter the ocean. Thus rapid changes in streamflow, flooding, high suspended sediment that creates turbid water, and bedload and debris movement that are common occurrences during winter present a second set of stark seasonal challenges to steelhead survival. At 1-3 years of age, juvenile steelhead in freshwater avoid volatile and harsh main river conditions during winter by seeking refuge in floodplain ponds, small floodplain tributaries. Floodplains near the estuary and along the South Fork Gualala River, including those within the Dogwood Timber Sale units, provide particularly important habitat for these fish, as they must migrate upstream out of the estuary in early winter or late fall once river temperatures have cooled, and before the bar is broken and the estuary becomes open to the sea with the first major rainfall. Many steelhead that have not escaped the estuary prior to the bar breaking are likely to be washed out to sea before they have reached sufficient size to survive the high predation intensity that occurs in the marine coastal zone (Higgins 1997).

Because of their typical 1-3 year freshwater residency, juvenile steelhead face adverse midsummer and midwinter conditions multiple times through their freshwater life. Thus, maintaining the habitats that offer respite and refuge from elevated summer water temperatures and harsh winter flood conditions are absolutely critical to steelhead survival and recovery. Any loss of or damage to these critical types of habitat of very limited availability—that is, small coolwater tributaries and the Gualala estuary in summer, and floodplain tributary streams, overflow channels and ponds in winter--are extremely likely to displace and result in increased juvenile mortality and reduced abundance of Gualala steelhead in the Gualala basin.

In sum, steelhead are vulnerable to alteration of spawning and incubation habitat, principally via erosion and sedimentation; warming of waters that restricts rearing habitat; loss of cover and pool-forming features due to the depletion of large wood following logging; direct mechanical disturbance or sediment delivery from roads and disturbed soils to seasonally wetted floodplain habitats and small tributary streams and overflow channels that provide shelter and food at high flows; and loss of rearing space and oxygen depletion in mainstem pools and smaller estuarine lagoons due to sedimentation or eutrophication following algae outbreaks.

3. Steelhead Status and Threats

This population segment of steelhead is listed under the US Endangered Species Act as a threatened species, or "likely to become endangered in the foreseeable future. Threats to these fish pertinent to this report and the Dogwood Timber Sale are "Land use activities associated with logging, road construction, urban development, mining, agriculture, ranching, and recreation..." which "have resulted in the loss, degradation, simplification, and fragmentation of NC steelhead habitat, and caused resulting declines in NC steelhead populations." (NMFS 2007, p. 7). Direct and cumulative impacts of these human activities on steelhead habitat include "alteration of stream bank and channel morphology; alteration of ambient stream water temperatures; degradation of water quality; elimination of spawning and rearing habitats; fragmentation of available habitats; elimination of downstream recruitment of spawning gravels and large woody debris; removal of riparian vegetation resulting in increased stream bank erosion; and increased sedimentation input into spawning and rearing areas…." (NMFS 2007, p. 8). The Recovery plan further recognizes "natural conditions," including floods and persistent drought, as persistent threats that interact with man-caused impacts to further impair habitat and survival (NMFS 2016, p.18).

Specific to the Gualala River and habitat elements impacted by the Dogwood Timber Sale, in the recovery plan NMFS (2016, p.53) summarized the following habitat conditions as the most

severely impactful category, “Poor” for steelhead: Shelter and pools for migrating adult steelhead in winter and juvenile steelhead in winter and summer; and flow and turbidity conditions for summer rearing. Summer temperature conditions were treated Fair because of the presence of some cool habitat in tributary streams, but the summer temperatures in the South Fork Gualala River itself are far higher than optimum and well exceed displacement temperatures for steelhead (Higgins 1997, CRWQCB 2001). Both timber harvest and streamside road density are identified in the steelhead Recovery Plan (p.52) as among the land use factors that contribute to poor habitat conditions in the Gualala watershed, with “Logging and Wood Harvesting” ranked as a “High” threat (p.65).

The environmental DNA detection study conducted in December of 2020 (Exhibit 3) provided evidence that during that season, steelhead of one life stage or another were or had been recently present (at unknown abundance) in virtually all surface water habitats sampled in the area of the estuary prior to the bar breaking), the lower Gualala River, and tributaries to South Fork Gualala River in the vicinity of the Dogwood Timber Sale. This finding corresponds with the above opinion that many smaller juvenile steelhead likely disperse upstream out of the estuary in fall or winter to avoid being flushed to sea when the bar breaks, and to seek suitable winter shelter in floodplain habitats and tributary streams in and along the North and South Forks of the Gualala River. In addition, it is certain that some young-of-year and yearling steelhead descend downstream from steeper headwater channels higher in the watershed to seek winter shelter in floodplain habitats in the lower miles of the North and South Forks.

V. IMPACTS OF GUALALA REDWOOD TIMBER, LLC'S LOGGING ON SALMONIDS

A. Categories and mechanisms of take:

1. Logging Removal of Large Trees Curtails Recruitment of Wood and Depletes Natural Wood Loading in Aquatic and Floodplain Habitats.

Dead and downed redwood trees are abundant in natural mature and old growth redwood forests on coastal river floodplains (Welsh et al. 2000, Benda et al. 2002, Benda and Bigelow 2013) and estuaries (Maser and Sedell 1994). In forests up and down the temperate Pacific Coast of North America, downed large wood, especially larger boles from mature trees, plays many vital roles in forming stream and floodplain habitat features that provide for survival of steelhead and coho salmon across the seasons (Abbe et al. 1996, Welsh et al. 2000, Collins 2012, Wohl 2013, 2014, Pollock and Beechie 2014). Importantly, although thinning of redwood forests is viewed by foresters as an acceptable practice from the standpoint of fiber production, it is well-established that thinning or selective logging in Pacific coastal riparian forests removes trees that would otherwise die naturally and contribute to riparian, floodplain, and aquatic ecosystem functions that strongly shape and form fish habitat (Pollock and Beechie 2014), and redwood forests are no exception (Benda et al. 2002, Benda and Bigelow 2013). Any trees that upon senescence, could fall into areas where they shape channel or floodplain flows, can play key roles in channel and pool formation, while also providing hiding cover for steelhead, coho, and other fishes. Indeed, this is the principle reason why commercial logging is not allowed within one tree-height distance of fish-bearing streams on federal forest lands in the Pacific Northwest and northern California. The absence of specific protections to ensure natural wood recruitment to streams is not curtailed by logging is also one reason why NMFS has withheld approval of the CalFire's ASP document for timber practices. As mentioned earlier in this report, wood-formed habitats on floodplains are essential to the survival of steelhead and coho salmon during winter stormflows and flooding of California coastal streams.

In the field on 22 April 2021, within units of the Dogwood timber sale I observed numerous redwood trees marked to be logged standing in close proximity to floodplain habitats

that are certainly important for overwintering of juveniles steelhead, and are also classic essential winter habitat for rearing coho salmon. Field cues confirmed that the Dogwood timber sale units lie predominantly (and some exclusively) within the 100-year floodplain of the South Fork Gualala River and Buckeye Creek. Marked trees were observed on the banks of some floodplain winter season habitat features, and as close as 20-30 meters of others; marked trees were observed within 15 meters of the annual active channel of the South Fork Guala River itself. All of these trees, if allowed to senesce and fall by way of natural self-thinning processes, would stand a good chance of contributing boles or branch debris to nearby winter steelhead and coho habitat, considering the average height of a 70-80-year-old redwood is about 50 meters (see review and summary by Graham 2020).

The floodplain habitats in closest proximity to marked trees and that would be most directly affected by logging those trees included 1) distinct channel swales and depressions that show vegetation, sedimentological, and topographic evidence of holding water and offering refuge from main river flows for periods of days to weeks during winter flooding (Units 5, 17, 21, 8, 9 and 10); 2) floodplain channels and wetlands with spring-fed flows that retain water throughout much or all of the year and are connected to the South Fork Gualala River during floods, allowing fish access (Unit 5); and 3) tributary streams to the South Fork Gualala River that appear to hold continuous perennial flow with potentially high-quality winter-long refuge habitat for steelhead and coho salmon. including Big Pepperwood Creek, Little Pepperwood Creek (Unit 5), and Buckeye Creek (Units 8,9 and 10).

In the near term, removal of these trees risks damage to channels and their banks for felling and yarding and mechanical equipment operation. In the longer term, removal of these trees starves steelhead and coho wintering habitat of the large wood needed to form and maintain complex channels, form pools for more persistent refuge habitat and overhead woody cover that protects salmonids in small streams from predation, downed trees that over time form natural log jams in the main river channel to aid in formation and diversion of natural flood flows into these floodplain refuge habitats. If allowed to grow freely, natural self-thinning processes would allow

many of these trees to die or be blown over, thus contributing to restoring the natural quantity and functions of downed wood that are an essential feature of older redwood floodplain forests. In my opinion, cutting of these trees directly curtails natural habitat recovery processes in these forests, preventing the return of high-quality wintering habitat that steelhead and coho salmon require to thrive in these coastal rivers with flashy winter flows. The historical condition of complex floodplains rich in downed wood and overflow channels—the river conditions in which steelhead and coho salmon evolved and require for good survival in freshwater—cannot be re-established, or its re-establishment will be greatly slowed, if logging replaces natural self-thinning processes and continues to remove redwood trees from the river's floodplains. In my opinion, logging such as that proposed in the Dogwood timber sale that causes the curtailment of natural self-thinning and dead wood recruitment processes is, when considered across the full temporal duration of its harm (the many decades to centuries it takes to replace mature redwood trees, and additional time for some of them to senesce and fall), the largest single source of adverse habitat modification and take of steelhead and coho salmon.

Thinning depletes large wood that is absolutely essential for habitat creation and maintenance. Benda and Bigelow (2014) conclude that the maximum rate of wood recruitment during the long history of a redwood floodplain forest occurs during this period when *self-thinning* of second growth stands occurs. Thus, thinning of second growth stands in roughly the 50-120 year age range is going to have the largest potential effect on depressing wood recruitment to the channels and floodplain, directly removing the natural dead and downed wood that is essential for recovery and established the wood loading that persists for centuries. In the field I observed some root wads and other pieces of wood artificially placed in tributary streams in the attempt to improve the simplified and wood-depleted fish habitat there (e.g., Little Pepperwood Creek). However the artificially placed wood I observed was of insufficient size and abundance to have substantial benefit to habitat recover in the S Fk Gualala River and its floodplains. Although these pieces might create some very localized benefit in smaller tributaries like Pepperwood Creek (if the stream retains water and sufficient temperatures to

support salmonids), they are grossly insufficient to shape channel form, divert flood flows, and create and maintain the natural complexity of floodplain channels and wetlands that steelhead and coho depend on for shelter during winter floods.

The long-term depletion of large wood caused by historical forest practices in the Gualala River, and that is furthered by continued logging in floodplains and riparian forests, has other more systemic effects that also modifies habitat in ways that take steelhead and coho salmon. Accumulations of large wood in the South Fork Gualala R and larger tributaries are essential to promote the subsurface exchange of water that connects floodplain soils and vegetation to the main channel; the flow of stream water into and out of this subsurface hyporheic or shallow groundwater flow system is a critical process that allows scrubbing of nitrogen and other nutrients from the water (Welsh et al. 2000, and reference therein). With present day, depleted wood loadings, rather than being absorbed by floodplain forests, nutrient loads remain in the stream channel where they promote algal blooms and the profusion of thick algal mats. Secondly, depleting wood on the river floodplain and in its channels reduces export of wood to the estuary. Dead wood in estuaries historically played key roles in providing habitat, co er, and an organic food source foe estuarine invertebrates that serve as the primary food source of steelhead, coho, and other fishes that rear in the estuaries (Maser and Sedell 1994), so the morphology and productivity of estuary rearing habitat has declined as wood has been depleted.

2. <u>Tractor logging and yarding directly damages floodplain channels and wetlands that are the key wintering habitats of steelhead and coho salmon.</u>

In the field I noted that a few flagged skid trail locations in Dogwood units appeared located to avoid sensitive floodplain habitats important to fish, including distributary channels, swales, some river-parallel floodplain channel features. However, other flagged skid trail locations were directly imposed on top of these same sensitive features, or crossed them with little regard for damage to soils, channel structure, or drainage. The result of operating on the latter flagged skid trails is certain to be direct alteration of the floodplain habitats, and delivery of

sediment from the disturbed skid trails into the floodplain habitats they cross. Because these essential habitats are both widespread within South Fork Gualala floodplains and sensitive to direct disturbance, any extensive ground-based logging operations across these floodplains are highly likely to adversely modify them.

Tractor yarding in riparian areas mobilizes sediment to streams, because of the close proximity of ground disturbance to surface waters (Anderson and Lockaby 2011), and increased erosion rates can persist at least five years after logging (Croke et al. 1999); for this reason, the banning of ground-based logging in near-stream and floodplain areas is an increasingly widespread example of a forest best management practice (Phillips et al. 2000).

Anywhere moderate to steep slopes occur, skid trails on those slopes are highly likely to trigger erosion and sediment transport in the geologic and soils setting of the Gualala Watershed. In Unit 1 of the Dogwood sale, where moderately steep slopes occur, I observed past and ongoing logging-related slope erosion in the form of eroding rills and small gullies on skid trails present from past logging activity in Unit 1. A large fraction of these skid trails run up and down slope rather than crossing the slope transversely, which is a sure-fire means of generating sustained rill and gully erosion of the compacted and disturbed skid trail surface. Moreover, the currently marked Dogwood project unit had its proposed skid trails flagged right on top of those same eroding older skid trails, rather than redirecting to less steep routes that could reduce erosion risk. One existing gullied channel originating from a skid trail presently contributes sediment to the small, intermittent flow tributary at the west edge of Unit 1, and by this route sediment it appears highly likely that sediment is a delivered to the lower river and estuary. Other rills and gullies from skid trails in Unit 1 disappeared into forest downslope of Unit 1, essentially on the floodplain of the Gualala River. Field time did not allow investigation of the ultimate delivery point of the sediment in the latter cases.

In this landscape, tractor yarding of slopes such as those within Dogwood Unit 1 can seldom be conducted in a way that does not generate significant erosion and sediment transport to downslope and downstream areas. Confining skid trails to gentler slopes can result in a large

increase the area of soil compacted and disturbed, whereas reoccupying older steeper skid trails can re-initiate or accelerate rill and gully erosion. Either way, increased erosion and sediment mobilization is inevitable with ground-based logging, and where sensitive aquatic habitats are adjacent or near, they are highly subject to impaired habitat conditions from sediment delivery.

3. Haul roads are configured such that they deliver chronic fine mineral sediment to river, tributary, and floodplain habitats.

It has long been known that vehicle traffic on gravel surfaced roads generates fine sediments, largely by the action of heavy vehicles pulverizing stones into fine particles that are available for transport by air as dust, or by water as sediment-laden runoff from the road surface and ditches (Reid and Dunne 1984). The rate and quantity of fine sediment generated by pulverization (Sheridan et al. 2006), and by deformation of the road surface and subgrade by haul during saturated soil conditions in wet weather is directly proportional to traffic intensity and especially heavily laden trucks carrying logging equipment or logs (VanMeerveld et al. 2014). Delivery of this road-generated fine sediment to waterways is an intrinsic, systemic problem wherever roads are superimposed on a floodplain and closely parallel and cross numerous aquatic and wetlands habitats—as roads slated to be used for haul in the Dogwood timber sale are. The state of California lists the Gualala Watershed as water quality impaired by excess sediment (CRWQCB 2001), and road and skid trail surface sediment are among sources of anthropogenic sources that must be controlled to meet water pollution targets recovery of steelhead and coho habitat. Fine sediment originating from roads and skid trails, and disturbed floodplain surfaces following logging is essentially always harmful to growth and survival of steelhead and salmon, and almost never beneficial—more so in streams already impaired by sustained elevated sediment loads caused by past disturbances and human alterations of the watershed.

The cumulative effect of past and ongoing practices in the Gualala Basin that contribute to habitat impairment by excess sediment create a context where even modest or small quantities of additional sediment load, especially chronic suspended fine sediments such as those

originating form logging roads, cause disproportionate harm to steelhead and salmon. These fishes seek winter habitat in channels, wetted swales, tributaries, and wetlands on the floodplain in order to escape turbid and sediment-laden conditions in the South Fork Gualala River; injecting sediment in to those floodplain habitats via roads and floodplain soil disturbance degrades those habitats by impairing the production of insects on which the fish depend for food, impairing the ability of salmonids to feed efficiently by reducing their ability to see food items, and increasing physiological stress by irritating gill structures. Steelhead and salmon sometimes are forced to abandon floodplain habitats when sediment conditions render them ill fit for survival, displacing thjem into less favorable habitat in the main river or elsewhere on the floodplain.

Fine sediment is generated and delivered from roads regardless whether log haul occurs during wet weather or dry weather. In the dry season, the fines are mobilized in the air as dust, which settles in roadside ditches and on vegetation, and is subsequently mobilized, transported and can reach surface waters during subsequent rainfall events. In the wet season, road surfaces are largely impermeable, so they retain standing or flowing water for prolonged periods during and after rainfall; in this situation the generated fine sediments create a slurry of sediment laden water that follows road grades and ditch lines to intersect and enter surface waters (Reid and Dunne 1984). When water is spread as a dust abatement measure during log haul in the dry season, as proposed for the Dogwood sale, the delivery of traffic-generated fine sediments is not contained, but merely delayed. The traffic-generated fines remain on the road surface and adjacent ditches, rather then spreading as dust, until they are mobilized in subsequent rain storms, thus entering surface waters in the wet season.

In the field I observed numerous locations where runoff from the main haul road for the Dogwood sale has in the recent past eroded and delivered sediment to floodplain swales, tributary streams, and (via water drafting access spurs) the South Fork Gualala River. The roads I observed in the sale area show relatively little evidence of the kind of design features and maintenance that are necessary to effectively direct sediment-laden road runoff away from

surface waters and wetlands and toward less sensitive slopes or floodplain areas where no salmonid winter habitat occurs. The roads showed long distances of uninterrupted slope that means an extreme length of road collects fine sediments and delivers them off of the road at low points, including stream crossings, road-adjacent floodplain swales and wetlands. The roadside ditches I observed in the field frequently connect and discharge to those same floodplain habitat features.

4. Logging that increases erosion and delivery of sediment also delivers nutrients to a highly nutrient loaded river, increasing harm from algae blooms and eutrophication in the river and estuary.

In field observations I could not help but notice the extremely, in fact shockingly, high pervasiveness and magnitude of algal blooms in the South Fork and mainstem Gualala River. Even after an episode of winter flooding should have scoured much of the algal growth from the streambed, massive algal mats were seen to be widespread in the river, and the water column was dense with drifting algal clots that had sloughed from the river bed. Under natural conditions, coastal rivers like the Gualala tend to be dominated by relatively clean beds with only localized areas of visible algal growth and virtually no algal sloughing into the water column, especially in spring and early summer. Nuisance algae blooms such as those presently evident in the South Fork Gualala River tend to form under a convergence of extreme conditions that include: nutrient loading far in excess of historical concentrations, reduced river flows, increased river temperatures, reduced stream surface shade caused by channel widening and loss of trees, or reduction in tree heights within 60 meter or so of stream banks, and nightly anoxia driven by decaying algal matter that causes die-offs of the larval insects and snails that normally consume streambed algae and keep blooms in check. The results of these changes, all known drivers of the algal bloom phemonenon that limnologists and ecologists refer to as eutrophication, can include exclusion of fishes from their usual rearing habitats in the river because of depleted oxygen conditions at night; stress to fishes and other biota from daily pH fluctuations that

accompany volatile changes in dissolved oxygen and carbon dioxide; and loss or reduced productivity of the invertebrate fauna that is the principle food source of juvenile salmonids.

What specific factors trigger the observed eutrophication in the South Fork and mainstem Gualala River have not been identified, as far as I am aware, but it is known that northern California coastal streams are principally limited by nitrogen availability (Hill and Knight 1988, Hill et al. 2009). The forms of nitrogen that can drive eutrophication and algal blooms derive principally from two sources in watersheds like the Gualala River: logged lands where decaying litter and root systems drive nutrient leaching from the soils to streams, and agricultural lands where removal of forest and fertilization combine to increase nitrogen loads. Vineyards in the Gualala headwaters could contribute to N loading of the river. Algal growth, however, requires balanced availability of nitrogen and phosphorus. Once nitrogen sources are elevated and algal growth starts to increaser, phosphorus can become the limiting nutrient. It appears likely that sustained elevation of nitrogen in the river creates a condition where the local delivery of phosphorus can spur more extreme algal proliferation and blooms. Among the principal local sources of phosphorus in the South Fork Gualala River in the Dogwood timber sale area would be soil erosion and road surface erosion that enters stream channels, by the same vectors as described above in the sediment section (in fact much phosphorus is delivered in the form of particulate, i.e., associated with mineral sediments). In my opinion, the disturbance of floodplain soils and delivery of sediment to streams and wetlands associated with the Dogwood timber sale is highly likely to exacerbate currently problematic algal blooms in the river.

Nutrient loading that drives algal blooms in coastal rivers can increase the export of sloughed and decaying algae into estuaries, and drive further eutrophication and oxygen depletion that harms fish rearing in the estuary (Paerl et al. 2018). Estuaries that are small in size with hydrology strongly influenced for much of the year by river inflow and less influenced by marine processes—such as the Gualala estuary after bar closure, when steelhead and coho salmon are dependent on the estuary for late spring, summer and fall rearing—are among those most vulnerable to adverse habitat modification by eutrophication, anoxia, and loss of

invertebrate food resources triggered by nutrient inputs from the river. Historical sediment loading from past logging and road-related erosion that has caused the estuary to grow much shallower compared to its depth in historical times (Higgins 1997) further increases vulnerability of the estuary to nutrient loading. The estuary receives continual season-long nutrient and organic matter loading through the constantly sloughing drifts of algal mats produced in the mainstem and South Fork Gualala River. Though the status of the estuary in relation to nutrient loading estuary has apparently not been specifically evaluated in the Gualala River, in my opinion current conditions are consistent with the Gualala estuary being on the cusp of eutrophic breakdown if elevated nutrient loads from the watershed continue or increase. Even relatively small contributions such as the loading of phosphorus that can be expected from the Dogwood timber sale could be sufficient to trigger extremely harmful habitat responses in the estuary, where it appears, a major fraction of the basin's anadromous fish rear today, given poor freshwater conditions in streams and rivers of the basin (Higgins 1997).

Although existing information does not support the fully informed analysis of nutrient loading risks to the river ecosystem that would be necessary to ascertain ongoing or imminent impact to steelhead and coho salmon, I include this habitat modification on my list of concerns here for two reasons: 1) in my opinion, the field observations I made of algal mats and algal drifts are extreme warning signs of eutrophication and imminent, if not incipient oxygen depletion in the river and potentially the estuary, and 2) in my view this threat to juvenile steelhead and coho salmon rearing in the river and estuary likely poses the greatest single threat of mass mortality and potential extirpation of their populations in the Gualala. Changes in the river's sediment, temperature, flows, large wood supply, and floodplain functions all conspire to increase the risk of extensive anoxia driven by sediment loading. For example, we know that exchange of flows between the redwood forest rivers and surface and subsurface waters of adjacent floodplains provide a critical natural means of "scrubbing" excess nutrients from the river (Welsh et al. 2000). However, with the depletion of large wood in the river and floodplains, and the and geomorphic consequences of massive sediment loading associated with past logging

and road effects, it is certain that interchange of flow between the river channel and floodplains, both surface and subsurface, is greatly curtailed compared to historic conditions, and this natural nutrient removal function is compromised. This complex web of ecological impact illuminates how many different aspects of human action interact to transform rivers in ways that put sensitive native species, like steelhead and coho salmon, at risk of extinction (Higgins 1997).

5. Logging on floodplains will produce summer stream temperature increases in streams where temperature already limits salmonid survival and productivity.

The South Fork Gualala River is listed by the North Coast Regional Water Quality Control Board as water quality impaired for sediment and temperature (https://www.waterboards.ca.gov/northcoast/water_issues/programs/tmdls/gualala_river/), largely because late spring and summer water temperatures exceed optimum temperature ranges for rearing coho salmon and steelhead. In a recent field experimental study of shade and stream temperature response to thinning of riparian coast redwood forests, Roon et al. (2021) reported that second growth redwood forests thinned to canopy closure between 70 and 80 percent resulted in summer water temperature increases of 1-2 degrees C, with increased temperatures persisting for more than 30 consecutive days. Downstream reaches below thinned stands also warmed as a result of canopy loss and increased solar insolation I thinned areas upstream. The post-cutting target canopy cover in the Dogwood timber sale is in the range of 70-80%, such that similar summer temperature increases are likely in tributary streams passing through cutting units of the Dogwood timber sale. These tributary streams include Big Pepperwood Creek, Little Pepperwood Creek, Buckeye Creek, and several smaller unnamed tributaries. An analysis of change in canopy after logging of Dogwood units 8, 9 and 10 (Exhibit 2) indicates that the levels of logging anticipated in the Dogwood sale can be expected to reduce canopy cover—hence stream shade within or adjacent to the unit boundaries—by about 6 to 14 percent. This magnitude of shade loss is well within the range reported by Roon et al. (2021) to produce

increases in already too-warm summer water temperatures. Such increases in solar insolation to wetted streams may also further spur algal blooms already facilitated by nutrient loads.

The junctions of shaded, cooler tributary streams offer coldwater thermal refugia for rearing salmon and steelhead where they join warmer mainstems like the South Fork Gualala River. Under unlogged conditions with high canopy cover and shade, these tributaries offer cooler habitat at a coldwater plume where they meet the river, and in the tributary itself where surface water connections allow fish movement into the tributary stream (Nielsen et al. 1994, Madej 2008, Greer et al. 2019). Water sources even 1-3 degrees F cooler than temperature-impaired mainstems can offer salmonid fish critical relief from thermal stress. My field observations on April 23d confirmed that the lower quarter to half mile of each of these major tributaries appears to be accessible to migrating juvenile salmon and steelhead in spring, so it appears likely that in many years at least they likely offer cooler thermal refuge in late spring and early summer when the main river is hottest. Also on the 23 April field trip I observed numerous trees marked for cutting within the immediate riparian areas of Big and Little Pepperwood Creeks, within 20-25 meters of the stream banks—easily close enough that cutting would result in canopy loss sufficient to measurably warm streams to the degree reported in Roon et al. (2021). Moreover, trees removed from within 60-70 m or so of the mainstem Gualala River will reduce morning shade on the river, speeding up the rate of daily warming in summer and reducing the morning cool period that steelhead and coho salmon require to survive summer thermal peaks.

By reducing canopy cover and warming these tributaries, the Dogwood THP is highly likely to shrink or eliminate cold water thermal refugia, thereby rendering summer habitat in the South Fork Gualala increasingly thermally hostile to salmon and steelhead. Canopy removal from logging withing the riparian areas of these tributaries is highly likely, therefore to take steelhead and impair potential coho salmon habitat by reducing the availability of critical summer coldwater refuge at tributary junctions with the South Fork Gualala River and in the lower reaches of the tributaries themselves. The effects on salmonid fish of such losses include

reduced growth, increased crowding and vulnerability to disease and predation, and displacement to remaining coldwater refugia downstream, in particular, at the mouth of the North Fork Gualala River, and in the Gualala River estuary, where fish are also be subject to crowding and predation by seabirds, otters, and other wildlife. Suitable summer rearing habitat will be reduced and fish restricted to more limited summer rearing area. In my opinion it is fairly certain that under logging allowed by the Dogwood THP, physiological thermal stress and the risk of predation will both be elevated by displacement from tributaries and thermal refugia at tributary junctions, with reduced growth and health of juvenile salmonids and increased mortality from crowding and resultant predation.

B. Opinion as to likelihood of take and adequacy of mitigation measures

1. None of above adequately accounted for in ASP or related rulemaking and guidance.

In the previous section, I focused on actions and effects that in my opinion are not adequately mitigated either in the specific plan for the Dogwood timber sale, or on the APS guidance document. In fact, the likely causes of take I have identified above correspond rather well with the technical and scientific concerns that NMFS's and the state water quality have raised about the ASP, and which have not been addressed to date. The concerns I discuss above are also frequently identified by independent researchers with whom I have who worked on water quality, salmonid habitat, and riparian and floodplain ecology in relation to logging and roads in northern California. The ASP and many parties that implement it in logging projects continue to avoid or deny, rather than identify and mitigate these sources of impact.

VI. CONCLUSION AND FINDINGS

The cumulative outcome of all of the effects covered above is in my opinion an extremely high probability of take of steelhead and coho salmon by adverse habitat alteration, which not only damages habitat, but more seriously curtails or reverses the natural processes of habitat recovery that are currently underway on the Gualala River floodplain and nearby slopes.

Even if the individual sources of harm were each small in probability of occurrence (they are not), the sum of probabilities aggregated across the timber sale produces a large probability of take. The chain of cause and effect of habitat modification extends from the floodplains of the South Fork Gualala and its tributaries downstream to the Gualala estuary. Importantly, none of the actions proposed in this timber sale has a foreseeable positive effect or benefit to habitat of protected steelhead and coho; at best they are effective in partially limiting the harms that will be done.

The sources of take of steelhead and coho salmon I identified can be summarized as follows:

1. Logging Removal of large trees Curtails Recruitment of Wood and Depletes Natural Wood Loading in Aquatic and Floodplain Habitats.

The depletion of wood recruitment to floodplain s and channels by selective logging is not offset by growth release of uncut trees, because thinning directly nullifies a portion of the natural self-thinning processes that produce dead and downed wood. Moreover, thinning is controversial in the forestry community. Thinning is a means of harvesting wood, but it is not a necessary means to "restore" floodplain redwood forests, which recent research establishes are perfectly capable of restoring themselves in the absence of thinning (Iberle et al. 2019).

2. Tractor logging and yarding directly damages floodplain channels and wetlands that are the key wintering habitats of steelhead and coho salmon.

Because these essential habitats are both widespread within South Fork Gualala floodplains and sensitive to direct disturbance, any extensive ground-based logging operations across these floodplains are highly likely to adversely modify them. Skid trail locations flagged on steeper slopes in Dogwood Unit 1 are already sites of ongoing erosion from past logging on the site.

3. Haul roads are configured such that they deliver chronic fine mineral sediment to river, tributary, and floodplain habitats.

Delivery of this road-generated fine sediment to waterways is an intrinsic, systemic problem wherever roads are superimposed on a floodplain and closely parallel and cross numerous aquatic and wetlands habitats. Roads in the sale area show relatively little evidence of the kind of design features and maintenance that are necessary to effectively direct sediment-laden road runoff away from surface waters and wetlands.

4. Logging on floodplains will produce summer stream temperature increases in streams where temperature already limits salmonid survival and productivity.

Canopy reduction of the magnitude anticipated in Dogwood units causes shade loss sufficient to measureably warm streams that are today already warmer than growth and survival optima for steelhead and coho salmon.

5. Logging that increases erosion and delivery of sediment also delivers nutrients to a highly nutrient loaded river, increasing harm from algae blooms and eutrophication in the river and estuary.

This threat to juvenile steelhead and coho salmon rearing in the river and estuary, though not sufficiently informed by existing analyses and data, is also not mitigated by proposed or current practices, and likely poses the greatest single threat of mass mortality and potential extirpation of their populations in the Gualala.

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Addendum to Expert Report on Take of Steelhead and Coho Salmon From Actions Proposed in the Dogwood Timber Sale

Photos taken in the field by Christopher A. Frissell
In units of the Dogwood timber sale
On 23 April 2021

With explanatory captions

Prepared by Christopher A. Frissell
18 May 2021

Photo 1. (Roll #P4230117) Redwood tree marked for logging removal in Dogwood Unit #17, within 20 m horizontal distance of the annual high flow channel of the South Fork Gualala River. This is one of many trees I observed that presently contribute canopy shade, and if unlogged would potentially contribute large wood debris to the wood-starved river channel.

Photo 2. (Roll #P4230105) Three large redwood boles marked for removal in the Dogwood Unit 17, within 40 m of the annual high flow channel of the South Fork Gualala River, and within 25 m of a floodplain swale that affords high water wintering habitat for steelhead and potentially coho salmon. These trees are close enough to the river channel that they certainly are a significant component of present canopy shade that moderates river temperature, particularly in the late morning hours. If unlogged and allowed to self-thin, they would contribute (through branch fall) beneficial dead wood structure to the adjacent floodplain high flow channel, and potentially could contribute to restoration of badly needed large wood in the main channel of the South Fork Gualala River.

Photo 3. (Roll # P4230107) Redwood tree marked for cutting immediate adjacent to the floodplain channel swale that affords high-water wintering habitat to steelhead and potentially coho salmon in Dogwood Unit #17. Felling and yarding mature trees in close proximity of these seasonal floodplain habitats poses extreme risk of direct damage to soils, banks, and channel structure, and removes those trees from the pool of trees that through self-thinning processes could contribute beneficial large woody structure and cover to the adjacent floodplain habitat feature.

Photo 5. (Roll # P4230108) This is the presently dry floodplain channel swale referred to in Photos 2 and 3. This channel feature is open at its mouth to the South Fork Gualala River, and shows evidence that during winter high flows and flood flow it is connected to the main river as a an extensive, quiet backwater habitat. Hydrologically this is an example of an ideal temporary winter refuge from high river flows for steelhead and potentially coho salmon. Large wood plays multiple key roles here: wood jams forming in the main river trigger the the lateral river flows diversions that initially create these floodplain channel swales, large wood within the swale provides local scour points for pool formation within the channeled swale, and small and large wood contributes the principle hiding cover for fish that shelter there.

Photo 6. (Roll # P4230067). Stumps of maturing redwoods already logged Dogwood Unit 10. These stumps are on the floodplain and within about 30 m of the channel of Buckeye Creek, close enough that the tree crowns like contributed canopy shade to Buckeye Creek, and near enough that the trees potentially would have contributed badly needed large wood to Buckeye Creek had they been not logged and the floodplain forest allowed to self-thin.

Photo 7. (Roll # P4230012). Example of a groundwater-fed floodplain springbrook of a type that provides ideal flow and cover conditions for wintering steelhead and coho salmon. This springbrook was photographed near the main haul route in Dogwood Unit #5. The persistence of water for many months, or sometimes all year, in these spring-fed channels allows the development of a highly productive macroinvertebrate community which then serves an excellent food source for salmonids during the season they occupy these habitats. During winter months groundwater flow feeding these small floodplain streams increases, increasing the volume of available habitat and forming reliable connections at the downstream end of the channel to allow juvenile steelhead and salmon a migration path to access, and later egress, these important floodplain habitats.

Photo 8. (Roll # P4230014) Another view of the floodplain springbrook seen in Photo7 in Dogwood Unit #5, showing the importance of boles of fallen redwood trees in providing large structure that helps form deeper pools in these channels, and redwood branches that and contribute persistent overhead cover for fishes.

Photo 9. (Roll # P4230019) This photo shows a recently occupied skid trail that branches off the main South Fork Gualala Road haul route for Dogwood timber sale in Unit #5. Just beyond the patch of deep shade between the two redwoods, this skid trail fords the floodplain springbrook seen in photos 7 and 8. There is no culvert or other crossing structure; heavy equipment travels through rather than around or over the springbrook. In such circumstances, skid trial use collapses streambanks, disturbs the streambed and destabilizes any wood debris present in the stream at the crossing site, and mobilizes clouds of fine sediment that cause turbidity plumes in the springbrook habitat downstream. I observed flag markers designating unbridged ford crossings of floodplain channels and swales in multiple units of the Dogwood timber sale.

Photo 10 (Roll # P42300220) Another logging road-related impact to the springbrook observed in Dogwood Unit #5 is this recently-built deposit of loose soil and gravel constructed as a short spur from the main haul route directly to a pool in the springbrook channel. This site, immediately upstream from the habitat depicted in Photo 7, appears to be perhaps intended as a drafting site for pumping water from the small springbrook for spreading on the main haul road as a dust abatement measure. Unfortunately the large pile of loose sand and gravel on the verge of the springbrook is certain to be a substantial source of sediment delivery to the springbrook channel, both during winter rains, and if this is a drafting site, again each time a water truck tank is overtopped during pumping. Beyond contributing to sedimentation of the springbrook that is certain to reduce invertebrate productivity and also damage the physical suitability of the springbrook for wintering fishes, the withdrawal of water from the stream is likely to further adversely impact its resident invertebrate community that is a primary food source for wintering salmonids.

Photo 11 (Roll # P4230048). Confluence of Big and Little Pepperwood Creeks within Unit #5 of Dogwood timber sale. Low-gradient seasonal or perennial flow tributaries like this one also constitute important winter habitat refugia when the main river is flooding in winter. As long as surface water persists, they may also afford important habitat for juvenile steelhead and coho salmon during other seasons as well. As with the floodplain springbrook pictured above, the sustained presence of water means these tributaries not only can afford physical refuge, but support productive invertebrate communities that provide an abundant food source for fishes sheltering or rearing there. Based on field inspection, I conclude that both forks of Pepperwood Creek seen here are depleted of the natural loading of large wood that is of critical importance for salmonid habitat in undisturbed redwood forest streams. Nevertheless, I observed numerous trees marked for cutting within close enough proximity to these streams that their removal would directly compromise and potentially delay for decades or a century the natural recovery of wood loading.

Photo 12 (Roll # P4230058). Pepperwood Creek at its confluence with the South Fork Gualala River, Dogwood Unit #5. This photo shows that Pepperwood Creek's confluence is unobstructed and low enough in gradient that the tributary is accessible to fish at all flow levels. When the river is at full flood, Pepperwood Creek provides a very long, deep, productive backwater sheltering habitat for steelhead and potentially coho, throughout its course within Dogwood Unit#5.

Photo 13, (Roll # P4230051) Physical traces of erosion and fine sediment deposition from the most recent winter storm reveal here that runoff from the surface of main haul road collects over many tens to hundreds of meters of distance over a gentle slope north of Pepperwood Creek, then flows laterally into this roadside ditch a few meters from the road crossing of Pepperwood Creek. Road sediment then travels the short distance through this ditch and is discharged into Pepperwood Creek.

Photo 14. This is the discharge point where runoff from the haul road enters Pepperwood Creek, Dogwood Unit #5. Pepperwood Creek's channel, hidden by ferns in patch of sunlight beyond the leaning hardwood tree, flows from middle left to upper right in this frame. The channel delivering sediment from the road ditch is wide with actively eroding banks, and floored by a sheet of easily mobilized sand and silt. This is clear evidence that a large quantity of sediment is delivered directly and likely almost continuously to Pepperwood Creek from this haul road during rainy weather, adversely altering fish habitat in the stream.

Photo 15. Extensive sheet wash erosion and transport of sediment from a large contributing area of the main haul road funneling into a major side spur constructed for drafting water from the South Fork Gualala River, Dogwood Unit #5. Sediment generated by traffic-generated erosion of the road surface flows a great distance with no drainage interruption or diversion. Therefore this constitutes a collection point that funnels a massive quantity of road sediment and discharges it directly to the South Fork Gualala River. Roads in close proximity to streams on floodplains pose extreme risk of sediment delivery hazards like this. Sediment pollution from such roads can only be avoided by painstaking design and maintenance procedures that ensure road sediment is discharged onto and trapped on ground surfaces well away from streams and other floodplain habitats. Field observations unequivocally revealed that such precautions have not been taken within the Dogwood timber sale and the main haul routes serving it.

Photo 16. (Roll # P4230031) View up the drafting road spur from the bed of the South Fork Gualala River toward the spur's junction with the main Dogwood haul route. This spur shows clear evidence of recent sediment transport, erosion, and direct discharge of collected road sediment into the river. At left is an actively eroding gully showing that not only does this spur collect a large volume of sediment generated by surface erosion of the main haul road, but additional sediment is generated by erosion as water and sediment concentrate and spill down the steeper slope of the drafting spur. Fresh erosion scars are plain evidence that gravel spread on the drafting spur to stabilize the running surface is in no way effective at reducing discharge of collected road sediment into the river.

Photo 17. (Roll # P4230120) Eroding skid trail originating from past logging activity in Dogwood Unit #1. This skid trail was constructed on a moderately steel slope running virtually straight down slope for a substantial distance. As a result of this design and compaction of the soils from equipment operation, the skid trail trace is still actively eroding today. A system of alternating rills and small gullies reveals ongoing erosion, and fresh deposits of soil where the gullies discharge onto the floodplain of the Gualala River at the base of the slope offer evidence of sediment delivery to a floodplain tributary stream at the west boundary of the cutting unit. Unfortunately, flags marking the proposed skid trail locations for the Dogwood sale show that the erosion-prone segment will not be redesigned or avoided—rather the skid trail is intended to be disturbed again and likely re-compacted by heavy equipment operations in this unit. Given easily observed conditions on the site today, it is virtually certain that ground-based logging using the proposed routes will further increase erosion at this site.

Photo 18. (Roll # P4230038). Extensive, thick algal mats show the results of an ongoing massive algae bloom in the South Fork Gualala River, in a reach adjacent to Dogwood Unit #5. Evident are massive, thick mats growing in place, as well as sloughing that generates dense drifts of algae in the water column. Such drifting clots of sloughed algae can be easily transported to, and accumulate in, the estuary. Algal growths of this magnitude and extent, especially in a relatively sloe-moving stream, are indicative of conditions ripe for hypoxic or even fully anoxic conditions during night time hours, that are lethal to fish and most of the food organisms that sustain salmonid growth. In my 39 years of studying coastal rivers in Oregon and California, I have not once seen a case of extreme eutrophication even remotely as severe as that observed in the South Fork and mainstem Gualala River.

Photo 19. Extreme biomass of vascular aquatic plants and algal mats in a backwater pool of the South Fork Gualala River, immediately downstream of the discharge point of road sediments at the drafting spur in Dogwood Unit #5 seen in photos 17-18. In my career I have only observed plant communities of this type, consisting of a high biomass of emergent and aquatic vascular plants layered with dense algal accumulations, in extremely nutrient polluted waters such as in livestock feedlot discharges, springs and pipe discharges polluted by human waste, and a handful of similar backwaters in a few Interior West streams polluted by runoff from streamside gravel-surfaced roads.

Photo 20. (Roll # P4230009) Relatively clean gravel bed of the North Fork Gualala River at its confluence with the South Fork. The North Fork streambed condition is quite representative of the normal extent of visible algal and plant growth for a coastal redwood stream. Algal growth is confined to a relatively thin film of brown algae (primarily diatoms) on streambed rocks, and accumulations of massive green and blue-green algae are prevented from forming because of constant grazing of rocks by stream insects and molluscs. Algae blooms tend to snowball to the magnitude seen in the South Fork only after most groups of grazing invertebrates have been eliminated by severe oxygen depletion (or in some cases, toxic chemicals).

EXHIBIT 1

Curriculum Vitae *19 June 2018* *20pp.*

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Birth: 1 December 1960, Chehalis, Washington

Education: Ph.D. in Fisheries Science, Oregon State University, 1992
M.S. in Fisheries Science, Oregon State University, 1986
B.A. with High Honors in Zoology, University of Montana, 1982

Professional Appointments:

Principal Scientist and founder, Frissell & Raven Hydrobiological and Landscape Sciences, LLC, Polson, MT, 2012-present (affiliated with Kier & Associates, M. Scurlock & Associates, and Pacific Watershed Associates).

Affiliate Research Professor and summer field course instructor, Flathead Lake Biological Station, The University of Montana, 2016-present

Director of Science and Conservation and Senior Staff Scientist, The Pacific Rivers Council, 2000-2012

Research Associate Professor, The University of Montana, Flathead Lake Biological Station, 1998-2000

Research Assistant Professor, The University of Montana, Flathead Lake Biological Station, 1993-1998

Research Assistant Professor, Department of Fisheries and Wildlife, Oregon State University, 1994-1997

Postdoctoral Research Associate (Faculty), Department of Fisheries and Wildlife, Oregon State University, 1992-1994

Research Assistant (Faculty), Oak Creek Laboratory of Biology, Department of Fisheries and Wildlife, Oregon State University, 1985-1992

Fields of Interest:

- Land-aquatic ecosystem linkages and cumulative impacts of natural processes and human activities on stream habitat and stream biota.
- Ecology, biogeography, and conservation biology of fishes and freshwater biota in relation to landscape and hydrologic change.
- Aquatic ecosystem conservation and restoration strategies.
- Geomorphic, hydrophysical, and landscape ecology considerations in design of integrated conservation reserves.
- Restoration and recovery planning and design for freshwater ecosystems and species.

Theses and Dissertations:

Frissell, C.A. 1992. Cumulative effects of land use on salmon habitat in southwest Oregon coastal streams. Doctoral dissertation, Oregon State University, Corvallis.

Frissell, C. A. 1986. A hierarchical stream habitat classification system: development and demonstration. M.S. thesis, Oregon State University, Corvallis.

Frissell. C. A. 1982. Colonization and development of community structure in coexisting Ephemerellid mayflies (Ephemeroptera, Ephemerellidae). Senior Thesis, Watkins Scholarship Program, The University of Montana, Missoula.

Professional Societies:

Society for Conservation Biology, 1991-present
American Fisheries Society, 1985-present
Ecological Society of America, intermittent
North American Benthological Society, intermittent

Graduate Students Mentored

Cavallo, B.J. M.S. in Organismal Biology and Ecology, The University of Montana, 1997. Thesis title: *Floodplain habitat heterogeneity and the distribution, abundance, and behavior of fishes and amphibians in the Middle Fork Flathead River Basin, Montana.*

Adams, S. B. Ph.D.in Organismal Biology and Ecology, The University of Montana, 1999. Dissertation title: *Mechanisms Limiting a Vertebrate Invasion: Brook Trout in Mountain Streams of the Northwestern USA*.

Hitt, N.P. M.S. in Organismal Biology and Ecology, The University of Montana, 2002, *Distribution and potential invasion of introduced rainbow trout in the upper Flathead River drainage.*

Carnefix, G. M.S. in Organismal Biology and Ecology, The University of Montana, 2002. Thesis title: *Movements and ecology of bull trout in Rock Creek, MT.*

Hastings, K. Ph.D.in Organismal Biology and Ecology, The University of Montana, 2005. Dissertation title: *Long-term persistence of isolated fish populations in the Alexander Archipelago.*

Reviewer for Journals and Agency Publications:

Canadian Journal of Fisheries and Aquatic Sciences, Conservation Biology, Ecological Applications, Environmental Management, Fisheries (AFS), Freshwater Biology, North American Journal of Fisheries Management, Oikos, Transactions of the American Fisheries Society, Fundamental and Applied Limnology, USDA Forest Service General Technical Reports

Member of Board of Editors for Journals:

Conservation Biology, 1996-2000

Appointments to Review Panels and Scientific Advisory Committees:

USEPA Bristol Bay Watershed Assessment Team, Subcontractor on road and pipeline impacts, through University of Alaska Anchorage and NatureServe, 2011-2012.

Independent Expert Review Panel for King County Water and Land Resources Division's Project Scoping and Implementation Practices. 2011-12. Subcontractor to MWH (Montgomery Watson Harza) for King County Dept. of Natural Resources and Parks, Seattle, WA. http://www.kingcounty.gov/environment/dnrp/publications/wlrd-expert-review-report.aspx

Umpqua Watersheds Science Advisory Council, Sponsored by Umpqua Watersheds, Inc., 16-17 November 2010, Roseburg, OR.

Wychus Creek Restoration Monitoring Plan Review Panel, sponsored by Upper Deschutes Watershed Council and Bonneville Environmental Foundation. 2 October 2009, Bend, OR.

Landscape Pattern Task Group, *State of the Nation's Ecosystems* report. 2003-2007. H. John Heinz III Center For Science, Economics and the Environment. Washington, DC. http://www.heinzctr.org/Programs/Reporting/Working%20Groups/Fragmentation/index.shtml

Science Review Team, King County Normative Flow Studies Project. 2002-2005, Seattle, WA. http://dnr.metrokc.gov/wlr/BASINS/flows/science-review-team.htm

Science Advisory Panel, Westside. Governor's Salmon Restoration Funding Board, Washington State, February 2000.

Ecological Work Group, Multi-species Framework Process and Subbasin Assessment Process, Northwest Power Planning Council 1998-2000.

Peer review panelist for U.S. Environmental Protection Agency/National Science Foundation Water and Watersheds Grants Program for 1997. 7-9 May 1997.

Scientific Group for the Governor's Bull Trout Restoration Team, State of Montana, 1994-2000

Oregon Department of Environmental Quality, 1992-95: Temperature Standards Review Subcommittee of the Technical Advisory Committee, Triennial Water Quality Standards Review

Scientific Assessment Panel for amphibian species, Eastside Oregon-Washington and Upper Columbia Basin EIS, US BLM and US Forest Service, 1994

Oregon Department of Forestry, 1990-93: Technical Advisory Group for the Forest Practices Monitoring Program; Wetlands Technical Group; Stream Protection Advisory Panel.

Peer-Reviewed Articles Published in Scientific Journals:

Hand, B.K., C. G. Flint, J. A. Stanford, C. A. Frissell, C. C. Mulhfeld, S. P. Devlin, B. P. Kennedy, R. L. Crabtree, W. A. McKee, Gordon Luikart. In Press. The Importance of a Social-Ecological Perspective for Riverscape Management in the Columbia River Basin**.** *Frontiers in Ecology and the Environment*.

DellaSala, D.A., R. Baker， D. Heiken, C. Frissell, J. R. Karr, S.K. Nelson, B. R. Noon, D. Olson, and J. Strittholt. 2015. Building on Two Decades of Ecosystem Management and Biodiversity Conservation Under the Northwest Forest Plan, USA. *Forests 6*(9):3326-3352. http://www.mdpi.com/1999-4907/6/9/3326/htm

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Williams, J. E., R. N. Williams, R. F. Thurow, L. Elwell, D. P. Philipp, F. A. Harris, J. L. Kershner, P. J. Martinez, D. Miller, G. H. Reeves, C. A. Frissell, and J. R. Sedell. 2011. Native Fish Conservation Areas: a vision for large-scale conservation of native fish communities. *Fisheries* 36:267-277. http://www.tu.org/sites/www.tu.org/files/documents/Williams%20et%20al.%202011%20Fisheries%20NFCA.pdf

Whiteley, A.R., K. Hastings, J. K. Wenburg, C. A. Frissell, J. C. Martin and F. W. Allendorf. 2010. Genetic variation and effective population size in isolated populations of coastal cutthroat trout. *Conservation Genetics* 11(5):1929-1943. DOI: 10.1007/s10592-010-0083-y

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Poole, G.C., J.A. Stanford, S.W. Running, and C.A. Frissell. 2006. Multiscale geomorphic drivers of groundwater flow paths: subsurface hydrologic dynamics and hyporheic habitat diversity. *Journal of the North American Benthological Society* 25(2): 288-303.

Poole, G. C., J. A. Stanford, S. W. Running, C. A. Frissell, W. W. Woessner, and B. K. Ellis. 2004. A patch hierarchy approach to modeling surface and sub-surface hydrology in complex flood-plain environments. *Earth Surface Processes and Landforms* 29**:** 1259–1284.

Articles Published in Scientific Journals, continued:

Karr, J. R., J. J. Rhodes, G. W. Minshall, F. R. Hauer, R. L. Beschta, C. A. Frissell, and D. A. Perry. 2004. The effects of postfire salvage logging on aquatic ecosystems in the American West. *BioScience* 54:1029-1033. http://www.sierraforestlegacy.org/Resources/Conservation/FireForestEcology/SalvageLoggingScience/Salvage-Karr04.pdf

Hitt, N.P., and C.A. Frissell. 2004. A case study of surrogate species in aquatic conservation planning. *Aquatic Conservation: Marine and Freshwater Ecosystems*. 14:625–633. Beschta, ,R.L., J. J. Rhodes, J.B. Kauffman, R.E. Gresswell, G.W. Minshall, J. R. Karr, D.A. Perry, F.R. Hauer, C. A. Frissell. 2004. Postfire Management on Forested Public Lands of the Western United States. *Conservation Biology* 18: 957–967. http://www.researchgate.net/publication/227654964_Postfire_Management_on_Forested_Public_Lands_of_the_Western_United_States?ev=prf_pub

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Williams, R.N., P.A. Bisson, D.L. Bottom, L.D. Calvin, C.C. Coutante, M.W. Erho Jr., C.A. Frissell, J.A. Lichatowich, W.J. Liss, W.E. McConnaha, P.R. Mundy, J.A. Stanford & R.R. Whitney. 1999. Return to the River: Scientific Issues in the Restoration of Salmonid Fishes in the Columbia River. *Fisheries* (Bethesda) 24(3):10-19Currens, K.P., F.W. Allendorf, D. Bayles, D.L. Bottom,. C.A. Frissell, D. Hankin, J.A. Lichatowich, P.C. Trotter, and T.A. Williams. 1998. Conservation of Pacific salmon: response to Wainwright and Waples. *Conservation Biology* 12:1148-1149.

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Frissell, C.A. 1999. Fisheries and watershed processes: strategies for protection and restoration. Invited paper, Annual Meeting of the Cal-Neva Chapter of the American Fisheries Society, 24-27 March, Redding, CA.

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Frissell, C.A. 1995. Birth in the fast lane: sediment transport, human disturbance, and reproductive strategies of salmonid fishes in Pacific Northwest streams. (Abstract). Invited paper for Symposium on Influence of Geomorphic Processes on Terrestrial and Aquatic Ecosystem patterns and Processes, Annual meeting of the Ecological Society of America, Snowbird, UT, 31 July-3 August.

Frissell, C.A. 1995. Resource management impacts on bull trout populations. Invited panel presentation for Searching for Solutions: Solving the Bull Trout Puzzle Science and Policy Conference, Andrus Center for Public Policy, Boise State University, Boise, ID, 1-2 June.

Frissell, C.A. 1995. Watershed dynamics: natural pattern and process and some consequences for ecosystem management. Invited presentations at Managing Terrestrial Ecosystems Relative to Past and Present Disturbances: A Workshop Integrating Fire, Range, Fish and Wildlife Habitat and the Practice of Silviculture in the Northern Region. U.S. Forest Service, Missoula, MT, 14-16 March.

Ebersole, J.L., C.A. Frissell, and W.J. Liss (co-presenters). 1995. Invasion of non-native fishes in northeast Oregon and western Montana streams: potential impacts of climate change. (Abstract). Oregon Chapter of the American Fisheries Society, Ashland, OR, 15-17 February.

Frissell, C.A. 1994. Watershed restoration strategies. (Invited presenter and session convenor) Watersheds '94 Expo, US Environmental Protection Agency and Center for Streamside Studies, University of Washington. Bellevue, WA, 27-30 September.

Frissell, C.A. 1994. A hierarchical approach to restoration of riverine ecosystems. Invited paper at Symposium on Aquatic Habitat Restoration in Northern Ecosystems, Alaska Chapter of the American Fisheries Society, Girdwood, AK, 20-22 September.

Selected Papers and Seminars Presented Since 1993, continued:

Frissell, C.A. 1994. An integrated, biophysical strategy for ecological restoration of large watersheds (Abstract). Annual Conference of The Universities Council on Water Resources, Big Sky, MT, 3-5 August.

Frissell, C.A., and J.A. Stanford. 1994. Designing a watershed reserve network to protect and restore aquatic biodiversity in the northern Rocky Mountains (Abstract). Annual meeting of the Montana Chapter of the American Fisheries Society, Billings, Montana, Billings, MT, 9 February.

Frissell, C.A. 1994. The Endangered Species Act: principles for the protection and recovery of fishes. Invited panel presentation, annual meeting of the Idaho Chapter of the American Fisheries Society, McCall, ID, 24-26 February.

Frissell, C.A., W.J. Liss, B. Doppelt, and D. Bayles. 1993. A new, ecologically based restoration strategy for Pacific salmon in the Pacific Northwest (Abstract). Annual meeting of the American Fisheries Society, Portland, OR, 29 August-2 September.

Technical Workshops Organized (selected):

Lead organizer and facilitator, New Science Implications for the Aquatic Conservation Strategy of the Northwest Forest Plan. Sponsored by the Coast Range Association, 2-3 December 2013, Portland, OR.

Co-organizer, with M. Scurlock and R. Kattelmann: SNEP Plus 15 Years: Ecological & Conservation Science for Freshwater Resource Protection & Federal Land Management in the Sierra Nevada. Sponsored by Pacific Rivers Council, Sierra Forest Legacy, UC Berkeley School Environmental Design, UC Davis Center for Watershed Science, and CaliforniaTrout; 12-13 December 2011, Davis, CA.

Organizer and facilitator, Workshop on Science for River and Watershed Conservation. Sponsored by Campaign for Montana's Headwaters, 7 October 2010, Flathead Lake Biological Station, Polson, MT.

Co-convener, with M. Scurlock and Kristen Boyles: Technical Workshop on Science for Forest Planning. Sponsored by Pacific Rivers Council and Earthjustice, 29 June 2010, Seattle, WA.

Organizer and panelist, Umpqua Independent Science Council. Sponsored by Pacific Rivers Council, 2010-2011.

Co-organizer and panelist, with Deanne Spooner and David Bayes: Workshop on Economics of ESA Critical Habitat Policy, sponsored by Pacific Rivers Council and San Francisco State University, October 4-5, 2007, San Francisco, CA.

Organizer and coordinator of Science Panel on Roads and Watersheds, sponsored by Pacific Rivers Council, 10-11 November 2006, Forest Grove, OR.

Organizer and coordinator of the Recovery Science Panel for the Western Native Trout Campaign. Sponsored by Pacific Rivers Council, meeting 2-3 March 2002, Portland, OR.

Technical Workshops Organized (selected), continued:

Organizer and coordinator of Biodiversity Workshop, Consortium for the Study of North Temperate Montane Ecosystems. A cooperative research venture of The University of Montana and Montana State University, supported by the NSF EPSCoR program. 4 February, 1997 Missoula, MT.

Scientific Workshop on Large Basin Restoration: Grande Ronde River (co-organizer). 21-22 March 1993, La Grande, OR. Sponsored by The Pacific Rivers Council.

Scientific Workshop on Large Basin Restoration: South Umpqua River. 16-18 September 1992, Roseburg, Oregon. Sponsored by The Pacific Rivers Council.

Scientific Workshop on Large Basin Restoration: Lower Rogue River. 21-23 October 1992, Gold Beach, OR. Sponsored by The Pacific Rivers Council.

Other Panels and Workshops Attended by Invitation since 1994 (selected):

Invited Review Panelist, Workshop on Linking Habitat Characteristics to Salmon Data. 29-30 September 1999, National Marine Fisheries Service, Northwest Fisheries Science Center, Seattle, WA.

Invited participant, Yellowstone to Yukon Aquatic Conservation Science Workshop. 20-22 August 1999, Flathead Lake Biological Station, The University of Montana, Polson, MT.

Invited Panelist, Workshop on Options for Restoring Salmon Habitat in the Mainstem Snake and Columbia Rivers. Pacific Northwest National Laboratory-Battelle, 19 August 1999, Kennewick, WA

Panelist at State of Oregon/National Marine Fisheries Service Memorandum of Agreement Committee Workshop: Cumulative Effects of State and Private Forest Practices on Salmon Habitat. 21April 1998, Salem, OR.

Invited participant in a scientific workshop, Multiple Stressors in Ecological Risk Management. Sponsored by the Society for Environmental Chemistry and Toxicology and the USEPA, 13-18 September 1997, Pellston, MI.

Society for Conservation Biology Workshop: Communicating with the Media (panel member). 9 June 1997, Victoria, British Columbia, Canada.

Invited speaker for a workshop, Continuing Education in Ecosystem Management. Sponsored by the University of Idaho. Catchment scale processes and linkages between landscape and stream conditions. 31 January 1997, Moscow, ID.

The Nature Conservancy, Aquatic Classification Workshop (invited presenter). 9-11 April 1996, Cedar Creek Farm, MO.

Kenai River Community Forum (keynote speaker and panelist). The Nature Conservancy of Alaska, USEPA and USFWS, 19-21 April, Soldotna, AK.

Conservation Biology and Management of Interior Salmonids (invited presenter and session co-moderator). USDA Forest Service Intermountain Research Station and Utah State University, 4-5 October 1995, Logan, UT.

Eastside Ecosystem Planning Workshop. Sierra Club Legal Defense Fund, 16 December 1994, Portland, OR.

Other Panels and Workshops Attended by Invitation since 1994, continued:

Co-instructor at workshop series on Watershed Restoration and the "Rapid Biotic Response Strategy" for Riverine Ecosystem Restoration, sponsored by The Pacific Rivers Council, 1993-95, California, Oregon, and Washington.

Fire/Salvage and Aquatic Ecosystems Policy Workshop. The Pacific Rivers Council, 15 December 1994, Portland, OR.

Panel on Forest Health Issues, Native Forest Network annual conference, 13 November 1994, Missoula, MT.

Workshop on Watershed/Fisheries Cumulative Effects Analysis, sponsored by Headwaters, The Pacific Rivers Council, USDA Forest Service, and Bureau of Land Management. 29 September-2 October, 1994, Ruch, OR.

Boise Funders' Scoping Meeting, sponsored by Bullit, Harder, and Lazar Foundations, 30-31 August 1994, Boise, Idaho.

Scientists Briefing for U.S. Senate staff on post-fire logging and forest management and freshwater resources. Washington, D.C., 18-19 September 2006.

Other Presentations and Outreach (Selected):

Invited testimony on federal land management and the future of salmon and aquatic biodiversity in the Pacific Northwest, to the U.S. House of Representatives, Subcommittee on National Parks and Public Lands, Washington, D.C., 11 March 1993.

Briefing for Congressional representatives and staff on federal lands management and conservation and recovery of salmonid fishes and riverine ecosystems, Washington, D.C., 22 January 1993.

Invited testimony to the 1991 Oregon State Legislature, on panel representing the Oregon Chapter of the American Fisheries Society, on the status of native fishes, impacts of forest practices on fish habitat, and the need or changes in environmental regulation.

Invited testimony to the Oregon Board of Forestry Forest Issues Forum, December 1990, on cumulative impacts of forest practices on native aquatic species and the need for changes in forest management.

Worked with Oregon Public Broadcasting to describe our research project and its significance in a 15-minute segment of the television program, Oregon Field Guide, first aired in June 1990.

Presented seminars, informal presentations, lectures, and discussions at research review meetings, as guest speaker in classrooms and public interest groups, at state board meetings, at workshops, and on field trips with foresters, geotechnical personnel, fishery and watershed managers, and conservationists.

EXHIBIT 2

Methods Used to Calculate Canopy Coverage from Photographs

5/14/2021

Curtis Bradley

Senior Scientist, GIS Specialist

Center for Biological Diversity

I am a Senior Scientist and Geographic Information Systems ("GIS") specialist at the Center for Biological Diversity, where I have worked for the past 20 years. I hold a Bachelor of Sciences in mechanical engineering and a Master of Sciences in watershed management, both from the University of Arizona. I have training in several GIS software applications and over 22 years of experience in GIS analysis and cartography.

1. Photographic-based Canopy Cover Methods

Dr. Dominick DellaSala provided me with a series of canopy photos from the transects he walked during our April 23rd field visit of the proposed logging units. I was asked to calculate the amount of canopy coverage that was present in each photo. Under the direction of Dr. Chris Frissell, who has experience with this type of classification, and was on the field trip, I performed this task as described herein.

Each of the photos was converted from Adobe Acrobat to .jpg format and the white border was removed using the crop tool in the Windows photo viewer application. I then used the cv2.cvtColor method within the Python Open CV library to convert each of the photographs to greyscale. Python is a programming language commonly used in GIS applications.

Greyscale images consist of individual pixels, each having a value ranging from 0 – 255. The lower the value, the darker the pixel. The objective was to determine the cutoff value between 0 and 255 that classified the darker pixels as vegetation and the lighter pixels as sky. I chose 3 different cutoff values of 175, 200, and 225 to apply to the photographs in Unit 1 as a proof of concept experiment. I applied these values to the greyscale images using the

cv2.threshold method in the Python Open CV library to produce a series of photos where all pixels were either black or white with black pixels representing vegetation and white pixels representing sky. I sent these classified images to Dr. Frissell and his opinion was that the 200 cutoff value was the most optimal to use in this effort.

I classified the images from the 6 units that Dr. DellaSala transected using the 200-cutoff value. I automated the process using Python and, after revieing the classified images, I found that there were 13 that were not classified correctly due to sun directly hitting the camera or clouds causing a sharp contrast in the sky. For these images, I applied lower threshold values of either 150 or 175 to correct.

I then calculated the percent canopy coverage for each classified image by determining the number of black pixels and dividing by the total number of pixels using the NumPy library in Python. The mean, minimum, and maximum of these values was reported for each transect.

2. LiDAR-based Canopy Cover Methods

I downloaded the "LiDAR Derived Rasters" datasets for the watersheds in the Project area from Sonoma County's website (http://sonomavegmap.org/data-downloads/). LiDAR data collected in late 2013 and provided by the University of Maryland and the Sonoma County Vegetation Mapping and LiDAR Program under grant from NASA's Carbon Monitoring System are available for unrestricted public use.

The Canopy Density datasets were clipped to the logging unit boundaries. Each 3-foot x 3-foot pixel within the logging units represented a value (0 – 1) of canopy cover. I calculated the mean value of all the cells within each logging unit and reported them in a table of average canopy cover per unit. Several of these units indicate canopy coverage before logging operations because these data were collected in 2013.

Averages	n	Photo Mean % CC	Photo Min % CC	Photo Max % CC	LiDAR (2013) Mean % CC	Photo - LiDAR		
Unit1	28	80.72	65.34	93.81	81.97	-1.26	ave photo-lidar lo	-10.67
Unit8	25	75.08	67.07	81.32	87.19	-12.11	ave photo-lidar ui	-0.29
Unit9	41	72.28	60.13	80.39	86.18	-13.90	Difference	-10.38
Unit10	32	74.41	62.01	94.29	80.41	-6.00		
Unit17	29	82.15	73.42	93.02	80.96	1.19		
Unit21	10	79.59	74.41	89.96	80.41	-0.81		

EXHIBIT 3

Using environmental DNA sampling to describe aquatic vertebrate habitat use of the Gualala River watershed during 2020 winter baseflows

A report describing sample collection, analysis, and results

Final Report of Sarah O'Neal
Freshwater Ecologist
Agua Dulce Freshwater Consulting
Conducted for Gross & Klein LLP
April 30, 2021

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Introduction

This report describes the results of a study that used environmental DNA (eDNA) to identify steelhead (*Oncorhyhnchus mykiss*) and coho (*O. kisutch*) salmon, and California red-legged frogs (*Rana dratonii*) use in the Gualala River watershed during late winter baseflows.

As explained below, the study was based on the analysis of 93 water samples taken from 34 unique locations in the Gualala River, during December 15 – 27, 2020. Results of that study indicate:

1. Steelhead use was nearly (if not entirely) ubiquitous throughout the sample area/lower Gualala River watershed.

2. Coho salmon—though less abundant than steelhead—were likely using estuarine (or nearby upstream) habitat.

3. California red-legged frogs were also less abundant than steelhead, but were likely widespread throughout the lower Gualala River watershed.

General Background

Gualala River Watershed

The Gualala River watershed drains nearly 800 km^2 of mixed Douglas fir (*Pseudotsuga menziesii*) and redwood (*Sequoia sempervirens*) dominated forest ranging from over 800-m elevation to its mouth at sea level (Higgins 1997). The estuary formed at the river mouth is lagoonal, meaning that it is shallow and frequently enclosed so that exchange with the ocean is limited to entirely obstructed at low river flows and/or tides (Figure 1; WCEE 2020). The estuary and the entire watershed upstream have hosted multiple life stages (i.e., spawning, incubating, and rearing stages) of steelhead (*Oncorhyhnchus mykiss*) and coho (*O. kisutch*) salmon, and California red-legged frogs (*Rana dratonii*)—all of which are listed under the federal Endangered Species Act as endangered (USFWS 2002, NMFS 2012, NMFS 2016).

Figure 1. Photographs of the estuary mouth prior to, during, and after the estuary sandbar breached (left and center photos taken December 26, 2020), right photo taken by T. Denniston (December 28, 2020).

Environmental DNA (eDNA)

Environmental DNA (eDNA) is a valuable method for expanding knowledge of species distribution, and particularly for detecting organisms in low abundance even without any obvious sign of their presence. Genetic material is constantly shed by organisms through cells or tissue from skin, excrement, decomposition, and other sources. Once collected from bulk environmental samples (i.e., air, soil, and water), DNA can be filtered and extracted in order to analyze for species presence using a number of laboratory techniques. Compared to traditional capture methods, eDNA collection and analysis has higher species detection probabilities and thus is particularly useful for detecting endangered, recovering, and recently introduced species (Goldberg et al. 2015, Laramie et al. 2015, McKelvey et al. 2016, Wilcox et al. 2016, Evans et al 2017, Duda et al. 2021). However, detection probabilities are affected by physical and chemical (e.g., flow, temperature, light, water chemistry), biological (e.g., species life stage, abundance), and field and lab methodological factors (Laramie et al. 2015, Strickler et al. 2015, Goldberg et al. 2016, Stoeckle et al. 2017, Tillotson et al. 2018). These factors, as relevant to this study, are discussed in the following sections.

Methods

Study Area

Samples were collected at publicly accessible sites throughout the basin from December 15 – 27, 2020 (Figure 2; Table 2), during which time the stream was near baseflow, with three substantial (1.06 -1.44" precipitation) rainstorms occurring just prior to and during the sampling period. Flows near the mouth of the North Fork Gualala River ranged from 6.3 – 384 cubic feet per second (cfs; Figure 3, USGS data), and flows in the South Fork Gualala River near Sea Ranch ranged from 5.7 – 216 cfs (Figure 3; USGS data). The lagoonal estuary at the Gualala River mouth remained closed throughout the sampling period until the third rain event breached the sandbar on December 27, 2020 (Figure 1). Prior to the breach, there was no detectable flow in the estuary.

Environmental DNA Field Methods

A total of 34 unique locations were sampled between December 15 and December 27, 2020 (Table 2). Three sites were revisited. Within the closed estuary, a subset of samples (at four sites in the estuary) were collected from the same location but at multiple water depths (Figure 6, Attachment 3). Negative field control samples were filtered at least twice daily to test for potential field contamination using store-bought, distilled water. In total, 113 individual filters (including 20 field blank filters) were submitted to contract laboratories for analysis.

During the December 20, 2020 estuary sampling event, a sonar fish finder (Deeper Chirp Smart Sonar, Vilnius, Lithuania) was used to identify high concentrations of fish to target sampling (Figure 4). Environmental DNA sample water was generally filtered in three replicate samples through a 1-µm pore size, encased nylon filter attached to a 60-mL syringe. When time and/or logistical factors were constraining, one or two field replicates were collected. Sterile nitrile gloves were worn at each unique site (and each unique depth) during sample collection. All

Figure 2. Map of locations of eDNA sample collection from December 15 – 27, 2020 in the Gualala R watershed.

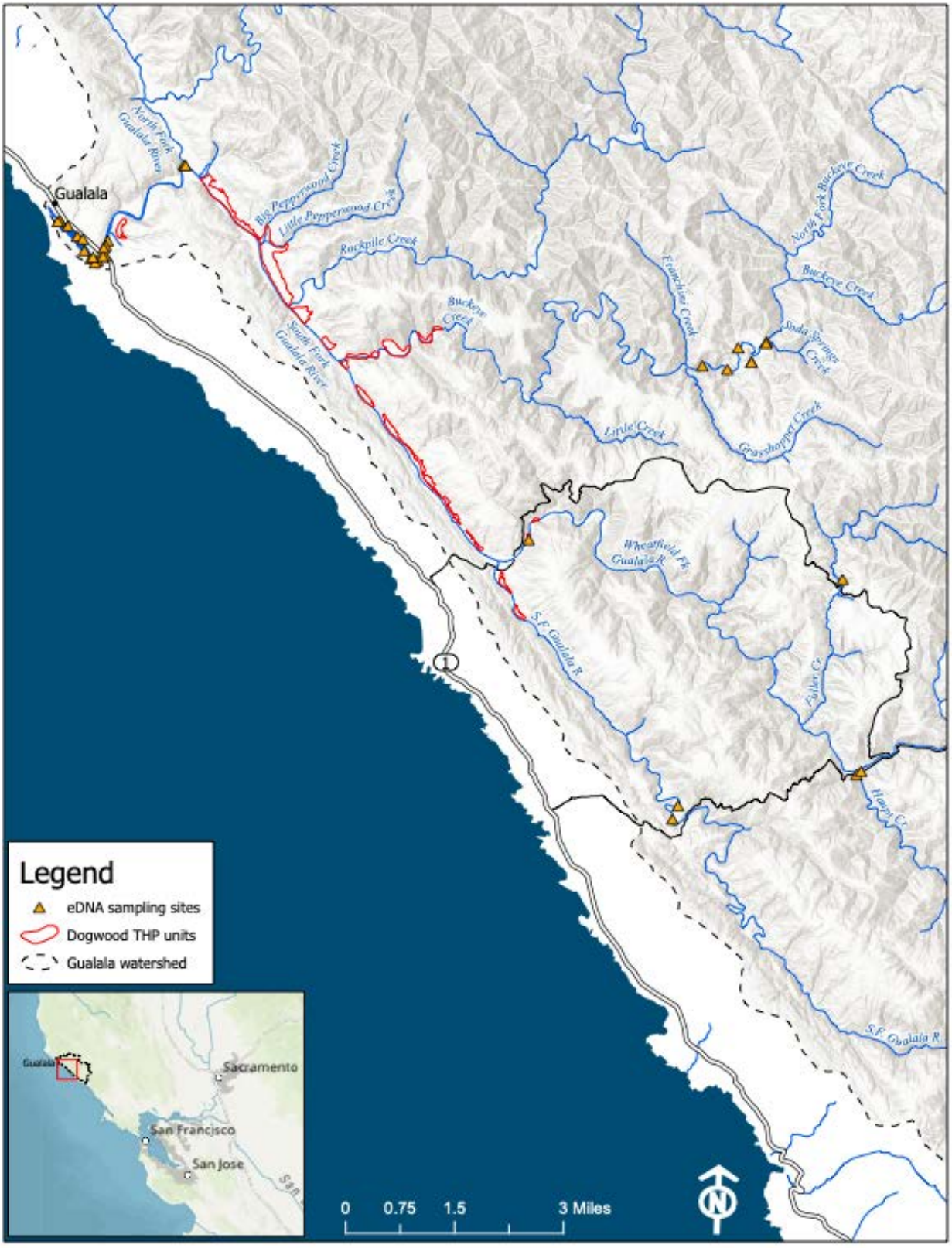
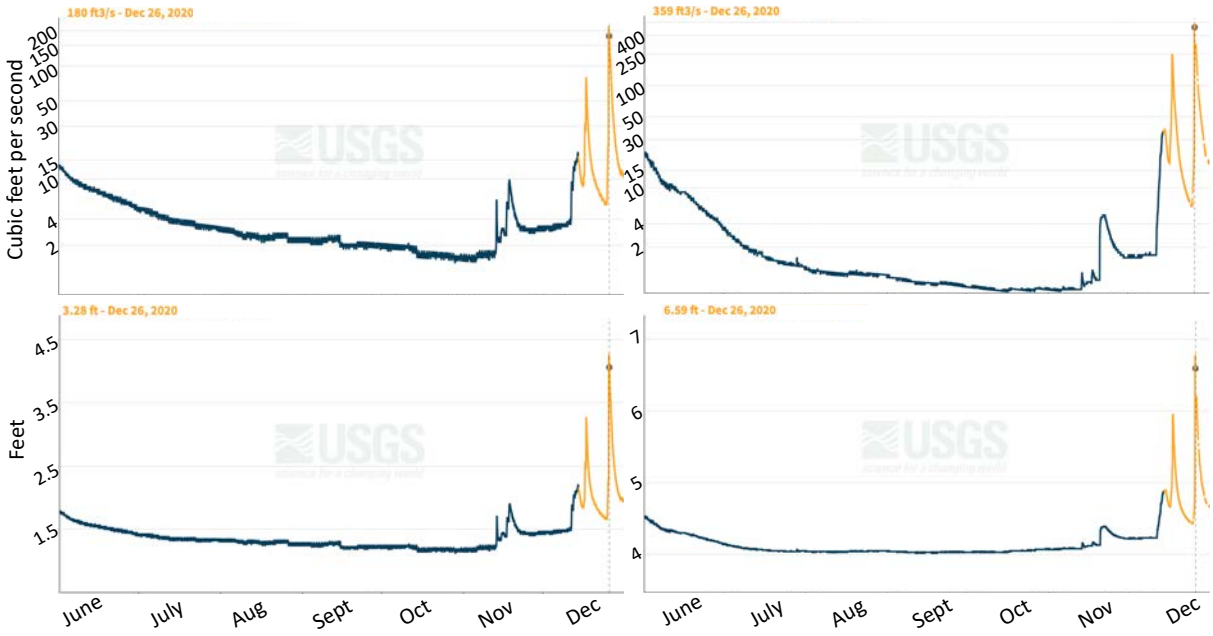


Figure 3. Streamflow (top) and river stage (bottom) data for the mouth of the North Fork Gualala R (left) and the South Fork Gualala R near Sea Ranch (right) from June 1st-December 31st, 2020. The sample period is highlighted in yellow. Note the logarithmic scale of streamflow data (top panel). Peak streamflow (180 ft^3/s and 359 ft^3/s, respectively, and depth increases of 1.5 ft and 2.6 ft, respectively) occurred on December 26, 2020, one day prior to the estuary opening and reconnecting to marine waters. The estuary remain closed for the rest of the sampling period.

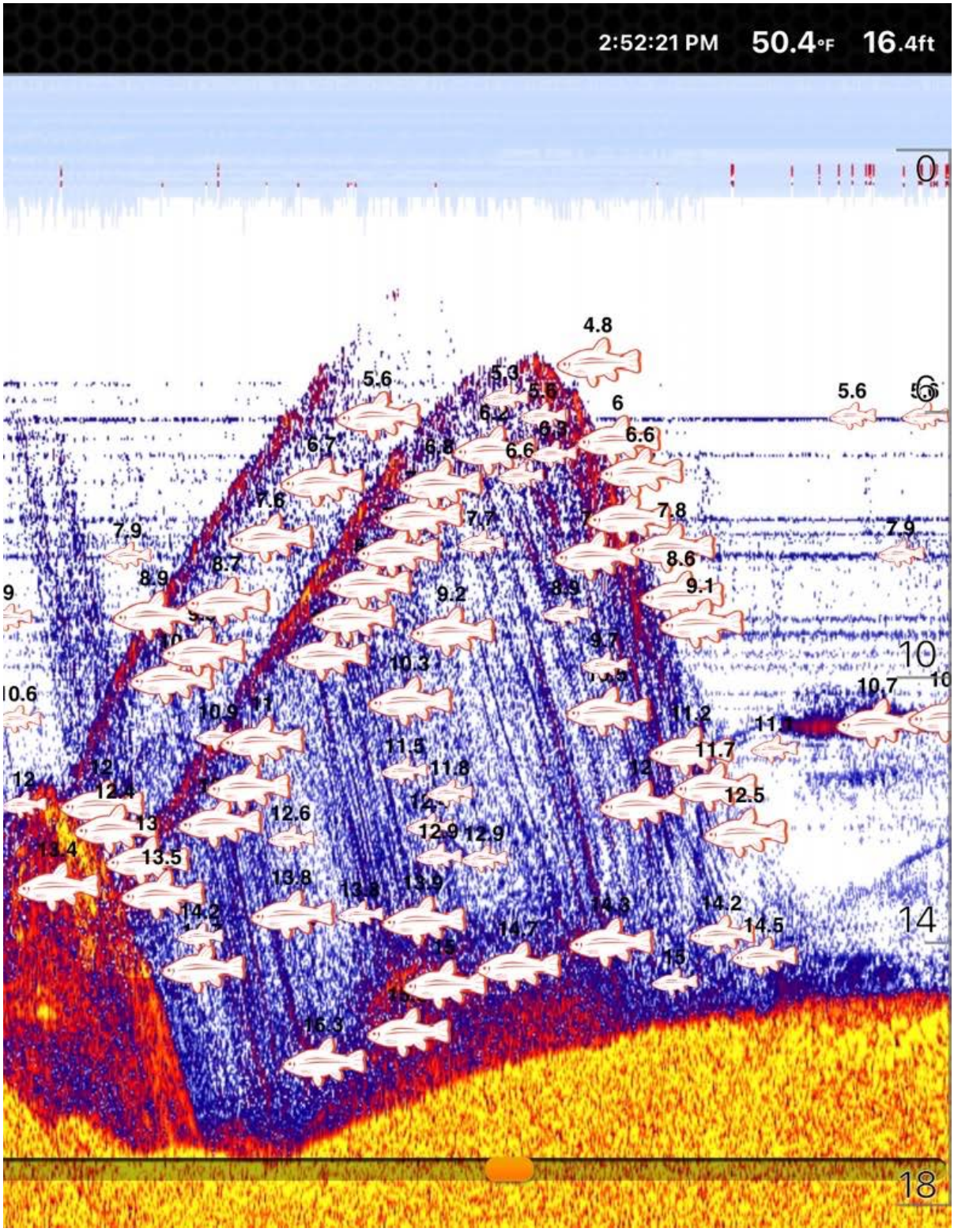


filters, filter casings, syringes, and storage containers were sterile upon arrival at each site, and none were reused during the study period. Water volumes filtered ranged from the maximum volume until filter obstruction up to 500 mL (range = 75-500 mL). In the estuary, samples were collected by foot (on December 17), from a drift boat (on December 20 and 23), and from the Highway 1 bridge (on December 27). All samples collected at depth were obtained using a clear, 5-L polycarbonate horizontal Van Dorn lake sampler (Aquatic Research Instruments, Hope, Idaho). For all estuary samples, salinity was measured concurrent with sample collection using a handheld analog refractometer (Cole-Parmer, Vernon Hills, Illinois) that was calibrated prior to sampling using store-bought distilled water (Attachment 3). Following filtration of all samples, tris-EDTA preservative was added to the filter using a separate, pre-loaded, sterile syringe. Samples were subsequently stored in individual sterile containers in a dark, cool location until shipment to contract laboratories for analysis. Approximately 60% of samples (n=70) were shipped to Jonah Ventures laboratory (Denver, CO), and the remaining samples (n=44) were shipped to Genidaqs laboratory (Sacramento, CA).

Environmental DNA Laboratory Methods

DNA was extracted by each laboratory in sterile, dedicated lab environments using pre-fabricated kits according to manufacturer's instructions with modifications as described in Attachment 1 (for Jonah Ventures samples) and Attachment 2 (for Genidaqs samples). After

Figure 4. Sonar fish finder image taken at Gualala River Estuary Site 14 on December 23, 2020—one of two locations where trace amounts of coho salmon DNA was detected. Numbers on the right-hand y-axis are depth measurements (in ft), and numbers associated with fish detected are also the depth of detection. Fish sizes (as represented by sizes of fish symbols) are relative and unquantifiable without physical fish samples.



extraction, quantitative polymerase chain reaction (qPCR) was used to interrogate extracted DNA using well established assays for the following mitochondrial genes and species as described in associated references:

- cytochrome *c* and NADH dehydrogenase subunit 2 for steelhead salmon/rainbow trout (Brandl et al. 2015 and Wilcox et al. 2015, respectively),
- cytochrome *b* and cytochrome *c* oxidase subunit for coho salmon (Duda et al. 2021 and Rasmussen et al. 2010, respectively), and
- cytochrome *b* for California red-legged frogs (unpublished assays from Jonah Ventures and Genidaqs).

All extracted samples were interrogated with qPCR for steelhead and coho DNA, and a subset of (surface water only) samples were interrogated for California red-legged (CRLF) frog DNA. In general, species specific assays were performed in triplicate for Jonah Ventures samples (Attachment 4), and in triplicate for Genidaqs samples prior to treatment for inhibition, and again in triplicate for Genidaqs samples after treatment for inhibition (Zymo Research, Irvine, CA; Attachment 5). Sample and reagent volumes as well as PCR procedures are described in Attachment 1 for Jonah Ventures samples, and according to Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin et al., 2009) for Genidaqs samples. Jonah Ventures PCR plates included a negative PCR control and gBlocks (double stranded synthetic gene sequences containing primer and probe sites, and diluted in a series of 7, 10-fold dilutions in order to quantify DNA concentrations). Genidaqs performed negative extraction controls (for samples extracted at the Genidaqs lab), and PCR plates included negative PCR controls, and positive PCR controls for each of the target species.

All PCR results were considered detections for samples amplifying at 40 or fewer denaturing cycles. Detections are differentiated as "trace" detections for cases where only one technical replicate was positive, "multiple" detections for cases where 2-4 technical replicates were positive, and "many" detections for cases where more than 4 technical replicates were positive. Replicates were considered within and among individual field replicates, and among sites sampled on multiple dates, as per Duda et al. 2021.

Results

Steelhead

Steelhead DNA was detected by at least one lab at all sites sampled (Attachments 4-5, Table 2). Detections were multiple to many with the exception of one site (at the mouth of Haupt Creek), where trace amounts of DNA were detected (Table 2).

Coho

Coho DNA was detected in trace amounts at two estuary sampling locations (Gualala River Estuary Sites 10 and 14): the Gualala River Estuary Site 11 sample was collected on December 20, 2020, at a depth of 5-m (17-ft), where the total estuary depth was 5.5-m (18-ft) and the

salinity was 3%; the Gualala River Estuary Site 14 sample was collected on December 23, 2020, at a depth of 3.5-m (12-ft) where the total estuary depth was 4-m (14-ft), the salinity was 0%, and the fish finder indicated high concentrations of fish (Figures 4-6; Table 2; Attachment 3).

California Red-Legged Frogs

California red-legged frog DNA was detected in trace amounts at three sampling locations (South Fork Gualala River downstream of Clipper Mill Bridge, mouth of North Fork Gualala River, and Gualala River Estuary Site 2—which was a sample collected on foot from the estuary surface; Figures 5 and 6; Table 2).

Controls

No DNA was detected in any field controls, extraction controls, or negative PCR controls (Table 2). Species-specific DNA was positively detected in all positive controls (Attachment 5). GBlocks used to develop calibration curves had acceptable r^2 values ranging from 0.997-0.998, which indicate a high goodness-of-fit, and thus reliability of estimates for the number of gene copies detected in each Jonah Ventures sample (Table 1, Attachment 4). GBlock efficiencies ranging from 98-105% further increase confidence in gene copy quantification (Table 1, Attachment 4).

Table 1. gBlock results (from Jonah Ventures laboratory only).

Assay	R^2	Efficiency (%)
Steelhead – NADH hydrogenase	0.998	98
Coho salmon – Cyt *c* oxidase	0.998	105
CA red-legged frog – Cyt *b*	0.997	98

Conclusions

Steelhead

Results for steelhead suggest that species use is nearly (if not entirely) ubiquitous throughout the sample area/lower Gualala River watershed. As no DNA was detected in any field or negative lab controls, alongside widespread locations of positive steelhead detections, it is reasonable to conclude similarly widespread distribution of steelhead throughout the study area during the sampling period.

Coho

While no single sample detected more than trace amounts of coho DNA, the combined evidence suggests coho were likely inhabiting the closed estuary during the sample period. The two samples in which traces of coho DNA were detected are located less than 500 m apart. Moreover, one sample with trace DNA detections (at Estuary Site 14) was collocated with an abundance of relatively small fish detected by sonar (Figure 4). The other site with trace coho DNA detections (at Estuary Site 10) was amongst the deepest sites sampled in the estuary, and

Figure 5. Map of locations where trace amounts of coho salmon DNA (green triangles) and California red-legged frog DNA (red triangles) was detected in the Gualala River watershed between December 15 – 27, 2020.

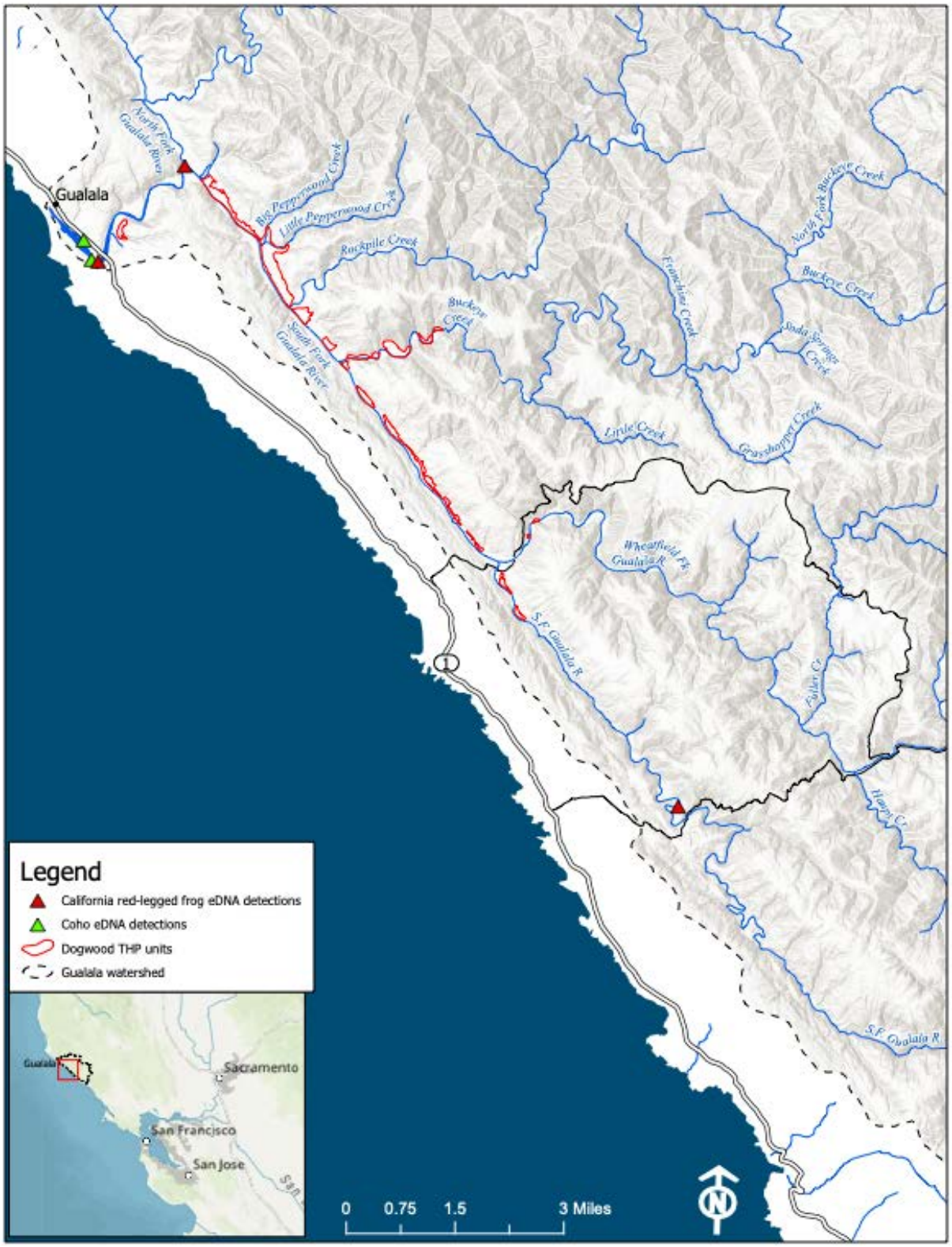


Table 2. Site locations, sampling dates, and detections for each species.

Site Description	Latitude	Longitude	Date/s	Steelhead	Coho	CRLF
Buckeye Cr - Site 1	38.74203	-123.36477	12/22/20	Multiple		
Buckeye Cr - Site 2	38.74148	-123.35836	12/22/20	Many		
Buckeye Cr - Site 3	38.74588	-123.35574	12/22/20	Many		
Buckeye Cr - Site 4	38.74309	-123.35226	12/22/20	Many		
Buckeye Cr (abv Soda Springs)	38.74729	-123.34856	12/22/20	Many		
Gualala R estuary - Site 1	38.76534	-123.52802	12/18/20	Multiple		
Gualala R estuary - Site 2	38.75866	-123.52015	12/20/20	Many		Trace
Gualala R estuary - Site 3	38.76267	-123.51771	12/20/20	Many		
Gualala R estuary - Site 4	38.76167	-123.51814	12/20/20	Many		
Gualala R estuary - Site 5	38.76118	-123.51862	12/20/20	Multiple		
Gualala R estuary - Site 6	38.75951	-123.51873	12/20/20	Many		
Gualala R estuary - Site 7	38.76506	-123.51949	12/20/20	Many		
Gualala R estuary - Site 8	38.76332	-123.52547	12/20/20	Many		
Gualala R estuary - Site 9	38.76053	-123.52357	12/20/20	Many		
Gualala R estuary - Site 10	38.75895	-123.52185	12/20/20	Many	Trace	
Gualala R estuary - Site 11	38.76614	-123.53056	12/20/20	Many		
Gualala R estuary - Site 12	38.75819	-123.52069	12/23/20	Many		
Gualala R estuary - Site 13	38.75924	-123.52129	12/23/20	Many		
Gualala R estuary - Site 14	38.76281	-123.52406	12/23/20	Many	Trace	
Gualala R estuary - Site 15	38.76630	-123.53004	12/23/20	Many		
Gualala R estuary – Site 16	38.75952	-123.51798	12/27/20	Many		
Gualala R estuary – Site 17	38.75971	-123.51830	12/27/20	Many		
Gualala R estuary – Site 18	38.75998	-123.51872	12/27/20	Many		
Gualala R estuary – Site 19	38.76017	-123.51904	12/27/20	Many		
Fuller Cr	38.70039	-123.32707	12/22/20	Many		
Haupt Cr (mouth)	38.66152	-123.32178	12/15/20	Trace		
North Fork Gualala R (mouth)	38.77837	-123.49891	12/17/20, 12/25/20	Many		Trace
South Fork Gualala R (mainstem blw Clipper Mill Br)	38.65136	-123.36826	12/18/20	Many		
South Fork Gualala R (floodplain blw Clipper Mill Br)	38.65408	-123.36908	12/18/20, 12/25/20	Many		Trace
South Fork Gualala R (abv Wheatfield Fork mouth)	38.74309	-123.35226	12/24/20	Many		
South Fork Gualala R (abv North Fork mouth)	38.77822	-123.49852	12/25/20	Many		
Soda Springs Cr (mouth)	38.74694	-123.34869	12/22/20	Multiple		
Wheatfield Fork Gualala R (abv Haupt Cr)	38.66223	-123.32063	12/15/20	Multiple		
Wheatfield Fork Gualala R (mouth)	38.70607	-123.40745	12/17/20, 12/24/20	Many		

the only site with any detectable salinity (Attachment 3). While coho salmon were likely not as abundant in the estuary or lower watershed as steelhead, the two trace detections of coho DNA within close proximity in the estuary support the likelihood of coho use of estuarine (or nearby upstream) habitat during the study period (Figures 5 and 6).

Potential False Negatives

False negatives (or the lack of detection of DNA in spite of a species' presence) can result from a multitude of environmental factors including low species abundance, low cellular shedding and/or metabolism rates, and instream degradation resulting from microbial action, UV light, temperature, and many other factors (Barnes and Turner 2016). False negatives can also result from sampling and analytical factors including the volume of water filtered relative to the pore size of individual filters. In the Gualala River watershed during the sample period, several rain events occurred which mobilized fine sediments that in turn quickly clogged 1-µm sample filters. While an ideal sample volume generally consists of 1000-5000 mL, volumes filtered at the two sites of detection were $\geq$ 250 mL due to filters clogging (e.g., Carim et al. 2016, Duda et al. 2021). In all likelihood, detections would have been higher with larger sample volumes. Given the known low abundance of coho in the watershed combined with low sample volumes during the December sampling event, results presented here could underestimate the prevalence and distribution of coho.

Potential False Positives

False positives are another consideration when interpreting eDNA data. False positives most commonly result from field or lab contamination, or transport of DNA in the environment. Field contamination generally occurs through sample exposure to previously exposed gear. To minimize field contamination, sterile gloves were worn at each site and all sample equipment was new upon arrival to the watershed (e.g., sample collection devices, boots and waders, electronics, etc.). In the case of estuary sampling, two items that were reused without sterilization between site locations were the drift boat and the Van Dorn sampler. Boats have been previously determined not to be a source of eDNA contamination (Carim et al. 2016), and the boat used for sampling sat dry-docked for over a year—during which time DNA would degrade. The Van Dorn sampling device was newly manufactured upon arrival in the watershed, thoroughly rinsed between sites, and sterilized and dried between sampling days. Moreover, the two detections of coho DNA were collected from the sampling device on different sampling days. Those factors combined with the lack of detections in all field control samples suggest field contamination did not occur during the course of the study.

Lab contamination can generally be detected by evaluating lab control samples and using DNA assays known to have species specificity. These criteria were employed in this effort, and all negative lab control samples detected no DNA, while positive control samples from voucher specimens did detect species-specific DNA.

Figure 6. Map of sample locations in the estuary, including locations where trace amounts of coho salmon DNA (green circles) and California red-legged frog DNA (red circles) was detected in the Gualala River watershed between December 20 – 23, 2020. Symbol sizes increases with the number of depths sampled at each location.

The last major cause of false positive detections is transport of DNA in the environment. Transport can occur as mediated by predators, and with water movement. Notably, a bald eagle and a river otter were observed in the estuary during the sample period (although other birds, fish, and additional taxa can also prey on salmonids). However, DNA deposited by a transient predator from outside of the watershed would unlikely appear at different locations, depths, and salinity levels in the estuary as found for coho DNA collected for this study. Fish predators which potentially could have appeared on the fish finder would most likely have been juvenile steelhead unless incubating coho eggs had been consumed by sticklebacks or sculpins which also inhabit the estuary (ECORP 2004). None of those taxa would originate from outside of the Gualala River watershed, particularly given the extended period for which the estuary was closed prior to sampling. While also unlikely due to low local coho abundances, the possibility exists that a predator consumed a coho from the marine environment outside of the estuary. However, coho in close proximity to the estuary in December would most likely be adults

returning to the Gualala River, again suggesting coho use of the watershed. The latter half of December is about two-thirds through the peak spawning season for Central California Coast coho (NMFS 2012).

Downstream transport of DNA via streamflow is the most thoroughly quantified environmental transport in the literature. It typically persists at detectable levels for hours to weeks (≤ 6 wks), and for about 200-m (0.1-mi) downstream (Barnes and Turner 2016, Spence et al. 2020). In the Gualala River estuary, however, limited potential for upstream transport from the ocean into the closed estuary exists via marine overwash, and potential (but even less likely) seepage through the sandbar. While less studied, DNA is documented persisting for less time in marine than freshwater environments (Barnes and Turner 2016). And, in the closed Gualala River estuary, seepage typically flows from the closed freshwater estuary to the saltwater marine environment in a downstream direction, particularly after storm events that increase closed estuary water levels like those that occurred during the study period (ECORP 2004). Moreover, significant ocean wave overwash is detectable with elevated salinity in the closed estuary (ECORP 2004). The only samples collected in the estuary with any detectable salinity (3%) were collected from its maximum depth which is documented retaining saline water throughout the year (ECORP 2004). Lastly, the samples in which coho DNA was detected in trace amounts were approximately 1.5-km and 2-km (0.9-mi and 1.2-mi) upstream of the lower extent of the closed estuary, which exhibited no detectable flow in any direction, and upstream flow at that time is implausible. Consequently, the likelihood of coho DNA originating from marine water is near zero.

Although at least as unlikely, positive eDNA sample results have on rare occasions resulted from contamination caused by humans not directly involved in sample collection (Merkes et al. 2014). Examples include recreational boating and dumping of food waste. If such contamination were to occur, however, positive detections would more likely be concentrated in time and space (for example, immediately downstream of a dumping site). Because coho detections in the estuary were in low concentrations, separated by several hundred meters, detected at different depths on different days, and both upstream of the Gualala stormwater outfall and city businesses, false positives generated by human activity are improbable.

Lastly, while juvenile coho are documented rearing in estuarine environments (including closed lagoonal estuaries), the majority of subyearling juveniles rear upstream in riverine habitats (Jones et al., 2014, Osteberback et al. 2018). Consequently, detections of coho salmon DNA in the estuary suggest additional upstream use of the watershed that was not captured during this sampling event.

California Red-Legged Frogs

Results for California red-legged frogs suggest limited but widespread use of the Gualala River watershed. Although frog results are subject to the same sources of false negative and false positive results as salmon (with the exception of marine sources), trace detections throughout the

lower watershed (using sterile sampling equipment, assays for multiple genes, and measuring no DNA in any field or lab negative controls) suggest a wide range of CRLF in the Gualala basin.

Statement of Qualifications

I am a Ph.D. Candidate in salmon ecology at the University of Washington School of Aquatic and Fishery Sciences, and an aquatic ecologist with extensive research experience regarding the utility of multiple methods of environmental DNA (eDNA) methodology for defining salmon habitat relative to traditional capture methods. I hold a Bachelor's of Science Degree in conservation biology from the University of Washington and a Master's Degree in freshwater ecology from the University of Montana's Flathead Lake Biological Station. My curriculum vitae is attached below (Attachment 6).

I have collected over a thousand eDNA samples from areas ranging from low to high abundances of fish, as determined with concurrent electrofishing. I have extracted DNA from the same number of samples and used 6 species-specific polymerase chain reaction (PCR) assays to determine fish distribution. I have conducted subsequent computational analyses using occupation models, spatial stream network modeling, and other quantitative tools. I have two manuscripts currently in progress concerning the analysis of eDNA analytical methods, have provided several presentations regarding eDNA to public and academic audiences, and currently participate in multiple working groups focused on eDNA methodology.

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Attachment 1

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Wild Rice qPCR Methods Document

Client: ONeal

1 Sample Processing

1.1 eDNA Filter Sample Processing

1.1.1 Standard Protocol

A High Quality 40W USB CO2 Laser Engraving Cutting Machine Engraver Cutter with CorelDraw Software (cat# DKJ-40W-US) (Amunstar Trading Company, Guangdong, China) was used for removal of plastic casing from filter. Once plastic casing was cut, sample barcodes were recorded and assigned a well within the 96 well plate or numbered extraction tube. The whole filter was removed and transferred to the extraction plate/tube using sterilized tweezers inside a laminar flow hood. Plates or tubes were immediately processed or stored in -20°C until the extraction process could be performed.

2 Extraction

2.1 Tube Extraction Protocol

2.1.1 Standard Protocol

Genomic DNA from samples was extracted using the MoBio PowerSoil Isolation Kit (Cat# 12888-100) according to the manufacturer's protocol. Approximately 0.25 grams of sample was used for genomic DNA extraction. Genomic DNA was eluted into 100µl and frozen at -20°C.

2.1.2 DNeasy update

Genomic DNA from samples was extracted using the DNeasy PowerSoil Kit (Cat# 12888-100) according to the manufacturer's protocol. Approximately 0.25 grams of sample was used for genomic DNA extraction. Genomic DNA was eluted into 100µl and frozen at -20°C.

3 qPCR Protocols

3.1 Coho Salmon Singleplex Assay, Cytochrome c Oxidase, subunit I gene

3.1.1 Standard Protocol

Forward primer: 5' CGCTCTTCTAGGGGATGAT 3'
Reverse primer: 5' CTCCGATCATAATCGGCATG3'
Probe: 5' ATTTACAACGTAATCGTC 3'
Primer/probe reference: Rasmussen et al., 2010

An amplicon from the cytochrome c oxidase (COI) gene was amplified via qPCR from genomic DNA samples using CohoSlmn FWD and REV primers, and CohoSlmn Probe. A standard curve was generated for each run to correspond to targeted region of the coho salmon COI gene. Each qPCR reaction is run in triplicate and contains 8.0 uL of QuantaBio PerfeCTa qPCR ToughMix Low ROX (Catalog Number 97065-966), 500 nM of each primer, 300 nM of probe, 2.0 uL of gDNA, and 6.8 uL of Nuclease-free H2O for a total reaction volume of 20 uL. qPCR

amplification was carried out on the Agilent AriaMx qPCR instrument with the following thermal profile conditions: 1 cycle of initial denaturation for 5 minutes at 95°C; followed by 50 cycles of 15 seconds at 95°C and 1 minute at 60°C.

3.2 Coho Salmon Assay Standard Curve, Cytochrome c Oxidase, subunit I gene

3.2.1 Standard Protocol

Coho Salmon gBlock:

ACTGATTCGAGCAGAACTGAGCCAGCCGGGCGCTCTTCTAGGGGATGATCAGATTTACAACGTAATCGT
CACAGCCCATGCCTTCGTTATGATTTTCTTTATAGTCATGCCGATTATGATCGGAGGCTTTGGAAACTGAT
TAATCCCCCTAATGA

A standard curve was tested in triplicate for each qPCR run. The targeted gene of interest, obtained from NBCI, was used to design a synthetic gBlock (from Integrated DNA Technologies) that contained the coho salmon primers and probe. A 10-fold dilution was carried out on the coho gBlock generating a 7-point standard curve ranging from 5.446 x 106 – 5.446 x 100 copies. Each qPCR reaction contains 8.0 uL of QuantaBio PerfeCTa qPCR ToughMix Low ROX (Catalog Number 97065-966), 500 nM of each primer, 300 nM of probe, 2.0 uL of coho salmon gBlock, and 6.8 uL of Nuclease-free H2O for a total reaction volume of 20 uL. qPCR was carried out on the Agilent AriaMx qPCR instrument with the following thermal profile conditions: 1 cycle of initial denaturation for 5 minutes at 95°C; followed by 50 cycles of 15 seconds at 95°C and 1 minute at 60°C.

3.3 Rainbow Trout Singleplex Assay, NADH dehydrogenase, subunit 2 gene

3.3.1 Standard Protocol

Forward primer: 5' AGTCTCTCCCTGTATATCGTC 3'
Reverse primer: 5' AGTTTAGTTCATGAAGTTGCGTGAGTA 3'
Probe: 5' /56-FAM/CCAA+CAA+CT+CTTTA+AC+CATC/3IABkFQ/ 3'
Primer/probe reference: Wilcox et al., 2015

An amplicon from the NADH dehydrogenase, subunit 2 (ND2) gene was amplified via qPCR from genomic DNA samples using rainbow trout FWD and REV primers, and rainbow trout Probe. A standard curve was generated for each run to correspond to targeted region of the rainbow trout cytochrome b gene. Each qPCR reaction is run in triplicate and contains 8.0 uL of QuantaBio PerfeCTa qPCR ToughMix Low ROX (Catalog Number 97065-966), 500 nM of each primer, 300 nM of probe, 2.0 uL of gDNA, and 6.8 uL of Nuclease-free H2O for a total reaction volume of 20 uL. qPCR amplification was carried out on the Agilent AriaMx qPCR instrument with the following thermal profile conditions: 1 cycle of initial denaturation for 5 minutes at 95°C; followed by 50 cycles of 15 seconds at 95°C and 1 minute at 60°C.

3.4 Rainbow Trout Assay Standard Curve, NADH dehydrogenase, subunit 2 gene

3.4.1 Standard Protocol

Rainbow Trout gBlock:

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CTACAATTCGCACCCTCTCTCACACTCCTCAGTCTCTCCCTGTATATCGTCATGACATCTTCAGCTTTCCTC
ACATTAAAAACCAACAACTCTTTAACCATCAATACTCTCGCAACTTCATGAACTAAATCCCCAACCCTTGC
CGCATTAACCGCTCTT

A standard curve was tested in triplicate for each qPCR run. The targeted gene of interest, obtained from NBCI, was used to design a synthetic gBlock (from Integrated DNA Technologies) that contained the Rainbow Trout primers and probe. A 10-fold dilution was carried out on the Rainbow Trout gBlock generating a 7-point standard curve ranging from 5.792 x 106 – 5.792 x 100 copies. Each qPCR reaction contains 8.0 uL of QuantaBio PerfeCTa qPCR ToughMix Low ROX (Catalog Number 97065-966), 500 nM of each primer, 300 nM of probe, 2.0 uL of Rainbow Trout gBlock, and 6.8 uL of Nuclease-free H2O for a total reaction volume of 20 uL. qPCR was carried out on the Agilent AriaMx qPCR instrument with the following thermal profile conditions: 1 cycle of initial denaturation for 5 minutes at 95°C; followed by 50 cycles of 15 seconds at 95°C and 1 minute at 60°C.

3.5 Red Legged Frog Singleplex Assay, Cytochrome b gene

3.5.1 Standard Protocol

Forward primer: 5’ ATCGGCTCCGACCTAGTT 3’
Reverse primer: 5’ AATGTGAAGAATCGGGTGAGA 3’
Probe: 5’ /5Cy5/GACTGAAAA/TAO/GCCCCCTCAGATC/3IAbRQSp/ 3’
Primer/probe reference: Jonah Ventures, 2019

An amplicon from the cytochrome b gene was amplified via qPCR from genomic DNA samples using RedFrog FWD and REV primers, and RedFrog Probe. A standard curve was generated for each run to correspond to targeted region of the California red legged frog cytochrome b gene. Each qPCR reaction is run in triplicate and contains 8.0 uL of QuantaBio PerfeCTa qPCR ToughMix Low ROX (Catalog Number 97065-966), 500 nM of each primer, 300 nM of probe, 2.0 uL of gDNA, and 6.8 uL of Nuclease-free H2O for a total reaction volume of 20 uL. qPCR amplification was carried out on the Agilent AriaMx qPCR instrument with the following thermal profile conditions: 1 cycle of initial denaturation for 5 minutes at 95°C; followed by 50 cycles of 15 seconds at 95°C and 1 minute at 60°C.

3.6 Red Legged Frog Assay Standard Curve, Cytochrome b gene

3.6.1 Standard Protocol

RedFrog gBlock:
CACTAACCTCCTCTCAGCTGCCCCATACATCGGCTCCGACCTAGTTCAATGGATCTGAGGGGGCTTTTCA
GTCGACAACGCCACTCTCACCCGATTCTTCACATTCCACTTCATCCTCCCATTYATCATTGCCG

A standard curve was tested in triplicate for each qPCR run. The targeted gene of interest, obtained from NBCI, was used to design a synthetic gBlock (from Integrated DNA Technologies) that contained the RedFrog primers and probe. A 10-fold dilution was carried out on the RedFrog gBlock generating a 7-point standard curve ranging from 1.851 x 106 –1.851 x 100

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copies. Each qPCR reaction contains 8.0 uL of QuantaBio PerfeCTa qPCR ToughMix Low ROX (Catalog Number 97065-966), 500 nM of each primer, 300 nM of probe, 2.0 uL of RedFrog gBlock, and 6.8 uL of Nuclease-free H2O for a total reaction volume of 20 uL. qPCR was carried out on the Agilent AriaMx qPCR instrument with the following thermal profile conditions: 1 cycle of initial denaturation for 5 minutes at 95°C; followed by 50 cycles of 15 seconds at 95°C and 1 minute at 60°C.

Attachment 2

OPEN ACCESS Freely available online PLoS one

Molecular Detection of Vertebrates in Stream Water: A Demonstration Using Rocky Mountain Tailed Frogs and Idaho Giant Salamanders

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Abstract

Stream ecosystems harbor many secretive and imperiled species, and studies of vertebrates in these systems face the challenges of relatively low detection rates and high costs. Environmental DNA (eDNA) has recently been confirmed as a sensitive and efficient tool for documenting aquatic vertebrates in wetlands and in a large river and canal system. However, it was unclear whether this tool could be used to detect low-density vertebrates in fast-moving streams where shed cells may travel rapidly away from their source. To evaluate the potential utility of eDNA techniques in stream systems, we designed targeted primers to amplify a short, species-specific DNA fragment for two secretive stream amphibian species in the northwestern region of the United States (Rocky Mountain tailed frogs, *Ascaphus montanus*, and Idaho giant salamanders, *Dicamptodon aterrimus*). We tested three DNA extraction and five PCR protocols to determine whether we could detect eDNA of these species in filtered water samples from five streams with varying densities of these species in central Idaho, USA. We successfully amplified and sequenced the targeted DNA regions for both species from stream water filter samples. We detected Idaho giant salamanders in all samples and Rocky Mountain tailed frogs in four of five streams and found some indication that these species are more difficult to detect using eDNA in early spring than in early fall. While the sensitivity of this method across taxa remains to be determined, the use of eDNA could revolutionize surveys for rare and invasive stream species. With this study, the utility of eDNA techniques for detecting aquatic vertebrates has been demonstrated across the majority of freshwater systems, setting the stage for an innovative transformation in approaches for aquatic research.

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Introduction

Freshwater systems are hotspots for both biodiversity and species endangerment [1], with freshwater fauna experiencing 123 documented extinctions in the 20th century [2]. Growing demand for water resources indicates that threats to freshwater species will further increase over the next century [3]. Stream species are particularly vulnerable to cumulative changes in land cover [4,5], climate [6], and biotic and abiotic inputs [7,8]. Migratory stream salmonids (e.g. bull trout, *Salvelinus confluentus*, and Chinook salmon, *Oncorhynchus tshawytscha*) are among the most imperiled North American fishes [9] and the most catastrophic documented amphibian population declines have been in streams [10]. Additionally, streams are increasingly being invaded, at great ecological and economic costs, by exotic species, including crayfish, aquatic mussels, and gastropods [11,12].

Investigations into the distribution and ecology of stream species are often hindered by the challenges of working in these systems. Stream species are difficult to inventory due to the complexity of topography and vegetation in streambeds and riparian areas, water turbidity and flow rate, low densities of individuals, cryptic coloration, and the use of microhabitats. Due to these and other factors, surveys for native and exotic species in streams can be expensive and inaccurate [13,14]. For example, a major challenge in amphibian decline research is that amphibians can be difficult to detect, especially in streams [15]. Electrofishing techniques have high success for detection of aquatic vertebrates in many cases [16], but can be time consuming and difficult to apply in streams, and may cause injury to target and non-target species [17].

Researchers have been using DNA from feces, urine, hair, feathers, shed skin, and eggshells to detect terrestrial vertebrate species for the past decade [18], and detection of microbial species using environmental DNA (eDNA) found in soil and seawater is revolutionizing species inventories [19] and enabling efficient disease detection [20]. Recently, the reliable detection of aquatic vertebrate species using eDNA in water was confirmed in wetlands [21] and in a large river and canal system [22]. Using eDNA to detect rare and secretive species in streams could increase accuracy and decrease costs of surveys, increase the number of sites sampled per unit effort, refine distribution and extinction records, and provide early detection of invasive species in these systems, without any risk to the species. However, the fast flow of streams may move shed cells away from their source at a rate prohibitive to eDNA collection. To evaluate the potential for using

eDNA to survey for stream species, we collected water samples from five small headwater streams in two seasons and tested them for DNA of two amphibian species (Rocky Mountain tailed frogs, *Ascaphus montanus*, and Idaho giant salamanders, *Dicamptodon aterrimus*) known to be present at the sites. To achieve this, we designed species-specific primers and tested multiple DNA extraction and PCR protocols designed to amplify low quality DNA templates.

Methods

In the first phase of the project, we collected one 10-L and two 5-L water samples from a headwater stream (Table 1) with known presence of two species of amphibians (Rocky Mountain tailed frogs and Idaho giant salamanders) in late September of 2010 using a flow-through filter with a peristaltic pump and 0.45 µm cellulose nitrate filter paper (State of Idaho Wildlife Collection Permit #030716 and Payette National Forest Research Permit #0105). Each filter was preserved in 95% ethanol in a separate 2 mL tube. We estimated the larval density of both species at this site using standard kick-sampling methodology [23] in July and August 2010. Density survey and water sample collections were made during base flow, measured as 0.23 m^3 s^{-1}, in the study stream.

We designed a set of species-specific primers for each species targeting a small region of the mitochondrial DNA (mtDNA) cytochrome *b* gene (obtained from GenBank) [24,25] (Table 2). The distribution of these two species is disjunct from their congeners along the Pacific coast to the west; therefore, we designed primers to be species-specific within our system (the northern Rocky Mountains region) but also to detect the congeners of each species for wider geographic applicability. Target fragment length was 78 base pairs for *Dicamptodon* and 85 base pairs for *Ascaphus*. This test was designed to amplify previously-published sequences characteristic of these species; no new sequence data was generated that had not already been published. All extractions and PCR set-up were done in a room dedicated to low-quantity DNA sources; no DNA from amphibians had previously been handled in this room.

In this first phase, we tested two DNA extraction and three PCR protocols for the detection of Rocky Mountain tailed frogs and Idaho giant salamanders using eDNA from these filter samples. First, we removed the filters from the ethanol and air-dried them overnight. We then divided each filter in half and extracted each half with either the DNeasy Tissue and Blood Kit (Qiagen, Inc.) or the UltraClean® Soil DNA isolation kit (MoBio Laboratories, Inc.). We then attempted to amplify DNA from each sample using a standard PCR protocol (PCR Protocol 1; Table S1). All reactions included a negative extraction control, negative PCR control, and positive controls for each of the target species. We ran PCR products on 3% agarose gels to determine success. When this first protocol produced no PCR products, we reran the reactions with a combination of each DNA sample and a positive control in each tube to determine whether PCR inhibitors were preventing amplification. For samples extracted using the Qiagen DNeasy kit, we also tested three PCR protocols (PCR Protocols 1, 2, and 3; Table S1) with a dilution series of each sample (1X, 0.1X, 0.01X, and 0.001X).

We sequenced products of the most successful combination of protocols using the BigDye system on a 3130xl capillary sequencer (Applied Biosystems). To streamline the assay for large-scale application, primers were labeled with fluorescent dyes and a PCR multiplex was created using primer sets for both species with PCR Protocol 3. We tested additional negative controls of DNA from sympatric amphibian species (*Ambystoma macrodactylum*, *Bufo boreas*, *Pseudacris sierra*, *Rana luteiventris*) with this multiplex, independently (1 reaction/species; approximately 5 – 100 ng DNA/reaction) and together with DNA from the target species, to verify the specificity of our diagnostic test.

In the second phase of the project, we collected a 5-L water filter sample from each of five headwater streams known to contain populations of Rocky Mountain tailed frogs and Idaho giant salamanders, including the original stream (Table 1). Streams were sampled in late March and early April 2011 using the same field collection techniques as above; density estimates for these streams were obtained July and August 2010 (State of Idaho Wildlife Collection Permit #030716 and Payette National Forest Research Permit #0105). We used the DNeasy extraction method and PCR Protocol 3 (Table S1) for one half of each filter and a modified extraction, with the addition of the use of a QIAshredder (Qiagen, Inc.) after overnight digestion with Proteinase K, and PCR Protocol 4 (Table S1) for the other half of each filter. We also tested the Qiagen Multiplex Plus PCR kit with this modified protocol (PCR Protocol 5). These samples were only run at full concentration. We tested whether field-estimated densities predicted PCR success for these samples using simple linear regression in R 2.13.0 [26].

Results

In the first phase of the project, we recovered the targeted DNA sequence from both species from all stream water filter samples

Table 1. Sampling sites, dates of sampling, PCR success for each species, and densities of Idaho giant salamanders (*Dicamptodon aterrimus;* DIAT) and Rocky Mountain tailed frogs (*Ascaphus montanus*; ASMO) where stream filter samples were taken, estimated using field methods in summer 2010.

Site	Latitude	Longitude	Date sampled	DIAT per m^2	DIAT PCR success (%)	ASMO per m^2	ASMO PCR success (%)
Phase 1							
Nasty Creek	44.877	−115.696	25Sept10	0.032	100	0.228	100
Phase 2							
Camp Creek	44.890	−115.706	27Mar11	0.036	100	0.097	16.7
Deadman Creek	44.966	−115.663	27Mar11	0.011	100	0.149	0
Goat Creek	44.759	−115.684	27Mar11	0.029	100	0.052	33.3
Nasty Creek	44.877	−115.696	03Apr11	0.032	100	0.228	33.3
Reegan Creek	44.949	−115.587	27Mar11	0.011	100	0.337	16.7

doi:10.1371/journal.pone.0022746.t001

Table 2. Primer sequences for species-specific amplification of short fragments of cytochrome *b*.

Species	Primer name	Primer sequence
Rocky Mountain tailed frog (*Ascaphus montanus*)	ASMO F	CGT CAA CTA TGG CTG GCT AA
	ASMO R	TCG GCC AAT GTG AAG ATA AA
Idaho giant salamander (*Dicamptodon aterrimus*)	Dicamp F	TCT GCA TCT TYC TAC ATA TYG GAC
	Dicamp R	ATC ACY CCG ACK TTT CAG GT

doi:10.1371/journal.pone.0022746.t002

only when using the DNeasy extraction method and PCR Protocol 3. For two of the three samples (one 5-L, one 10-L) the correct fragment was also detectable at 0.1X DNA concentration for Rocky Mountain tailed frogs and down to 0.001X DNA concentration for Idaho giant salamanders. Tests for inhibition with PCR Protocol 1 showed that samples from the DNeasy extraction method were inhibited (but the extraction negative control was not), while samples from the MoBio extraction were not inhibited, indicating the lack of target species DNA in the results of these extractions. PCR multiplexing with fluorescently-tagged primers provided clear and efficient detection of amplified fragments (Fig. 1), with all samples and positive controls at >8000 fluorescent units, even when DNA from the target species was mixed with DNA from non-target species. None of the negative controls, including DNA from four sympatric amphibian species, tested positive.

In the second phase of the project, where samples were collected in the early spring, we detected Idaho giant salamanders in all filter samples using PCR Protocol 3 but did not detect Rocky Mountain tailed frogs. Amplifications for Idaho giant salamanders were weak ($\bar{x}$ = 203, 95% C.I. 25 – 382 fluorescent units) compared with samples collected in early fall. With the addition of the QIAshredder kit and using PCR protocol 4, we detected both species in all but one of the streams, with strong signal for Idaho giant salamanders in all reactions ($\bar{x}$ = 5962, 95% C.I. 4555 – 7369 fluorescent units) and detection probability across 6 PCR replicates for Rocky Mountain tailed frogs ranging from 0 to 33% (Table 1; for successful amplifications, $\bar{x}$ = 7636, 95% C.I. 6176 – 9097 fluorescent units). Substitution of the Qiagen Multiplex Plus PCR kit for the Qiagen Multiplex PCR kit in PCR Protocol 5 did not improve performance (Table S1). There was no evidence that the probability of detection of Rocky Mountain tailed frogs in a PCR replicate was related to field-estimated density from the previous summer (PCR Protocol 4; $F_{1,3} = 0.036$, $P = 0.86$).

Discussion

Using filter samples taken from stream water, we developed an efficient protocol for detecting targeted DNA sequences for two secretive amphibian species, demonstrating that the recovery of amphibian DNA from stream water is possible even when amphibian populations are at low densities. The rapid field collection protocol, relatively simple field equipment and low cost (supply cost per sample with 6 PCR replicates = $10.11) make this technique widely applicable to broad-scale inventory and monitoring efforts. The probability of detection of eDNA across densities likely varies with species, stream size, and discharge rate, and by season, as suggested by this study. However, the potential impact of this technique for inventorying species in stream systems is far-reaching, including detection of rare or imperiled vertebrates.

We found that only the DNeasy Blood & Tissue kit with the Qiagen Multiplex PCR kit detected eDNA for both species in water filter samples. We did not successfully extract DNA from the filter samples using the UltraClean® Soil DNA isolation kit (MoBio Laboratories, Inc.); possibly the PowerWater DNA Isolation kit (MoBio Laboratories, Inc.) used to detect eDNA of Asian carp [22] would have yielded better results. Our results indicate that using the Qiagen Multiplex PCR kit improves species detection in water filter samples over a protocol using Amplitaq Gold DNA polymerase and BSA; this latter combination was used to establish that the detection of aquatic vertebrates using eDNA in water samples was possible [21].

Although we only sampled one stream in the first phase of our project, our results suggest that detection of Rocky Mountain tailed frogs and Idaho giant salamanders using eDNA may be more difficult when samples are taken in early spring rather than early fall. This could be due to decreased metabolism during cold weather or changes in behavior of the target species, such as moving into the hyporheic zone. For Idaho giant salamanders, we were able to compensate for this by modifying protocols, but for Rocky Mountain tailed frogs, detectability was still relatively low in early spring samples. This difference between species may be due to species-specific seasonal changes in density; while streams in the spring are likely to have one fewer Rocky Mountain tailed frog tadpole cohort than in the early fall due to timing of metamorphosis [27], the difference in overall population density is likely less extreme for Idaho giant salamanders because they are commonly neotonic [28]. This result demonstrates that sampling design for eDNA needs to be informed by the ecology of target species to maximize detection probabilities.

Our approach was to design species-specific primers to detect species of interest; these kinds of targeted primers can be multiplexed to test for many species in a single PCR reaction. However, when the species list is large or inventory for unknown species is the goal of sampling, universal primers and next-generation sequencing techniques could be applied [19]. Using these tools, researchers would sample a stream, river, or wetland, use primers that work across taxa to amplify DNA from this sample, and compare the sequences to those available in a reference library [29]. If sequences are recovered that do not match any in the library, sequences that are closest matches could be used to determine the probable taxonomic group of the unknown species and additional field surveys could be conducted to attempt to locate the species. Next-generation sequencing is currently prohibitively expensive for large survey efforts, but costs will likely be greatly reduced in the near future as the technology improves [30].

The success of eDNA for detecting vertebrates efficiently across freshwater systems indicates that this new tool has the potential to revolutionize surveys for aquatic species with the techniques currently available. The ability to survey for species across taxa with a single water sample would greatly enhance data

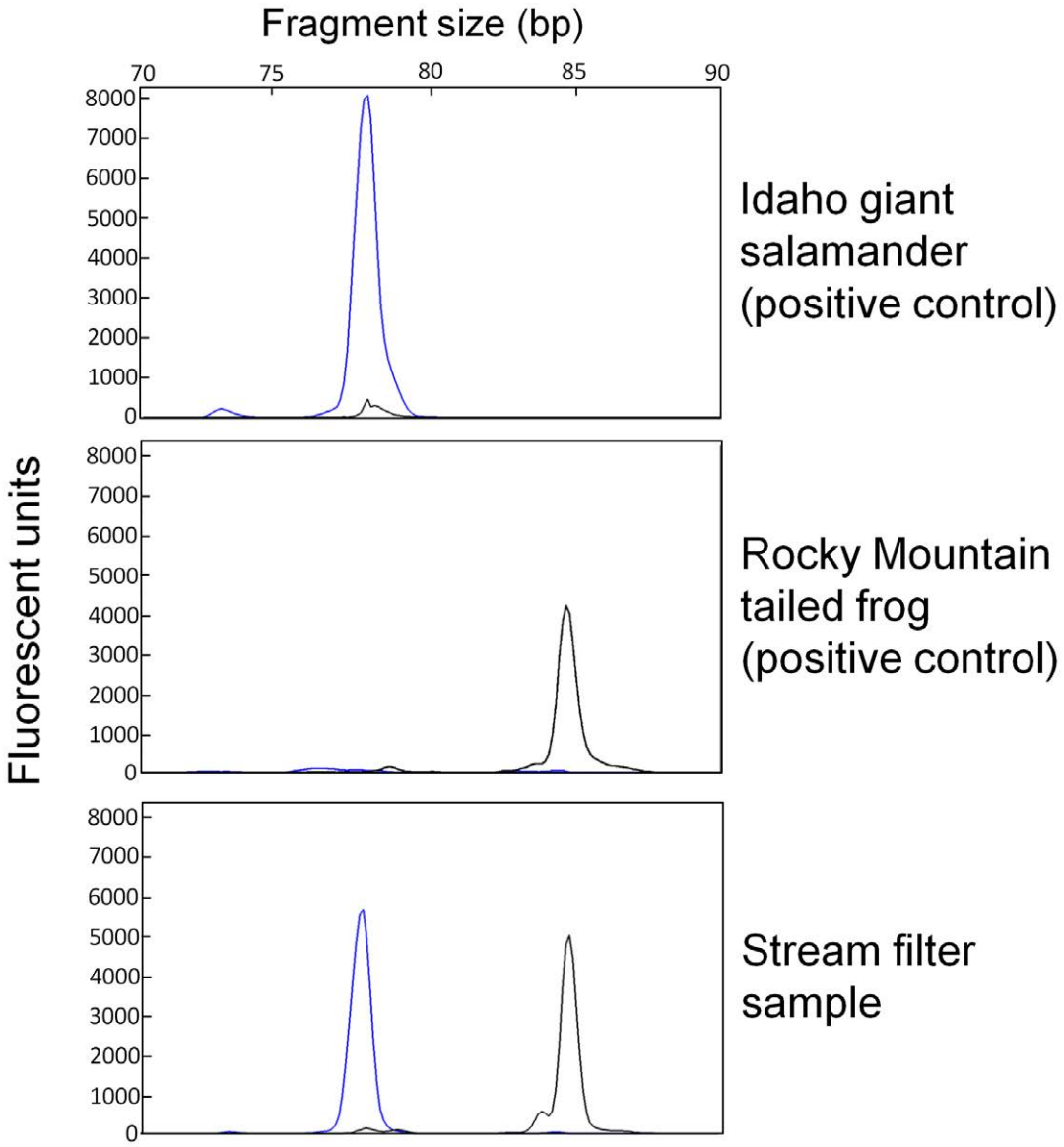

Figure 1. Electropherograms of species-specific PCR amplification of DNA in positive controls and stream water. The blue peak indicates the species-specific fragment for Idaho giant salamanders (*Dicamptodon aterrimus*) and the black peak indicates the species-specific fragment for tailed frogs (*Ascaphus montanus*). All reactions were diluted to produce these images.
doi:10.1371/journal.pone.0022746.g001

availability for aquatic species and benefit resource managers and many fields of research, including community ecology, biogeography, evolutionary biology, conservation biology, and invasion biology. eDNA techniques could be used to form cost-efficient multi-species inventory and monitoring programs for sensitive species, in combination with occupancy models [31] to estimate probabilities of detection. With next-generation sequencing, DNA sequences of a community of aquatic vertebrates could be analyzed simultaneously, exponentially increasing the data available for analysis without disturbing sensitive species. Other applications include early detection of invasive species [21,22], determining whether invasive species have been successfully removed through management actions, detecting rare individuals surviving after catastrophic population declines, and discovering new species in rapid bioassessement surveys. Sensitivity of these techniques to density of individuals and covariates of detection probability such as water temperature and discharge will need to be determined for study systems individually; however, this technique shows great potential for increasing our knowledge of aquatic systems.

Supporting Information

Table S1 PCR protocols and results for amplifying DNA of Rocky Mountain tailed frogs (*Ascaphus montanus*) and Idaho giant salamanders (*Dicamptodon aterrimus*) from stream water.
(DOCX)

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Author Contributions

Conceived and designed the experiments: CSG DSP RSA LPW. Performed the experiments: CSG RSA. Analyzed the data: CSG DSP RSA LPW. Contributed reagents/materials/analysis tools: CSG DSP LPW. Wrote the paper: CSG DSP RSA LPW.

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Text

Attachment 3

Attachment 3 - Sample ID's and estuary field data - p. 1 of 3

Sample ID	Site name	Sample depth (ft)	Estuary depth (ft)	Sample salinity (%)
05M4KYV8	North Fork Gualala R (mouth)	--	--	--
095ITR0P	North Fork Gualala R (mouth)	--	--	--
0BDDEWOC	Gualala R estuary - Site 12	8	20.5	0
0F4DHR4R	South Fork Gualala R (abv North Fork mouth)	--	--	--
0VCTK0PF	Gualala R estuary - Site 12	5	20.5	0
1H0BJ7ME	Gualala R estuary - Site 3	3	6	0
1NUFQEHO	Gualala R estuary - Site 16	6	8	0
1V7B7BMP	Wheatfield Fork Gualala R (mouth)	--	--	--
1WGLD9M5	Gualala R estuary - Site 15	7	12	0
2R1A2CYM	Gualala R estuary - Site 12	20	20.5	3
2UUNLQXE	Gualala R estuary - Site 10	17	18	3
39M0J44W	Gualala R estuary - Site 14	12	14	0
3AIWQ0V7	Gualala R estuary - Site 12	15	20.5	3
3HIXC2ZM	Gualala R estuary - Site 1	1.5	3	0
3LE1AARQ	Gualala R estuary - Site 18	10	12	0
3Q12YK1D	Buckeye Cr - Site 2	--	--	--
42FZZWZS	South Fork Gualala R (abv Wheatfield For mouth)	--	--	--
47SWK8U7	Gualala R estuary - Site 19	5	6	0
4THH8PZW	Gualala R estuary - Site 8	4	8	0
53PNZQ71	Buckeye Cr (abv Soda Springs)	--	--	--
5KDNKS8Z	Gualala R estuary - Site 19	5	6	0
5PH5SX8Y	South Fork Gualala R (abv North Fork mouth)	--	--	--
5SXS0780	Gualala R estuary - Site 17	5	6	0
64VV6LYI	Gualala R estuary - Site 12	20	20.5	3
7AHQ8ZMS	Wheatfield Fork Gualala R (mouth)	--	--	--
7CRW044N	Gualala R estuary - Site 18	10	12	0
7F1OORAN	South Fork Gualala R (floodplain blw Clipper Mill Br)	--	--	--
8MGMHAG6	South Fork Gualala R (floodplain blw Clipper Mill Br)	--	--	--
8Y71F1LZ	Gualala R estuary - Site 8	0	8	0
9T62H5S6	South Fork Gualala R (floodplain blw Clipper Mill Br)	--	--	--
A0L88KVI	Wheatfield Fork Gualala R (mouth)	--	--	--
AX4ZF68W	Wheatfield Fork Gualala R (mouth)	--	--	--
C63NAY22	Gualala R estuary - Site 9	2	8	0
CJV66V15	South Fork Gualala R (mainstem blw Clipper Mill Br)	--	--	--
CLPBQAG9	Gualala R estuary - Site 1	1.5	3	0
CTER2MGM	Gualala R estuary - Site 18	10	12	0
D2D5DJ0O	Gualala R estuary - Site 12	20	20.5	3
D8I300WL	Gualala R estuary - Site 2	1.5	3	0
DP8QDL9O	Gualala R estuary - Site 16	6	8	0
EJ7DDIOZ	Gualala R estuary - Site 14	12	14	0
EW39X0SK	Gualala R estuary - Site 14	12	14	0
EWP6XTYI	Gualala R estuary - Site 3	5.5	6	0
FCL19JPY	Buckeye Cr - Site 3	--	--	--
FUUPIWXL	Gualala R estuary - Site 6	4	7	0
G55VU339	South Fork Gualala R (abv Wheatfield For mouth)	--	--	--

Attachment 3 - Sample ID's and estuary field data - p. 2 of 3

Sample ID	Site name	Sample depth (ft)	Estuary depth (ft)	Sample salinity (%)
GDNQL0ZO	South Fork Gualala R (mainstem blw Clipper Mill Br)	--	--	--
HKAB1QU1	Wheatfield Fork Gualala R (abv Haupt Cr)	--	--	--
HNCZSV1E	Gualala R estuary - Site 19	5	6	0
HO4FDT7E	Gualala R estuary - Site 16	6	8	0
HRFFW57K	Gualala R estuary - Site 3	5.5	6	0
HTV6CKOV	Haupt Cr (mouth)	--	--	--
I8B5D89O	Gualala R estuary - Site 9	6	8	0
IYU7HP0R	Gualala R estuary - Site 7	5	10	0
J1KSHEIH	Fuller Cr	--	--	--
JHTXY52F	South Fork Gualala R (mainstem blw Clipper Mill Br)	--	--	--
JR1Y8WNQ	Gualala R estuary - Site 17	5	6	0
K2DHF8C3	Wheatfield Fork Gualala R (mouth)	--	--	--
K3TFQV0R	Wheatfield Fork Gualala R (abv Haupt Cr)	--	--	--
KKSXDM2X	Buckeye Cr (abv Soda Springs)	--	--	--
L0AUE7UM	Gualala R estuary - Site 12	8	20.5	0
L3AQAVF1	Wheatfield Fork Gualala R (abv Haupt Cr)	--	--	--
L45P6CA5	Buckeye Cr - Site 4	--	--	--
L4T7R48H	South Fork Gualala R (abv Wheatfield For mouth)	--	--	--
LE5NTJ46	Soda Springs Cr (mouth)	--	--	--
M2SX85PS	Fuller Cr	--	--	--
M5DCMVXT	North Fork Gualala R (mouth)	--	--	--
NPJSTL2H	Gualala R estuary - Site 2	1.5	3	0
NRFV9YVO	Gualala R estuary - Site 14	9	12	0
NSBLUQLY	Fuller Cr	--	--	--
OGMN6FAF	Gualala R estuary - Site 15	7	12	0
OOXYG66M	Gualala R estuary - Site 1	1.5	3	0
PNYIECBT	Gualala R estuary - Site 17	5	6	0
PQ0ECPRW	South Fork Gualala R (floodplain blw Clipper Mill Br)	--	--	--
T1CSCIGM	Gualala R estuary - Site 11	9.5	10	0
TCK7OOY3	South Fork Gualala R (floodplain blw Clipper Mill Br)	--	--	--
TKOPYDA1	Gualala R estuary - Site 13	0.5	1.5	0
TPMCDA86	Haupt Cr (mouth)	--	--	--
U23WIPU3	Gualala R estuary - Site 2	1.5	3	0
UKA30BZY	North Fork Gualala R (mouth)	--	--	--
UP080OK5	Soda Springs Cr (mouth)	--	--	--
UR0BCE3E	Soda Springs Cr (mouth)	--	--	--
UVPK5GMO	North Fork Gualala R (mouth)	--	--	--
VL3DNA37	Buckeye Cr - Site 1	--	--	--
VNJS4EUS	Gualala R estuary - Site 15	7	12	0
VQKGDGNM	North Fork Gualala R (mouth)	--	--	--
WPYP33K4	Wheatfield Fork Gualala R (mouth)	--	--	--
WQWEPN8B	Gualala R estuary - Site 5	0.5	2	0
WR0VHYHR	Gualala R estuary - Site 12	8	20.5	0
WRU3122	South Fork Gualala R (abv North Fork mouth)	--	--	--
X18WXS58	Buckeye Cr (abv Soda Springs)	--	--	--

Attachment 3 - Sample ID's and estuary field data - p. 3 of 3

Sample ID	Site name	Sample depth (ft)	Estuary depth (ft)	Sample salinity (%)
XV1IQ261	South Fork Gualala R (floodplain blw Clipper Mill Br)	--	--	--
YQ4Z3OW9	Haupt Cr (mouth)	--	--	--
YT0A2AQR	Gualala R estuary - Site 4	5.5	6	0

Attachment 4

Jonah Ventures qPCR Report

JONAH VENTURES

KNOWLEDGE IN SEQUENCE

February 09 2021

For more information go to www.jonahventures.com

info@jonahventures.com

ClientID = 1391

Last Name = OnealSarah

BatchID = JVB0086

Number samples analyzed = 70

#Histograms Shown are histograms for the distribution of the data for each assay. x-axis is log transformed copies per 100 mL (if data on water volume provided) after adding 1 to show zeros. y-axis represents count of samples in each category.

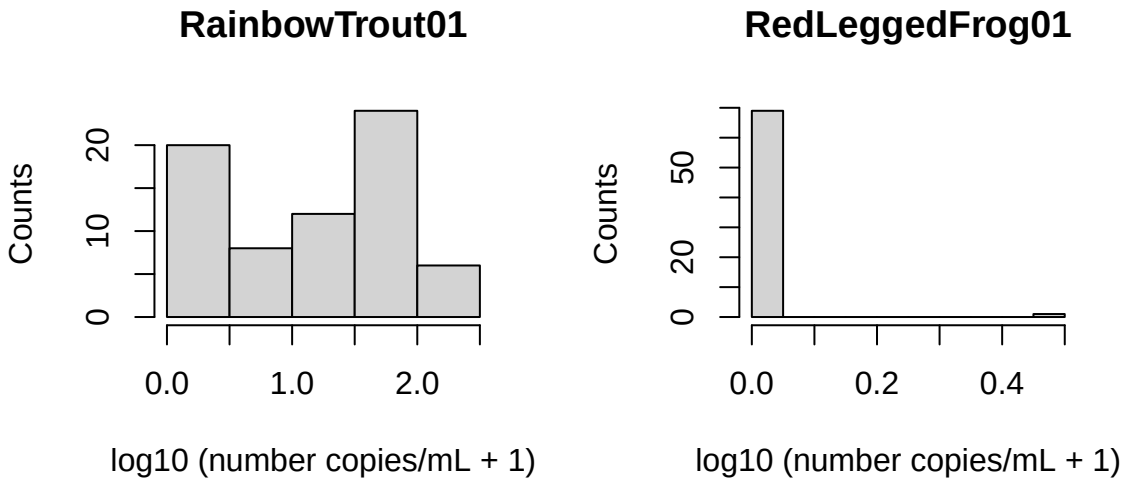

Table 1. Average Number of Copies for Each Assay

This table provides data on the average number of copies that were detected for each sample for each assay.

RunID = an internal identification number. Can be used by the client to look up which calibration curve applies to each sample

SampleID = Jonah Sample ID provided by client.

Subsequent columns represent the number of copies per filtered 100 mL of water (if data provided) for each assay averaged across internal replicates. The column name denotes the assay name.

	SampleID	RainbowTrout01	RedLeggedFrog01
1	05M4KYV8	305	0
2	095ITR0P	307	0
3	0BSVFNMK	0	0
4	0F4DHR4R	0	0
5	0VCTK0PF	69	0
6	1NUFQEH0	53	0
7	1V7B7BMP	19	0
8	2R1A2CYM	0	0
9	39M0J44W	60	0
10	3HIXC2ZM	5	0
11	3LE1AARQ	123	0
12	3NCVYI0U	0	0
13	42FZZWZS	12	0
14	4THH8PZW	34	0
15	5165X3XK	0	0
16	53PNZQ71	12	0
17	5KDNKS8Z	35	0
18	5PH5SX8Y	2	0
19	5SXS0780	81	0
20	5XL5EJ0L	0	0
21	7AHQ8ZMS	109	0
22	7F1I0RAN	14	0
23	8MGMHAG6	27	0
24	A0L88KVI	0	0
25	AX4ZF68W	46	0
26	BDZ55WDJ	0	0
27	CB41TIFF	0	0
28	CJV66V15	71	0
29	CLPBQAG9	6	0
30	CQGXT9OK	0	0
31	CTER2MGM	62	0
32	D2D5DJ0O	0	0
33	D8I30OWL	10	0
34	EJ7DDIOZ	71	0
35	EWP6XTYI	28	0
36	FCL19JPY	73	0
37	GDNQL0ZO	78	0
38	GE8TENKI	0	0
39	HKAB1QU1	3	0
40	HNCZSV1E	29	0
41	HO4FDT7E	62	0
42	HRFFW57K	20	0

43	HTV6CKOV	0	0
44	I8B5D89O	63	0
45	IYU7HPOR	57	0
46	J1KSHEIH	9	0
47	JR1Y8WNQ	84	0
48	K3TFQVOR	0	0
49	KKSXDM2X	47	0
50	LOAUE7UM	32	0
51	L4T7R48H	38	0
52	LE5NTJ46	0	0
53	M2SX85PS	95	0
54	M5DCMVXT	177	0
55	NPJSTL2H	20	0
56	NRFV9YVO	35	0
57	OGMN6FAF	25	0
58	PQOECPRW	46	2
59	QGMMFUO4	0	0
60	T1CSCIGM	6	0
61	TKOPYDA1	9	0
62	TPMCDA86	0	0
63	UPO8OOK5	0	0
64	UVPK5GMO	224	0
65	VL3DNA37	34	0
66	VNJS4EUS	29	0
67	WQWEPN8B	3	0
68	WROVHYHR	42	0
69	XV1IQ261	8	0
70	YBOGFANO	0	0

Table 2. Percent of Replicates Above Detection Limit for Each Assay

This table provides data on what percentage of the replicates that were run were above the detection limit. The detection limit is as high as the lowest positive on the calibration curve, but can be up to an order of magnitude lower. For example, a calibration curve might generate a positive at 100 copies and no positive for 10 copies, but the actual detection limit would be 11 copies. See Table 3 for the range of number of copies for each assay.

RunID = an internal identification number. Can be used by the client to look up which calibration curve applies to each sample

SampleID = Jonah Sample ID provided by client.

Subsequent columns show the percent of all replicates that were above detection limit for each assay. The column name denotes the assay name.

	RunID	SampleID	RainbowTrout01	RedLeggedFrog01
1	JVQ0158	05M4KYV8	100.0	0.0
2	JVQ0158	095ITR0P	100.0	0.0
3	JVQ0158	0BSVFNMK	0.0	0.0
4	JVQ0158	0F4DHR4R	0.0	0.0
5	JVQ0158	0VCTK0PF	100.0	0.0
6	JVQ0158	1NUFQEH0	100.0	0.0
7	JVQ0158	1V7B7BMP	100.0	0.0
8	JVQ0158	2R1A2CYM	33.3	0.0
9	JVQ0158	39M0J44W	100.0	0.0
10	JVQ0158	3HIXC2ZM	33.3	0.0
11	JVQ0158	3LE1AARQ	100.0	0.0
12	JVQ0158	3NCVYIOU	0.0	0.0
13	JVQ0158	42FZZWZS	66.7	0.0
14	JVQ0158	4THH8PZW	33.3	0.0
15	JVQ0158	5165X3XK	0.0	0.0
16	JVQ0158	53PNZQ71	100.0	0.0
17	JVQ0158	5KDNKS8Z	66.7	0.0
18	JVQ0158	5PH5SX8Y	66.7	0.0
19	JVQ0158	5SXS0780	100.0	0.0
20	JVQ0158	5XL5EJ0L	0.0	0.0
21	JVQ0158	7AHQ8ZMS	100.0	0.0
22	JVQ0158	7F1OORAN	100.0	0.0
23	JVQ0158	8MGMHAG6	100.0	0.0
24	JVQ0158	A0L88KVI	0.0	0.0
25	JVQ0158	AX4ZF68W	66.7	0.0
26	JVQ0158	BDZ55WDJ	0.0	0.0
27	JVQ0158	CB41TIFF	0.0	0.0
28	JVQ0158	CJV66V15	66.7	0.0
29	JVQ0158	CLPBQAG9	33.3	0.0
30	JVQ0158	CQGXT9OK	0.0	0.0
31	JVQ0158	CTER2MGM	100.0	0.0
32	JVQ0158	D2D5DJ0O	0.0	0.0
33	JVQ0158	D8I30OWL	66.7	0.0
34	JVQ0158	EJ7DDI0Z	100.0	0.0
35	JVQ0158	EWP6XTYI	66.7	0.0
36	JVQ0158	FCL19JPY	100.0	0.0
37	JVQ0158	GDNQL0ZO	100.0	0.0

38	JVQ0158	GE8TENKI	0.0	0.0
39	JVQ0158	HKAB1QU1	66.7	0.0
40	JVQ0158	HNCZSV1E	66.7	0.0
41	JVQ0158	HO4FDT7E	66.7	0.0
42	JVQ0158	HRFFW57K	66.7	0.0
43	JVQ0158	HTV6CKOV	0.0	0.0
44	JVQ0158	I8B5D89O	100.0	0.0
45	JVQ0158	IYU7HPOR	100.0	0.0
46	JVQ0158	J1KSHEIH	100.0	0.0
47	JVQ0158	JR1Y8WNQ	100.0	0.0
48	JVQ0158	K3TFQVOR	0.0	0.0
49	JVQ0158	KKSXDM2X	100.0	0.0
50	JVQ0158	LOAUE7UM	100.0	0.0
51	JVQ0158	L4T7R48H	100.0	0.0
52	JVQ0158	LE5NTJ46	0.0	0.0
53	JVQ0158	M2SX85PS	100.0	0.0
54	JVQ0158	M5DCMVXT	100.0	0.0
55	JVQ0158	NPJSTL2H	100.0	0.0
56	JVQ0158	NRFV9YVO	100.0	0.0
57	JVQ0158	OGMN6FAF	100.0	0.0
58	JVQ0158	PQOECPRW	100.0	0.0
59	JVQ0158	QGMMFUO4	0.0	0.0
60	JVQ0158	T1CSCIGM	66.7	0.0
61	JVQ0158	TKOPYDA1	100.0	0.0
62	JVQ0158	TPMCDA86	0.0	0.0
63	JVQ0158	UPO8OOK5	0.0	0.0
64	JVQ0158	UVPK5GMO	100.0	0.0
65	JVQ0158	VL3DNA37	100.0	0.0
66	JVQ0158	VNJS4EUS	100.0	0.0
67	JVQ0158	WQWEPN8B	100.0	0.0
68	JVQ0158	WROVHYHR	100.0	0.0
69	JVQ0158	XV1IQ261	66.7	0.0
70	JVQ0158	YBOGFANO	0.0	0.0
71	JVQ0159	O5M4KYV8	0.0	0.0
72	JVQ0159	O95ITROP	0.0	0.0
73	JVQ0159	OBSVFNMK	0.0	0.0
74	JVQ0159	OF4DHR4R	0.0	0.0
75	JVQ0159	1V7B7BMP	0.0	0.0
76	JVQ0159	3HIXC2ZM	0.0	0.0
77	JVQ0159	42FZZWZS	0.0	0.0
78	JVQ0159	53PNZQ71	0.0	0.0
79	JVQ0159	5PH5SX8Y	0.0	0.0
80	JVQ0159	5XL5EJOL	0.0	0.0
81	JVQ0159	7AHQ8ZMS	0.0	0.0
82	JVQ0159	7F1OORAN	0.0	0.0
83	JVQ0159	8MGMHAG6	0.0	0.0
84	JVQ0159	AOL88KVI	0.0	0.0
85	JVQ0159	AX4ZF68W	0.0	0.0
86	JVQ0159	BDZ55WDJ	0.0	0.0
87	JVQ0159	CB41TIFF	0.0	0.0
88	JVQ0159	CJV66V15	0.0	0.0
89	JVQ0159	CLPBQAG9	0.0	0.0
90	JVQ0159	CQGXT9OK	0.0	0.0
91	JVQ0159	D8I30OWL	0.0	0.0

92	JVQ0159	FCL19JPY	0.0	0.0
93	JVQ0159	GDNQL0ZO	0.0	0.0
94	JVQ0159	GE8TENKI	0.0	0.0
95	JVQ0159	HKAB1QU1	0.0	0.0
96	JVQ0159	HTV6CKOV	0.0	0.0
97	JVQ0159	J1KSHEIH	0.0	0.0
98	JVQ0159	K3TFQV0R	0.0	0.0
99	JVQ0159	KKSXDM2X	0.0	0.0
100	JVQ0159	L4T7R48H	0.0	0.0
101	JVQ0159	LE5NTJ46	0.0	0.0
102	JVQ0159	M2SX85PS	0.0	0.0
103	JVQ0159	M5DCMVXT	0.0	0.0
104	JVQ0159	NPJSTL2H	0.0	0.0
105	JVQ0159	PQ0ECPRW	0.0	33.3
106	JVQ0159	QGMMFU04	0.0	0.0
107	JVQ0159	TKOPYDA1	0.0	0.0
108	JVQ0159	TPMCDA86	0.0	0.0
109	JVQ0159	UP080OK5	0.0	0.0
110	JVQ0159	UVPK5GMO	0.0	0.0
111	JVQ0159	VL3DNA37	0.0	0.0
112	JVQ0159	WQWEPN8B	0.0	0.0
113	JVQ0159	XV1IQ261	0.0	0.0
114	JVQ0159	YBOGFAN0	0.0	0.0
115	JVQ0161	O5M4KYV8	0.0	0.0
116	JVQ0161	O95ITR0P	0.0	0.0
117	JVQ0161	OBSVFNMK	0.0	0.0
118	JVQ0161	OF4DHR4R	0.0	0.0
119	JVQ0161	OVCTKOPF	0.0	0.0
120	JVQ0161	1NUFQEHO	0.0	0.0
121	JVQ0161	1V7B7BMP	0.0	0.0
122	JVQ0161	2R1A2CYM	0.0	0.0
123	JVQ0161	39M0J44W	0.0	0.0
124	JVQ0161	3HIXC2ZM	0.0	0.0
125	JVQ0161	3LE1AARQ	0.0	0.0
126	JVQ0161	3NCVYIOU	0.0	0.0
127	JVQ0161	42FZZWZS	0.0	0.0
128	JVQ0161	4THH8PZW	0.0	0.0
129	JVQ0161	5165X3XK	0.0	0.0
130	JVQ0161	53PNZQ71	0.0	0.0
131	JVQ0161	5KDNKS8Z	0.0	0.0
132	JVQ0161	5PH5SX8Y	0.0	0.0
133	JVQ0161	5SXS0780	0.0	0.0
134	JVQ0161	5XL5EJ0L	0.0	0.0
135	JVQ0161	7AHQ8ZMS	0.0	0.0
136	JVQ0161	7F1OORAN	0.0	0.0
137	JVQ0161	8MGMHAG6	0.0	0.0
138	JVQ0161	A0L88KVI	0.0	0.0
139	JVQ0161	AX4ZF68W	0.0	0.0
140	JVQ0161	BDZ55WDJ	0.0	0.0
141	JVQ0161	CB41TIFF	0.0	0.0
142	JVQ0161	CJV66V15	0.0	0.0
143	JVQ0161	CLPBQAG9	0.0	0.0
144	JVQ0161	CQGXT9OK	0.0	0.0
145	JVQ0161	CTER2MGM	0.0	0.0

146	JVQ0161	D2D5DJ0O	0.0	0.0
147	JVQ0161	D8I30OWL	0.0	0.0
148	JVQ0161	EJ7DDIOZ	0.0	0.0
149	JVQ0161	EWP6XTYI	0.0	0.0
150	JVQ0161	FCL19JPY	0.0	0.0
151	JVQ0161	GDNQL0ZO	0.0	0.0
152	JVQ0161	GE8TENKI	0.0	0.0
153	JVQ0161	HKAB1QU1	0.0	0.0
154	JVQ0161	HNCZSV1E	0.0	0.0
155	JVQ0161	HO4FDT7E	0.0	0.0
156	JVQ0161	HRFFW57K	0.0	0.0
157	JVQ0161	HTV6CKOV	0.0	0.0
158	JVQ0161	I8B5D89O	0.0	0.0
159	JVQ0161	IYU7HPOR	0.0	0.0
160	JVQ0161	J1KSHEIH	0.0	0.0
161	JVQ0161	JR1Y8WNQ	0.0	0.0
162	JVQ0161	K3TFQVOR	0.0	0.0
163	JVQ0161	KKSXDM2X	0.0	0.0
164	JVQ0161	LOAUE7UM	0.0	0.0
165	JVQ0161	L4T7R48H	0.0	0.0
166	JVQ0161	LE5NTJ46	0.0	0.0
167	JVQ0161	M2SX85PS	0.0	0.0
168	JVQ0161	M5DCMVXT	0.0	0.0
169	JVQ0161	NPJSTL2H	0.0	0.0
170	JVQ0161	NRFV9YVO	0.0	0.0
171	JVQ0161	OGMN6FAF	0.0	0.0
172	JVQ0161	PQOECPRW	0.0	0.0
173	JVQ0161	QGMMFUO4	0.0	0.0
174	JVQ0161	T1CSCIGM	0.0	0.0
175	JVQ0161	TKOPYDA1	0.0	0.0
176	JVQ0161	TPMCDA86	0.0	0.0
177	JVQ0161	UPO80OK5	0.0	0.0
178	JVQ0161	UVPK5GMO	0.0	0.0
179	JVQ0161	VL3DNA37	0.0	0.0
180	JVQ0161	VNJS4EUS	0.0	0.0
181	JVQ0161	WQWEPN8B	0.0	0.0
182	JVQ0161	WROVHYHR	0.0	0.0
183	JVQ0161	XV1IQ261	0.0	0.0
184	JVQ0161	YBOGFANO	0.0	0.0

Table 3. All data (copies/100ml)

	ClientID	RunID	Assay	SampleID	Rep1	Rep2	Rep3
1	1391	JVQ0158	RainbowTrout01	05M4KYV8	322	342	252
2	1391	JVQ0158	RainbowTrout01	095ITROP	389	226	307
3	1391	JVQ0158	RainbowTrout01	0BSVFNMK	0	0	0
4	1391	JVQ0158	RainbowTrout01	0F4DHR4R	0	0	0
5	1391	JVQ0158	RainbowTrout01	0VCTK0PF	56	76	75
6	1391	JVQ0158	RainbowTrout01	1NUFQEHO	75	69	14
7	1391	JVQ0158	RainbowTrout01	1V7B7BMP	15	27	14
8	1391	JVQ0158	RainbowTrout01	2R1A2CYM	0	0	1
9	1391	JVQ0158	RainbowTrout01	39M0J44W	31	84	65
10	1391	JVQ0158	RainbowTrout01	3HIXC2ZM	0	16	0
11	1391	JVQ0158	RainbowTrout01	3LE1AARQ	136	85	147
12	1391	JVQ0158	RainbowTrout01	3NCVYIOU	0	0	0
13	1391	JVQ0158	RainbowTrout01	42FZZWZS	22	0	13
14	1391	JVQ0158	RainbowTrout01	4THH8PZW	0	0	101
15	1391	JVQ0158	RainbowTrout01	5165X3XK	0	0	0
16	1391	JVQ0158	RainbowTrout01	53PNZQ71	6	22	7
17	1391	JVQ0158	RainbowTrout01	5KDNKS8Z	80	25	0
18	1391	JVQ0158	RainbowTrout01	5PH5SX8Y	4	0	2
19	1391	JVQ0158	RainbowTrout01	5SXS0780	112	31	101
20	1391	JVQ0158	RainbowTrout01	5XL5EJ0L	0	0	0
21	1391	JVQ0158	RainbowTrout01	7AHQ8ZMS	94	121	113
22	1391	JVQ0158	RainbowTrout01	7F1OORAN	7	28	8
23	1391	JVQ0158	RainbowTrout01	8MGMHAG6	13	29	38
24	1391	JVQ0158	RainbowTrout01	A0L88KVI	0	0	0
25	1391	JVQ0158	RainbowTrout01	AX4ZF68W	41	0	97
26	1391	JVQ0158	RainbowTrout01	BDZ55WDJ	0	0	0
27	1391	JVQ0158	RainbowTrout01	CB41TIFF	0	0	0
28	1391	JVQ0158	RainbowTrout01	CJV66V15	105	0	107
29	1391	JVQ0158	RainbowTrout01	CLPBQAG9	0	17	0
30	1391	JVQ0158	RainbowTrout01	CQGXT9OK	0	0	0
31	1391	JVQ0158	RainbowTrout01	CTER2MGM	101	58	27
32	1391	JVQ0158	RainbowTrout01	D2D5DJ0O	0	0	0
33	1391	JVQ0158	RainbowTrout01	D8I30OWL	0	12	19
34	1391	JVQ0158	RainbowTrout01	EJ7DDIOZ	107	54	53
35	1391	JVQ0158	RainbowTrout01	EWP6XTYI	0	53	30
36	1391	JVQ0158	RainbowTrout01	FCL19JPY	80	105	35
37	1391	JVQ0158	RainbowTrout01	GDNQL0ZO	158	37	38
38	1391	JVQ0158	RainbowTrout01	GE8TENKI	0	0	0
39	1391	JVQ0158	RainbowTrout01	HKAB1QU1	5	5	0
40	1391	JVQ0158	RainbowTrout01	HNCZSV1E	57	31	0
41	1391	JVQ0158	RainbowTrout01	HO4FDT7E	56	130	0
42	1391	JVQ0158	RainbowTrout01	HRFFW57K	17	44	0
43	1391	JVQ0158	RainbowTrout01	HTV6CKOV	0	0	0
44	1391	JVQ0158	RainbowTrout01	I8B5D89O	10	94	86
45	1391	JVQ0158	RainbowTrout01	IYU7HPOR	13	71	86
46	1391	JVQ0158	RainbowTrout01	J1KSHEIH	9	14	3
47	1391	JVQ0158	RainbowTrout01	JR1Y8WNQ	101	88	62
48	1391	JVQ0158	RainbowTrout01	K3TFQVOR	0	0	0
49	1391	JVQ0158	RainbowTrout01	KKSXDM2X	44	61	35
50	1391	JVQ0158	RainbowTrout01	LOAUE7UM	54	17	25
51	1391	JVQ0158	RainbowTrout01	L4T7R48H	37	42	34

52	1391	JVQ0158	RainbowTrout01	LE5NTJ46	0	0	0
53	1391	JVQ0158	RainbowTrout01	M2SX85PS	108	73	104
54	1391	JVQ0158	RainbowTrout01	M5DCMVXT	110	206	214
55	1391	JVQ0158	RainbowTrout01	NPJSTL2H	7	35	18
56	1391	JVQ0158	RainbowTrout01	NRFV9YVO	36	31	38
57	1391	JVQ0158	RainbowTrout01	OGMN6FAF	48	17	9
58	1391	JVQ0158	RainbowTrout01	PQ0ECPRW	67	15	57
59	1391	JVQ0158	RainbowTrout01	QGMMFU04	0	0	0
60	1391	JVQ0158	RainbowTrout01	T1CSCIGM	0	7	10
61	1391	JVQ0158	RainbowTrout01	TKOPYDA1	4	16	8
62	1391	JVQ0158	RainbowTrout01	TPMCDA86	0	0	0
63	1391	JVQ0158	RainbowTrout01	UP080OK5	0	0	0
64	1391	JVQ0158	RainbowTrout01	UVPK5GMO	223	181	268
65	1391	JVQ0158	RainbowTrout01	VL3DNA37	39	49	13
66	1391	JVQ0158	RainbowTrout01	VNJS4EUS	11	11	66
67	1391	JVQ0158	RainbowTrout01	WQWEPN8B	3	3	4
68	1391	JVQ0158	RainbowTrout01	WR0VHYHR	39	68	18
69	1391	JVQ0158	RainbowTrout01	XV1IQ261	7	16	0
70	1391	JVQ0158	RainbowTrout01	YBOGFAN0	0	0	0
71	1391	JVQ0159	RedLeggedFrog01	05M4KYV8	0	0	0
72	1391	JVQ0159	RedLeggedFrog01	095ITR0P	0	0	0
73	1391	JVQ0159	RedLeggedFrog01	0BSVFNMK	0	0	0
74	1391	JVQ0159	RedLeggedFrog01	0F4DHR4R	0	0	0
75	1391	JVQ0159	RedLeggedFrog01	1V7B7BMP	0	0	0
76	1391	JVQ0159	RedLeggedFrog01	3HIXC2ZM	0	0	0
77	1391	JVQ0159	RedLeggedFrog01	42FZZWZS	0	0	0
78	1391	JVQ0159	RedLeggedFrog01	53PNZQ71	0	0	0
79	1391	JVQ0159	RedLeggedFrog01	5PH5SX8Y	0	0	0
80	1391	JVQ0159	RedLeggedFrog01	5XL5EJ0L	0	0	0
81	1391	JVQ0159	RedLeggedFrog01	7AHQ8ZMS	0	0	0
82	1391	JVQ0159	RedLeggedFrog01	7F1OORAN	0	0	0
83	1391	JVQ0159	RedLeggedFrog01	8MGMHAG6	0	0	0
84	1391	JVQ0159	RedLeggedFrog01	A0L88KVI	0	0	0
85	1391	JVQ0159	RedLeggedFrog01	AX4ZF68W	0	0	0
86	1391	JVQ0159	RedLeggedFrog01	BDZ55WDJ	0	0	0
87	1391	JVQ0159	RedLeggedFrog01	CB41TIFF	0	0	0
88	1391	JVQ0159	RedLeggedFrog01	CJV66V15	0	0	0
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91	1391	JVQ0159	RedLeggedFrog01	D8I30OWL	0	0	0
92	1391	JVQ0159	RedLeggedFrog01	FCL19JPY	0	0	0
93	1391	JVQ0159	RedLeggedFrog01	GDNQL0ZO	0	0	0
94	1391	JVQ0159	RedLeggedFrog01	GE8TENKI	0	0	0
95	1391	JVQ0159	RedLeggedFrog01	HKAB1QU1	0	0	0
96	1391	JVQ0159	RedLeggedFrog01	HTV6CKOV	0	0	0
97	1391	JVQ0159	RedLeggedFrog01	J1KSHEIH	0	0	0
98	1391	JVQ0159	RedLeggedFrog01	K3TFQV0R	0	0	0
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108	1391	JVQ0159	RedLeggedFrog01	TPMCDA86	0	0	0
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114	1391	JVQ0159	RedLeggedFrog01	YBOGFAN0	0	0	0
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119	1391	JVQ0161	CohoSalmon01	0VCTK0PF	0	0	0
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132	1391	JVQ0161	CohoSalmon01	5PH5SX8Y	0	0	0
133	1391	JVQ0161	CohoSalmon01	5SXS0780	0	0	0
134	1391	JVQ0161	CohoSalmon01	5XL5EJ0L	0	0	0
135	1391	JVQ0161	CohoSalmon01	7AHQ8ZMS	0	0	0
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145	1391	JVQ0161	CohoSalmon01	CTER2MGM	0	0	0
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148	1391	JVQ0161	CohoSalmon01	EJ7DDIOZ	0	0	0
149	1391	JVQ0161	CohoSalmon01	EWP6XTYI	0	0	0
150	1391	JVQ0161	CohoSalmon01	FCL19JPY	0	0	0
151	1391	JVQ0161	CohoSalmon01	GDNQL0ZO	0	0	0
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153	1391	JVQ0161	CohoSalmon01	HKAB1QU1	0	0	0
154	1391	JVQ0161	CohoSalmon01	HNCZSV1E	0	0	0
155	1391	JVQ0161	CohoSalmon01	HO4FDT7E	0	0	0
156	1391	JVQ0161	CohoSalmon01	HRFFW57K	0	0	0
157	1391	JVQ0161	CohoSalmon01	HTV6CKOV	0	0	0
158	1391	JVQ0161	CohoSalmon01	I8B5D89O	0	0	0
159	1391	JVQ0161	CohoSalmon01	IYU7HP0R	0	0	0

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162	1391	JVQ0161	CohoSalmon01	K3TFQV0R	0	0	0
163	1391	JVQ0161	CohoSalmon01	KKSXDM2X	0	0	0
164	1391	JVQ0161	CohoSalmon01	L0AUE7UM	0	0	0
165	1391	JVQ0161	CohoSalmon01	L4T7R48H	0	0	0
166	1391	JVQ0161	CohoSalmon01	LE5NTJ46	0	0	0
167	1391	JVQ0161	CohoSalmon01	M2SX85PS	0	0	0
168	1391	JVQ0161	CohoSalmon01	M5DCMVXT	0	0	0
169	1391	JVQ0161	CohoSalmon01	NPJSTL2H	0	0	0
170	1391	JVQ0161	CohoSalmon01	NRFV9YVO	0	0	0
171	1391	JVQ0161	CohoSalmon01	OGMN6FAF	0	0	0
172	1391	JVQ0161	CohoSalmon01	PQ0ECPRW	0	0	0
173	1391	JVQ0161	CohoSalmon01	QGMMFU04	0	0	0
174	1391	JVQ0161	CohoSalmon01	T1CSCIGM	0	0	0
175	1391	JVQ0161	CohoSalmon01	TKOPYDA1	0	0	0
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178	1391	JVQ0161	CohoSalmon01	UVPK5GMO	0	0	0
179	1391	JVQ0161	CohoSalmon01	VL3DNA37	0	0	0
180	1391	JVQ0161	CohoSalmon01	VNJS4EUS	0	0	0
181	1391	JVQ0161	CohoSalmon01	WQWEPN8B	0	0	0
182	1391	JVQ0161	CohoSalmon01	WR0VHYHR	0	0	0
183	1391	JVQ0161	CohoSalmon01	XV1IQ261	0	0	0
184	1391	JVQ0161	CohoSalmon01	YBOGFAN0	0	0	0

Calibration curves

Each assay in each run is associated with a calibration curve based typically on a series of 7 10-fold dilutions of a known standard.

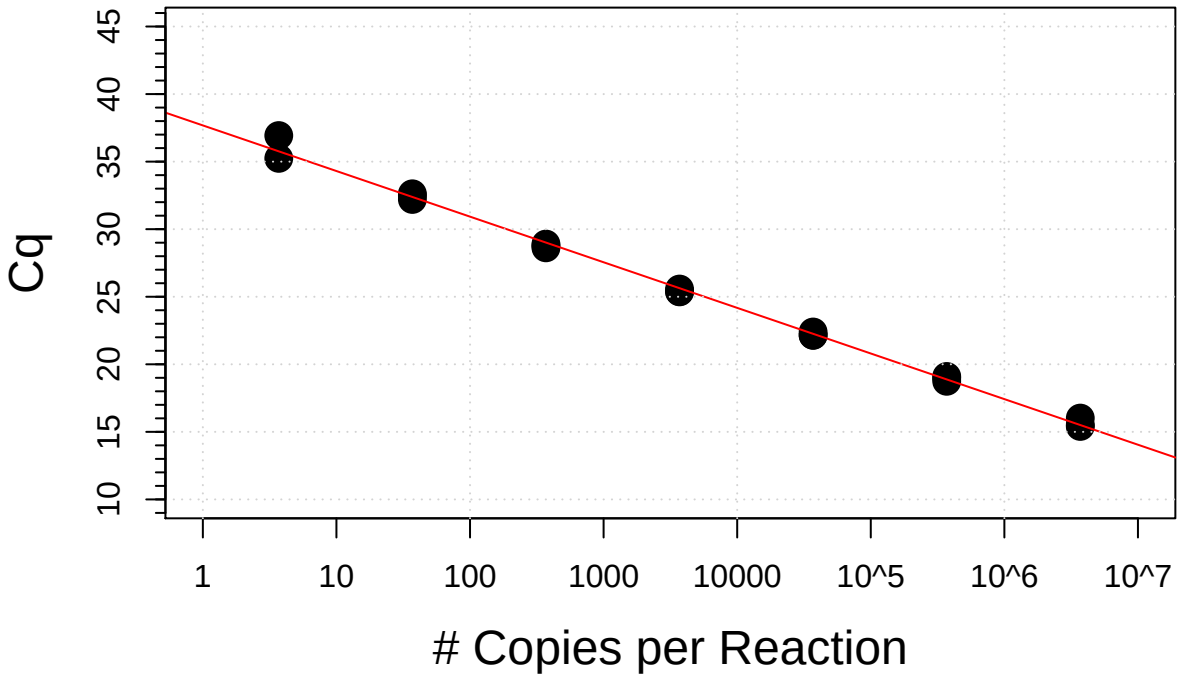
The calibration curve shown is for the number of cycles vs. the log10 of the number of copies in the standard. A linear regression is applied to this relationship and the r2 intercept and slope extracted for further analyses.

RunID = an internal identification number. Can be used by the client to match the calibration curve applies with each sample for an assay

Assay = the name of the assay for the calibration curve in the particular run

r2 = the coefficient of determination, or goodness of fit for the linear relationship. Typically, r2 should be > 0.98.

efficiency = how close to a doubling of product with each PCR cycle. For a 10-fold dilution, 100% efficiency is for ~3.3 cycles per 10-fold dilution. A range of values is acceptable here, but we try to keep efficiency between 85% - 110%.

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[1] "Assay = RedLeggedFrog01"
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[1] "efficiency = 98%"
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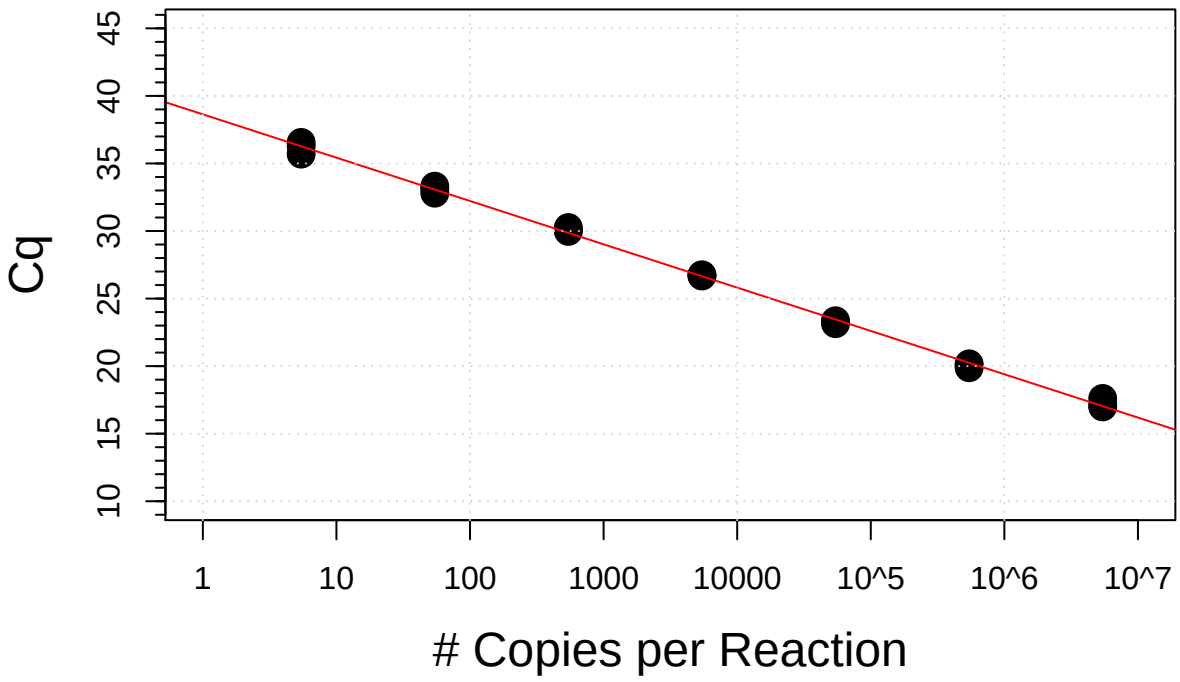


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[1] "Assay = CohoSalmon01"
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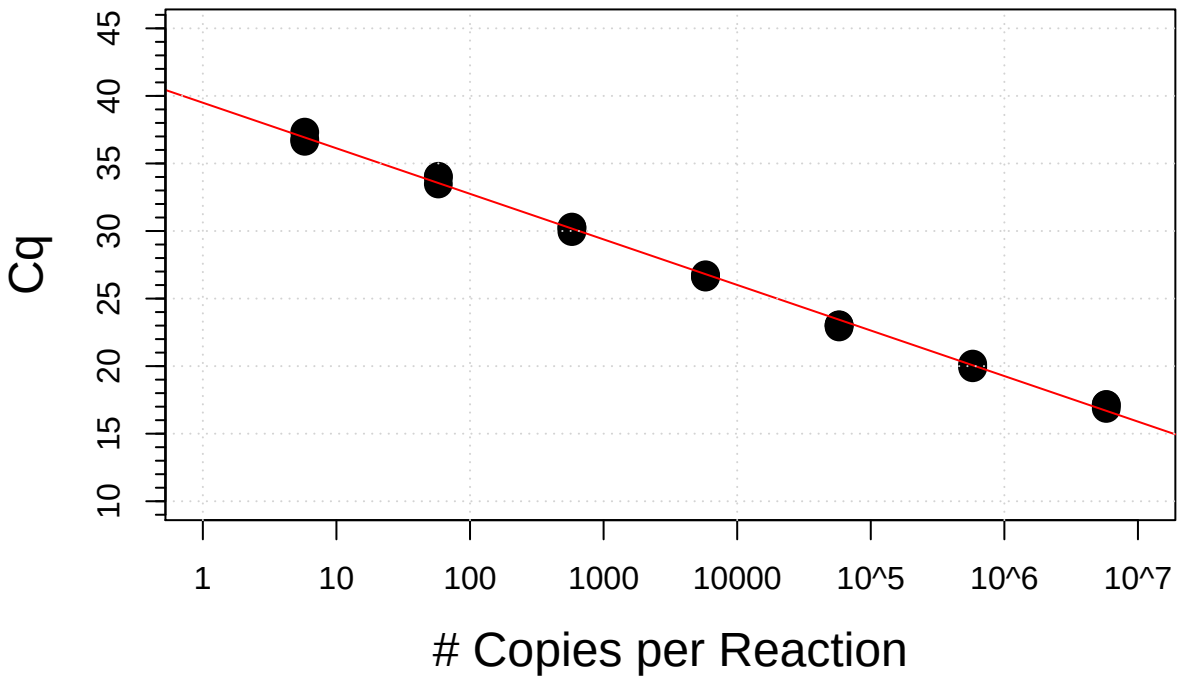


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[1] "Calibration Curve"
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[1] "Assay = RainbowTrout01"
[1] "r2 = 0.998"
[1] "efficiency = 98%"
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#Assays

```
[1] "Assay Name = RainbowTrout01"
[1] "Assay Description = Mitochondrial gene NADH dehydrogenase subunit 2"
[1] "Assay Thermal Profile = 1 cycle at 95 deg C for 5 minutes. 50 cycles at 95 deg"
[2] "C for 15 seconds. 60 deg C for 1 minute"
[1] "Assay Reference = Wilcox TM, Carim KJ, McKelvey KS, Young MK, Schwartz MK"
[2] "(2015) The Dual Challenges of Generality and Specificity When Developing"
[3] "Environmental DNA Markers for Species and Subspecies of Oncorhynchus. PLoS ONE"
[4] "10(11): e0142008. doi:10.1371/journal.pone.0142008"

[1] "Assay Name = RedLeggedFrog01"
[1] "Assay Description = Mitochondrial Cytochrome b"
[1] "Assay Thermal Profile = 1 cycle at 95 deg C for 5 minutes. 50 cycles at 95 deg"
[2] "C for 15 seconds. 60 deg C for 1 minute"
[1] "Assay Reference = Jonah Ventures"
```

Attachment 5

CRAMER FISH SCIENCES
An Employee Owned Consulting Company

GENIDAQS℠

Molecular Diagnostic Report

Report Status: Final
Report No: 3/3/2021
Contract No: GKLSAN-2021

Client

Name: Sarah O'Neal

Address: Aqua Dulce Freshwater Consulting

Contact: Sarah O'Neal

Service Description

Sample type:
Syring Filter
Analysis type:
Quantitative PCR
Received date:
1/25/2020
Reporting date:
3/3/2021

Location Description

Gualala River

Molecular Diagnostics

Samples	Laboratory Tests			Supplemental Sample Information
Identifier	Coho	RBT	CRLF	Comments
1_0BDDEWOC	ND	+		
2_1H0BJ7ME	ND	+		
3_1WGLD9M5	ND	+	ND	
4_2TK5P6JC	ND	ND	ND	
5_2UUNLQXE	+	+		1/6 technical PCR replicates positive for coho
6_3AIWQ0V7	ND	+		
7_3Q12YK1D	ND	+	ND	
8_47SWK8U7	ND	+		
9_64VV6LYI	ND	+		
10_7CRW044N	ND	+		
11_8Y71F1LZ	ND	+		
12_9T62H5S6	ND	+	ND	
13_AQL0EATG	ND	ND	ND	
14_C63NAY22	ND	+		
15_D5SDGT4B	ND	ND	ND	
16_DP8QDL9O	ND	+		

Samples	Laboratory Tests			Supplemental Sample Information
Identifier	Coho	RBT	CRLF	Comments
17_E2JCZW5K	ND	ND	ND	
18_E45EVFHV	ND	ND	ND	
19_EW39X0SK	+	+	ND	1/6 technical PCR replicates positive for coho
20_FUUPIWXL	ND	+		
21_G55VU339	ND	ND	ND	
22_JHTXY52F	ND	+	ND	
23_K2DHF8C3	ND	ND	ND	
24_KMTSBSXW	ND	ND	ND	
25_L3AQAVF1	ND	+	ND	
26_L45P6CA5	ND	+	ND	
27_NSBLUQLY	ND	+	ND	
28_OOXYG66M	ND	+	ND	
29_PC0CPSG2	ND	ND	ND	
30_PNYIECBT	ND	+		
31_QAZ9T3MY	ND	ND	ND	
32_QUK06CLQ	ND	ND	ND	
33_TCK7OOY3	ND	ND	ND	
34_U23WIPU3	ND	+	+	1/6 technical PCR replicates positive Red-legged Frog
35_UKA30BZY	ND	+	+	1/6 technical PCR replicates positive Red-legged Frog
36_UR0BCE3E	ND	+	ND	
37_VQKGDGNM	ND	+	ND	
38_WPYP33K4	ND	+	ND	
39_WRU3122	ND	+	ND	
40_X18WXS58	ND	+	ND	
41_YQ4Z3OW9	ND	+	ND	
42_YT0A2AQR	ND	+		
43_YT55RQ45	ND	ND	ND	

Molecular Diagnostics

RESULTS

Methods General

1. Field sample collections completed by Aqua Dulce Freshwater Consulting using syringe filters.
2. Total DNA was isolated from each eDNA sample (filter) following Goldberg et al. 2011.
3. DNA templates were interrogated for the presence of target species mitochondrial DNA (barcodes) using quantitative PCR.
4. 3 technical replicates (PCR) were completed from each filter DNA extraction.
5. An additional 3 technical replicates (PCR) were completed for each filter DNA extraction after treatment with Zymo OneStep PCR Inhibitor Removal Kit.
6. If any technical replicate tests positive for target DNA, then sample is considered (+), otherwise sample is non-detected (ND).
7. No template controls (NTC) are added to each plate to ensure plate to plate consistency.

Laboratory test (assay)

Coho Assay Publication: Duda, JJ, Hoy, Chase, DM et al. Environmental DNA is an effective tool to track recolonizing migratory fish following large-scale dam removal. Environmental DNA, 2021: 121–141. Geniday Lab manager Cheryl Dean 3/10/2020 16:12.

Code	Common Name	Species Name	Publication
Coho	Coho	Oncorhynchus kisutch	~~Gregg Schumer (August 2017 Cramer Fish Sciences - Genidaqs; unpublished)~~
CRLF	California Red legged Frog	Rana draytonii	Gregg Schumer (August 2017 Cramer Fish Sciences - Genidaqs; unpublished)
RBT	Rainbow trout/Steelhead	Oncorhynchus mykiss	Brandl, S., Schumer, G., Schreier, B.M., Condrad, J.L., May, B. Baerwald, M.R. (2015). Ten real-time PCR assays for detection of fish predation at the community level in the SF Estuary-Delta. Molecular Ecology. 15, 278-284. doi: 10.1111/1755-0998.12305

References

Goldberg CS, Pilliod DS, Arkle RS, Waits LP (2011) Molecular Detection of Vertebrates in Stream Water: A Demonstration Using Rocky Mountain Tailed Frogs and Idaho Giant Salamanders. PLoS ONE 6(7): e22746. doi:10.1371/journal.pone.0022746

CRAMER FISH SCIENCES
An Employee Owned Consulting Company

GENIDAQS℠
Aquatic Species Sampling Simplified

Molecular Diagnostic Report

Report Status: Final
Report No: 4/2/2021
Contract No: GKLSAN-2021

Client

Name: Sarah O'Neal
Address: Aqua Dulce Freshwater Consulting
Contact: Sarah O'Neal

Service Description

Sample type: Syring Filter
Analysis type: Quantitative PCR
Received date: 3/24/2021
Reporting date: 4/2/2021

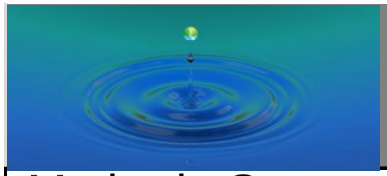
Location Description

Gualala River

Molecular Diagnostics

Samples	Laboratory Tests		Supplemental Sample Information
Identifier	Coho	CRLF	Comments
1_05M4KYV8	ND	ND	
2_095ITR0P	ND	ND	
3_0VCTK0PF	ND		
4_2R1A2CYM	ND		
5_39M0J44W	ND		
6_4THH8PZW	ND	ND	
7_7F1OORAN		ND	
8_CB41TIFF	ND	ND	
9_CLPBQAG9	ND	ND	
10_CQGXT9OK	ND	ND	
11_D8I300WL	ND	ND	
12_EJ7DDIOZ	ND		
13_EWP6XTYI	ND		
14_HRFFW57K	ND		
15_I8B5D89O	ND		
16_IYU7HP0R	ND		
17_L0AUE7UM	ND		
18_M5DCMVXT	ND	ND	

Molecular Diagnostics	Samples	Laboratory Tests		Supplemental Sample Information
	Identifier	Coho	CRLF	Comments
	19_NPJSTL2H	ND	ND	
	20_NRFV9YVO	ND		
	21_OGMN6FAF	ND		
	62_PQ0ECPRW	ND	ND	
	22_QGMMFU04	ND	ND	
	23_T1CSCIGM	ND		
	24_TKOPYDA1	ND	ND	
	25_UVPK5GMO	ND	ND	
	26_VNJS4EUS	ND		
	27_WQWEPN8B	ND	ND	
	28_WR0VHYHR	ND		
	29_XV1IQ261		ND	
	30_YBOGFAN0	ND	ND	



RESULTS

Methods General

1 DNA receive as extracted in liquid. Extraction techniqe and DNA quantity unknown.
2 DNA templates were interrogated for the presence of target species mitochondrial DNA (barcodes) using quantitative PCR.
3 6 technical replicates (PCR) were completed from each filter DNA extraction.
4 If any technical replicate tests positive for target DNA, then sample is considered (+), otherwise sample is non-detected (ND).
5 Positive control from voucher specimen (2ng/ul extracted DNA) are added to each place to document PCR success.
6 No template controls (NTC) are added to each plate to ensure plate to plate consistency.

Laboratory test (assay)

Code	Common Name	Species Name	Publication
Coho	Coho	Oncorhynchus kisutch	Duda, JJ, Hoy, MS, Chase, DM, et al. Environmental DNA is an effective tool to track recolonizing migratory fish following large-scale dam removal. Environmental DNA. 2021; 3: 121– 141. https://doi.org/10.1002/edn3.134
CRLF	California Red-legged Frog	Rana draytonii	Gregg Schumer (August 2017 Cramer Fish Sciences - Genidaqs; unpublished)

Molecular Diagnostic Report

Report Status: Final
Report No: 4/6/2021
Contract No: GKLSAN-2021

Client

Name: Sarah O'Neal
Address: Aqua Dulce Freshwater Consulting
Contact: Sarah O'Neal

Service Description

Sample type:
Syring Filter
Analysis type:
Quantitative PCR
Received date:
3/24/2021
Reporting date:
4/6/2021

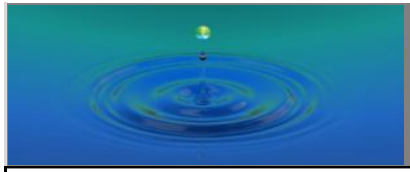
Location Description

Gualala River

Molecular Diagnostics

Samples	Laboratory Tests	Supplemental Sample Information
Identifier	RBT	Comments
35_3LE1AARQ	+	
40_5SXS0780	+	
41_5XL5EJ0L	ND	
42_7AHQ8ZMS	+	
48_FCL19JPY	+	
49_GDNQL0Z0	+	
61_M2SX85PS	+	

Attachment 5 - Genidaqs results, 2 of 7
Case 3:20-cv-06453-JD Document 61-5 Filed 05/20/21 Page 138 of 152



RESULTS

Methods General

1 DNA receive as extracted in liquid. Extraction techniqe and DNA quantity unknown.
2 DNA templates were interrogated for the presence of target species mitochondrial DNA (barcodes) using quantitative PCR.
3 3 technical replicates (PCR) were completed from each filter DNA extraction.
4 If any technical replicate tests positive for target DNA, then sample is considered (+), otherwise sample is non-detected (ND).
5 Positive control from voucher specimen (2ng/ul extracted DNA) are added to each place to document PCR success.
6 No template controls (NTC) are added to each plate to ensure plate to plate consistency.

Laboratory test (assay)

Code	Common Name	Species Name	Publication
RBT	Rainbow trout/Steelhead	Oncorhynchus mykiss	Brandl, S., Schumer, G., Schreier, B.M., Condrad, J.L., May, B. Baerwald, M.R. (2015). Ten real-time PCR assays for detection of fish predation at the community level in the SF Estuary-Delta. Molecular Ecology. 15, 278-284. doi: 10.1111/1755-0998.12305

Attachment 5 - Genidaq results.pdf - 7 of 7
Case 3:20-cv-06453-JD Document 61-5 Filed 05/20/21 Page 139 of 152

Attachment 6

CURRICULUM VITAE
for
SARAH LOUISE O'NEAL

sarahlouiseoneal@gmail.com
127 17TH ST SW, OLYMPIA, WA 98501
(360) 918-4352

EDUCATION

June, 1999, University of Washington, Seattle, WA
B.S. Ecology, Evolution and Conservation Biology GPA 3.91, Cumulative GPA 3.89

May, 2008, Flathead Lake Biological Station, University of Montana, Missoula and Polson, MT
Advisor: Dr. Jack A. Stanford
M.S. Limnology GPA 3.86

In progress, University of Washington, Seattle, WA
Advisor: Dr. Daniel Schindler
PhD Candidate School of Aquatic and Fishery Sciences Current GPA: 3.66

GRANTS/AWARDS

- Graduated *magna cum laude*
- Annual Dean's List, four consecutive years
- University of Washington Undergraduate Scholar Award, three consecutive years
- Mary Gates Undergraduate Research Training Grant
- Phi Beta Kappa
- Charles Levitan Scholarship
- Jessie M. Bierman Scholarship
- EPA Science to Achieve Results (STAR) research fellowship
- American Fisheries Society Cultural Diversity Travel Award
- National Fish Habitat Partnership grant recipient

PROFESSIONAL AFFILIATIONS

American Fisheries Society, local and national chapters member
Association for the Sciences of Limnology and Oceanography
Ecological Society of America
National Lake Management Society
Society for Freshwater Science
Washington Mountaineers
Washington State Lakes Protection Association

ADDITIONAL SKILLS

Trained in wilderness first aid and CPR
Trained in swiftwater safety
Trained in mountaineering and mountain oriented first aid by The Mountaineers (Seattle, WA)
Trained in jet and prop motor boat operation and safety by U.S. Coast Guard and others
Trained in electrofishing and snorkel survey techniques by Washington Trout

Set and drift gillnet commercial fisherman
Conversational in Spanish
Proficient in all Microsoft programs
Proficient in R

PROFESSIONAL EXPERIENCE

PRINCIPAL: September 2013 through present
Agua Dulce Freshwater Consulting, Missoula, Montana and Olympia, Washington
Ecological contracting for non-profit and aboriginal organizations including Trout Unlimited, Susitna River Coalition, Bristol Bay Heritage Land Trust, Center for Science in Public Participation, Bristol Bay Regional Seafood Development Association, and others. Conduct technical reviews, interpret freshwater ecological information (including fisheries information) to a wide variety of audiences. And conduct public, legislative, and legally oriented research, outreach and education. Design and execute monitoring studies and other freshwater ecological scientific inquiries.

FISHERIES BIOLOGIST: February 2013 through September 2013
US Geological Survey, Bozeman, Montana
Conducted field and desktop research to support a salmon reintroduction project above large hydropower dams in the lower Columbia River basin in Washington State.

ASSOCIATE DIRECTOR: June 2010 through December 2012
Fisheries Research and Consulting, Anchorage, Alaska
Planned and led field studies to determine baseline salmonid habitat conditions in Alaska's remote Bristol Bay watershed. Conducted data analysis and dissemination. Presented information to Bristol Bay residents, sportsman's groups, media, and academic audiences regarding potential risks to fisheries from copper-sulfide mining. Conducted extensive literature reviews regarding natural resources in Bristol Bay, risk factors threatening the region, and related general ecology in order to assist with federal decisions regarding permitting for large-scale hard rock mining activities. Successfully prepared and submitted grant applications fact sheets, literature reviews, and other gray literature.

POPULATION BIOLOGIST: December 2008 through June 2010
State of the Salmon Program, Portland, Oregon
Coordinated and planned large international, interdisciplinary conferences regarding Pacific salmon conservation. The work included collaborating with experts in salmon science, management, policy, and conservation to coordinate program content, as well as cultivate sponsorship relationships, manage volunteers, and attend to a vast array of details in preparation for and execution of the events. Success of the events required careful budget management, fundraising, media outreach, extraordinary organizational skills and attention to detail, and a fundamental understanding of the science underpinning our efforts. Additionally conducted research regarding salmon management with respect to harvest and hatchery practices; and conducted data collection, analysis, and dissemination regarding threats posed by a large, proposed mining project in Southwest Alaska.

RESEARCH ASSISTANT: May 2008 through November 2008
Flathead Lake Biological Station, Polson, Montana
Assisted with a comparative study between the Elk and North Fork of the Flathead rivers in southeastern British Columbia regarding the effect of open-pit coal mining on the freshwater ecology of the two neighboring watersheds with respect to the proposed initiation of mining

(Professional experience continued)

activity in the Flathead system. Additionally assisting with data collection for the Salmonid Rivers Observatory Network (SaRON) project assessing the health of salmon populations and their freshwater habitat in rivers throughout the Pacific Rim.

GRADUATE RESEARCH ASSISTANT, Tierra del Fuego: October 2005 through May 2008
University of Montana, Missoula, Montana and Rio Grande, Argentina
Successfully launched an ongoing project regarding life history and management of a large anadromous brown trout population in Argentine Tierra del Fuego. The project entailed coordinating data collection and collaboration amongst dozens of fishing guides, several local field technicians, other local landowners and stakeholders, government agencies, a local university, and fishing lodges and their clients. Local Ecological Knowledge was collected, the compilation of which informed the analysis of historical fishing records and general population trends. Additionally involved data collection, analysis, and interpretation at all trophic levels within rivers and streams (including algae, macroinvertebrates, juvenile and adult fish), as well as habitat data (including chemistry, discharge, and substrate metrics). Data resulting from this effort produced population and mortality estimates of the sea trout population generated by mark-recapture and fish scale analysis, as well as an assessment of the general health of the juvenile population and their freshwater habitat. The brown trout life history analysis was the subject of my master's thesis.

GRADUATE RESEARCH ASSISTANT, SaRON: May 2005 through October 2005
University of Montana, Missoula, Montana
Conducted fisheries and freshwater ecology research in British Columbia, Canada, and southwestern Alaska as part of the Salmonid Rivers Observatory Network (SaRON) project. Work was conducted with a wide variety of stakeholders, including university colleagues, First Nations and native employees, government agencies, and non-profit organizations. Involved collection any analysis of aquatic ecological data describing physical, chemical, and biological characteristics of watersheds.

TEACHING ASSISTANT: June 2004 through May 2005
University of Montana, Missoula, Montana
Lab instructor for introductory biology course for majors covering topics ranging from cellular structure to molecular genetics. Lectured to lab sections, administered and graded exams, supervised students' lab work, evaluated term papers. Additionally instructed freshwater ecology labs, lead field trips, supervised lab work including aquatic organism identification and quantification, and organized and presented lectures.

FISHERIES TECHNICIAN (VOLUNTEER): February 2005 through May 2005
State of Montana Fish Wildlife and Parks Department, Missoula, Montana
Gill netting a large reservoir in a research and exclusion effort focused on Northern Pike in the former Milltown Reservoir. Field fish identification and morphometric measurements, PIT tagging, radio tagging, and subsequent radio tracking.

WATER QUALITY ANALYST (Lakes and Streams): February 2000 through May 2004
Washington State Department of Ecology, Olympia, Washington
Data collection, analysis, and interpretation for lakes and rivers water quality assessments, Total Maximum Daily Load studies (TMDLs). Generated approved Quality Assurance Project Plans (documents detailing study plans and design). Supervised up to four staff in data collection efforts. Created maps using ArcView and ArcGIS. Worked with private landowners as well as

(Professional experience continued)

government agencies to coordinate data collection efforts. Processed data using statistical analysis (largely in SYSTAT and Excel) and wrote reports pertinent to results of the data collected. Lakes reports evaluated general lake health by compiling chemical, physical, and biological data. A nutrient criterion was determined and justified for each lake assessed. Data compilation required coordination with state Fish and Wildlife officials on fish habitat issues, identification of phytoplankton and zooplankton, knowledge of statistics, quality assurance, and limnological principles.

WASHINGTON CONSERVATION CORPS CORPSMEMBER: October 1999 through February 2000
Washington State Department of Ecology, Olympia, Washington:
Project Lead for the Chehalis River Best Management Practices Evaluation project which included field sample collection, taking flow measurements and calculating discharge, determining several parameters onsite, data management and analysis, and technical writing. Field Technician and Data Manager for the Dungeness River TMDL which included extensive work in Ecology's Environmental Information Management database, exceptional organization, and supervision of field assistants. Other miscellaneous field work and data collection including ArcView experience.

AQUATIC BOTANIST: Summers 1998, 1999, and 2000
Washington State Department of Ecology, Olympia, Washington
Assessed plant communities in lakes throughout the state of Washington paying specific attention to both rare plants and noxious weeds. Required familiarity with aquatic plant species and macroalgae, frequent use of botanical keys, and maintenance of an herbarium. Also demanded proficiency in small and large boat operation and field first aid. Interacted frequently with property owners and lake users in order to convey the importance of both native and nonnative plants to the health of a lake. Contributed to, and led, studies regarding the effectiveness of herbicides on aquatic weeds which involved both field work as well as lab and computer work. Field and lab work required knowledge of sampling techniques and project design, while computer work required the use of a large database (using Access) in addition to statistical analysis. Supervised up to two employees during both general surveys as well as herbicide effectiveness studies. Additionally evaluated fish and wildlife habitat and trained colleagues in those evaluation techniques. Assisted in field work and data analysis for TMDL studies.

RESEARCH ASSISTANT: September 1998 – June 1999
U.S. Forest Service & University of Washington, Seattle & Leavenworth, Washington
Researched the role of fire in the ecology in the life history of two rare plant species endemic to the Wenatchee Mountains. Lab, greenhouse, and field components all required attention to detail and careful reporting.

TECHNICAL WRITER: Summers 1996 and 1997
Washington State Labor and Industries, Tumwater, Washington
Wrote and designed several technical manuals for the purpose of training new hires after observing and analyzing colleagues work activities. Required extensive coordination and interaction with those observed in order to ensure accuracy. Exercised judgment and skills to provide a both clear and attractive presentation. Additionally performed legal research.

PUBLICATIONS

Peer Reviewed

O'Neal, S.L., D.C. Whited, D. Young, M.S. Hoy, and D.E. Schindler. TBD. Applying environmental DNA to spatial stream network models for informing management decisions in remote environments. TBD. *In progress.*

O'Neal, S.L., M.S. Hoy, C.O. Ostberg, D. Young, M. Geist, and D.E. Schindler. TBD. Documenting habitat for anadromous species: Exploring affordable methods in remote Alaska streams. TBD. *In progress.*

O'Neal, S.L., D.E. Schindler, A.R. Spanjer, H.S. Barrett, M.I. Chow, A.P. Smits, R. Evrard, and T.S. Lee. 2021. Theory and practice: Defining indirect effects of disturbance for science and policy. *In progress.*

Sexton, E.K., C.J. Sergeant, J.W. Moore, A.R. Westwood, D.M. Chambers, M.V. McPhee, S.A. Nagorski, S.L. O'Neal, J. Weitz, A. Berchtold, M. Capito, C.A. Frissell, J. Hamblen, F.R. Hauer, L.A. Jones, G. Knox, R. Macnair, R.L. Malison, V. Marlatt J. McIntyre, N. Skuce, and D. Whited. 2020. Canada's mines pose transboundary risks. Science 368:376-377.

Woody, C.A., S. O'Neal, D. Rinella, D. Bogan, D. Merrigan, and M. Geist. 2014. Environmental baseline and mining in remote Alaska. Alaska Park Science 13(2): 49-53.

O'Neal, S. and R.M. Hughes. 2012. Fisheries and hard rock mining: AFS symposium synopsis. *Fisheries* 37(2): 54-55.

O'Neal, S. and J.A. Stanford. 2011. Partial migration in a robust brown trout (*Salmo trutta*) population in a Patagonian River. *Transactions of the American Fisheries Society*. 140: 625-635.

Ciancio, J.E., M.A. Pascual, F. Botto, M. Amaya, S. O'Neal, and O. Iribarne. 2008. Anadromy detection in sympatric anadromous and resident introduced salmonids in Patagonia. *Journal of Fish Biology* 72: 1708–1719.

Parsons, J.K., K.S. Hamel, S.L. O'Neal, A. Moore. 2004. The Impact of Endothall on the Aquatic Plant Community of Kress Lake, Washington. *Journal of Aquatic Plant Management* 42:109-114.

Other

O'Neal, S. 2019. Technical comments regarding fish and aquatic habitat in the Pebble Project Draft Environmental Impact Statement. Expert comments to US Army Corps of Engineers regarding fish and habitat impacts for Trout Unlimited, AK.

Zamzow, K., A. Maest, C. Frissell, and S. O'Neal. 2019. Fugitive dust issues in the Pebble Project draft EIS. Expert comments for Trustees for Alaska.

Zamzow, K., A. Sobolewski, A. Maest, C. Frissell, and G. Reeves. Selenium issues in the Pebble Project draft EIS. Expert comments for Trustees for Alaska.

(Other publications continued)

O'Neal, S. 2018. Pebble Project Department of the Army Application for Permit POA-2017-271. Expert comments to US Army Corps of Engineers regarding Clean Water Act Section 404 permit application by Pebble Limitied Partnership for Trout Unlimited, AK.

O'Neal, S. 2015. A review of the Tulsequah Chief Ecological Risk Assessment. Expert comments to the Canadian federal government for Rivers Without Borders.

O'Neal, S. 2015. BC Mines and the Taku Watershed. Juneau Empire. October 6. Juneau, AK.

O'Neal, S. 2013. Comments on Chapter 15 of Kerr-Sulphurets-Mitchell (KSM) Project Environmental Assessment, Fish and Aquatic Habitat. Expert comments to British Columbia provincial government for Trout Unlimited, AK.

O'Neal, S. 2012. Review of EPA work on Pebble was sound. Anchorage Daily News guest editorial. August 29. Anchorage, AK.

O'Neal, S. 2011. A review of PLP Environmental Baseline Documents: Resident fish and juvenile salmon habitat, distribution and assemblage. Fisheries Research and Consulting. Anchorage, AK. 21 pp.

O'Neal, S. 2011. A review of PLP Environmental Baseline Documents: Aquatic macroinvertebrates (Bristol Bay drainages). Fisheries Research and Consulting. Anchorage, AK. 13 pp.

O'Neal, S. and C.A. Woody. 2011. Canada's Fraser River: Reasons for sockeye salmon declines with a comparison to Bristol Bay. Fisheries Research and Consulting. Anchorage, AK. 27 pp.

Woody, C.A. and S.L. O'Neal. 2010. Fish surveys in headwater streams of the Nushagak and Kvichak River drainages in Bristol Bay, Alaska, 2008-2010. The Nature Conservancy. Anchorage, AK. 48 pp.

O'Neal, S., J.A. Stanford, and A.L. Liberoff. October 2007. Population Status and Ecology of Brown Trout; Rio Grande, Tierra del Fuego, Argentina. Flathead Lake Biological Station. University of Montana. Polson, MT.

O'Neal, S. and J.A. Stanford. October 2006. Population Status and Ecology of Brown Trout; Rio Grande, Tierra del Fuego, Argentina. Flathead Lake Biological Station. University of Montana. Polson, MT.

Carroll, J., S.L. O'Neal, and S. Golding. April 2006. Wenatchee River Basin Dissolved Oxygen, pH, and Phosphorus Total Maximum Daily Load Study. Washington State Department of Ecology, Environmental Assessment Program. Olympia, WA.

Carroll, J. and S. O'Neal. Data Summary: Wenatchee River Basin Dissolved oxygen, pH and Fecal Coliform Total Maximum Daily Load. Washington State Department of Ecology, Environmental Assessment Program. Olympia, WA.

(Other publications continued)

Carroll, J. and S. O'Neal. December 2005. Wenatchee River Basin Fecal Coliform Bacteria Total Maximum Daily Load Study. Washington State Department of Ecology, Environmental Assessment Program. Olympia, WA.

Bilhimer, D., J. Carroll, S. O'Neal, G. Pelletier. August 2003. Quality Assurance Project Plan: Wenatchee River Temperature, Dissolved Oxygen, pH, and Fecal Coliform Total Maximum Daily Load Year 2 Technical Study. Washington State Department of Ecology, Environmental Assessment Program.

Bilhimer, D., J. Carroll, S. O'Neal, G. Pelletier. July 2002. Quality Assurance Project Plan: Wenatchee River Temperature, Dissolved Oxygen, pH, and Fecal Coliform Total Maximum Daily Load Year 1 Technical Study. Washington State Department of Ecology, Environmental Assessment Program.

Sargeant, D., S. O'Neal, W. Ehinger. April 2002. Chehalis Best Management Practices Evaluation Project, Final Report for Water Quality Sites. Washington State Department of Ecology, Environmental Assessment Program.

Parsons, J. and S. O'Neal. September 2001. Aquatic Plants Technical Assistance Program 2000 Activity Report. Washington State Department of Ecology, Environmental Assessment Program.

O'Neal, S., D. Hallock, K. Smith. Water Quality Assessments of Selected Lakes within Washington State: 1999. July 2001. Washington State Department of Ecology, Environmental Assessment Program.

Smith, A.K., D. Hallock, S. O'Neal. December 2000. Water Quality Assessment of Selected Lakes within Washington State: 1998. Washington State Department of Ecology, Environmental Assessment Program

MEDIA

Osborn, K. 2020. Pebble Mine and Bristol Bay with Sarah O'Neal. Fisherwomen podcast. October 29, 2020. San Francisco, CA.
https://www.podomatic.com/podcasts/fisherwomenpod/episodes/2020-10-29T03_00_00-07_00

Braun, A. 2020. Secret recording portray regulators as easing Pebble Mine's path to approval. Hakai Magazine. September 30, 2020. Victoria, BC, Canada.
https://www.hakaimagazine.com/news/secret-recordings-portray-regulators-as-easing-pebble-mines-path-to-approval/

Bauman, M. 2020. Corps' final EIS on Pebble mine draws criticism from Native leadership. The Cordova Times. August 1, 2020. Cordova, AK.

Higgins, P. 2020. Radio interview on Monday Morning Magazine. KMUD. May 31, 2020. Eureka, CA.

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University of Washington School of Aquatic and Fishery Sciences. 2020. SAFS researchers and graduates coauthor international letter addressing transboundary mining pollution. May 4, 2020. Seattle, WA. https://fish.uw.edu/2020/05/safs-researchers-and-graduates-coauthor-international-letter-addressing-transboundary-mining-pollution/

Minemyer, D. and M. Ross. 2019. KTUU, Anchorage NBC affiliate. Pebble Mine opponents, supporters prepare for final public hearing on draft environmental impact statement. April 15, 2019. Anchorage, AK.

Lumpkin, R. 2019. Every Least Thing. Gray's Sporting Journal. March/April Issue.44(1): https://www.grayssportingjournal.com/every-least-thing/.

Shea, M.R. 2019. Gold Digger. Men's Journal. January Issue 28(1): 74-81. https://www.mensjournal.com/features/the-former-clintonite-trying-to-build-the-countrys-most-controversial-mine/.

Lewis, R. 2015. Activists: Canada mine approvals threaten Alaska fishing communities. Aljazeera America. July 10. http://america.aljazeera.com/articles/2015/7/10/bc-mines-threaten-alaskan-fishing-communities.html

DeMarban, A. 2014. Manager says increasingly expensive Susitna dam could help salmon. Alaska Dispatch News. December 18. Anchorage, AK. https://www.adn.com/energy/article/susitna-dam-costs-rise-manager-says-it-may-help-salmon/2014/12/19/

Denning, A. 2014. Transboundary group wants a voice in BC mining process. KTOO Public Radio. November 14. Juneau, AK. https://www.ktoo.org/2014/11/14/transboundary-group-wants-voice-bc-mining-process/

Dobbyn, P. 2014. Building a dam in paradise: Salmon in balance on the Susitna River. Pacific Fishing Magazine. September 2014: 6-12. http://www.pacificfishing.com/PF_Past_Issues/PF_Sept14_ebook.pdf

Dobbyn, P. 2014. Hot dam! Anchorage Press. July 17. Anchorage, AK. https://www.anchoragepress.com/news/hot-dam/article_80e0e2c2-502e-5aed-aa1d-1e8d459db175.html

American Fisheries Society. 2012. Spotlight on women in fisheries. Fisheries 37(3): 132-135. https://doi.org/10.1080/03632415.2012.669617

Fiorucci, D. 2012. Fish biologist alleges huge discrepancy in Pebble salmon estimates. KTUU Channel 2 News. Septemeber 30. Anchorage, AK.

Lempinen, E.W. 2011. In fight over Alaskan mine, public interest turns to science, AAAS News and Notes. Science 334(6055): 469-473. https://www.aaas.org/news/fight-over-alaskan-mine-public-interest-turns-science

Kean, S. 2010. Fishing for gold in the last frontier state. Science 327: 263-265. https://science.sciencemag.org/content/327/5963/263

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Dobbyn, P. 2010. Fighting to save the best of the last in Alaska's Bristol Bay. Trout Magazine Spring 2010: 24–27.

Wiswell, G. 2010. Rio Irigoyen searuns at the far end of the world. Wild on The Fly Issue 14: http://www.wildonthefly.com/content/news-features?article=2.

Behnke, R. 2009. Old fish in new waters: Sorting out nature from nurture. Trout Magazine Winter 2009: 56–58.

Randolph, J. 2009. Tierra del Fuego beneath the wind and whitecaps: A game plan for sea run success. Fly Fisherman December 2009: 30–35.

Caranci, M. 2009. Rio Grande brown trout. Fly Fisherman December 2009: 33 (inset).

O'Neal, S. 2008. Merwin: A world record brown trout? Field and Stream, November 2008. Invited letter to the editor. https://www.fieldandstream.com/merwin-world-record-brown-trout

PRESENTATIONS

Invited

O'Neal, S. October 2019. Pebble Mine: A case study in science and policy. Advancing scientific knowledge of mining impacts on salmonid-bearing watersheds workshop. Polson, MT.

O'Neal, S. August 2019. Theory and practice: Defining indirect effects of development for science and policy. World Salmon Forum, Seattle, WA.

Herendeen, E., S. O'Neal, D. Berg, A. Boone, and M. Jackson. November 2018. Bristol Bay Means Business: Town Hall with business leaders about the proposed Pebble Mine. Pacific Marine Expo, Seattle, WA.

O'Neal, S., M. Hoy, M. Geist, D. Young, C. Ostberg, T. Simmons, D. Menning, and J. Duda. May 2018. Documenting habitat for anadromous species: Exploring affordable methods for protecting Alaska's salmon streams. American Fisheries Society, Western Division. Anchorage, AK.

Williams, N., M. Dischner, S. O'Neal, and A. Williams. May 2018. Safeguarding Bristol Bay. American Fisheries Society, Western Division. Anchorage, AK.

O'Neal, S., D. Young, C. Ostberg, J. Duda, M. Hoy, and M. Geist. March 2017. Documenting habitat for anadromous species: Exploring affordable methods for protecting Alaska's salmon streams. American Fisheries Society, Alaska Chapter. Fairbanks, AK.

O'Neal, S. March 2015. Sculpin Matter? Southwest Alaska Interagency Meeting (SWIM). Dillingham, AK.

O'Neal, S. November 2014. Potential impacts of Susitna Dam to salmon. Pacific Marine Expo. Seattle, WA.

O'Neal, S. April 2013. Global conservation of salmon and trout. Invited keynote. University of Montana Western Research Symposium. Dillon, MT.

O'Neal, S. and C.A. Woody. March 2012. Fish density and distribution in headwater streams in Bristol Bay, Alaska. Western Alaska Interdisciplinary Science Conference. Dillingham, AK.

Contributed

O'Neal, S. October 2014. Costs and benefits of large hydropower mitigation. Joint annual meeting of the Alaska chapters of the American Water Resources Association and the American Fisheries Society. Juneau, AK.

Woody, C.A. and S. O'Neal (presenter). October 2014. Coho salmon biodiversity, distribution, and density in a proposed mining area, Bristol Bay, Alaska. Joint annual meeting of the Alaska chapters of the American Water Resources Association and the American Fisheries Society. Juneau, AK.

O'Neal, S., C.A. Woody and K. Zamzow. February 2013. Freshwater baseline inventory and monitoring in headwater streams of a proposed hard rock mining district in Bristol Bay, Alaska. Montana Chapter of the American Fisheries Society Annual Meeting. Fairmont Hot Springs, MT.

Woody, C.A., S. O'Neal (presenter), and D. Bogan. November 2011. Freshwater baseline inventory and monitoring in Nushagak and Kvichak headwater streams. American Fisheries Society Alaska Chapter. Girdwood, AK.

O'Neal, S.L. (presenter and symposium organizer) and K. Zamzow. September 2011. Fish presence and water quality in a proposed copper mining district, Alaska. American Fisheries Society (national chapter, Fisheries and Hardrock Mining Symposium). Seattle, WA.

Woody, C.A., S. O'Neal (presenter), and D. Bogan. September 2011. Freshwater baseline inventory and monitoring in Nushagak and Kvichak headwater streams. American Association for the Advancement of Science Arctic Science Conference. Dillingham, AK.

O'Neal, S. and C.A. Woody. September 2010. Proposed copper-sulfide mining in Bristol Bay: identified risks to fisheries. Wild Trout Symposium X. West Yellowstone, MT.

O'Neal, S. and J. Stanford. June 2008. Life history variation of introduced brown trout in a Patagonian River. Oral presentation. North American Benthological Society Annual Meeting, Salt Lake City, UT.

O'Neal, S. May 2008. Lessons to learn from all out invasion: Life history of brown trout in a Patagonian river. Master's thesis defense. Presented to the Department of Biological Sciences and the Flathead Lake Biological Station, University of Montana, Missoula, MT.

O'Neal, S. October 2006. Brown trout invasion of the Rio Grande, Tierra del Fuego: The role of life history. Oral presentation. October 18, 2006. Presented to the Department of Biological Sciences, University of Montana, Missoula, MT.

Outreach

O'Neal, S. February 2020. Presentations to Alaska and Washington State Congressional delegations regarding shortcomings of Pebble Mine Preliminary Final Environmental Impact Statement. Washington, DC.

O'Neal, S. June 2019. Presentations to Alaska, Washington State, and West Virginia Congressional delegations regarding shortcomings of Pebble Mine draft Environmental Impact Statement. Washington, DC.

O'Neal, S. April 2019. Testimony to US Army Corps of Engineers regarding risks of mine development to Bristol Bay fisheries. Anchorage, AK.
(Outreach continued)

O'Neal, S. April 2018. Testimony to US Army Corps of Engineers regarding risks of mine development to Bristol Bay fisheries. Nondalton, AK.

O'Neal, S. March 2018. Defining fish habitat in Alaska's waters. Alaska State Legislature informational presentation. Juneau, AK.

O'Neal, S. March 2018. Defining fish habitat in Alaska's waters. United Fishermen of Alaska informational presentation. Juneau, AK.

O'Neal, S. April 2014. Presentations to Alaska State legislators regarding salmon ecology and hydropower development. Juneau, AK.

O'Neal, S. October 2014. Risks of hard rock mining to salmon, BC-Alaska transboundary rivers. Public presentations in communities throughout southeast Alaska: Juneau, Ketchikan, Wrangell, Petersburg, and Sitka.

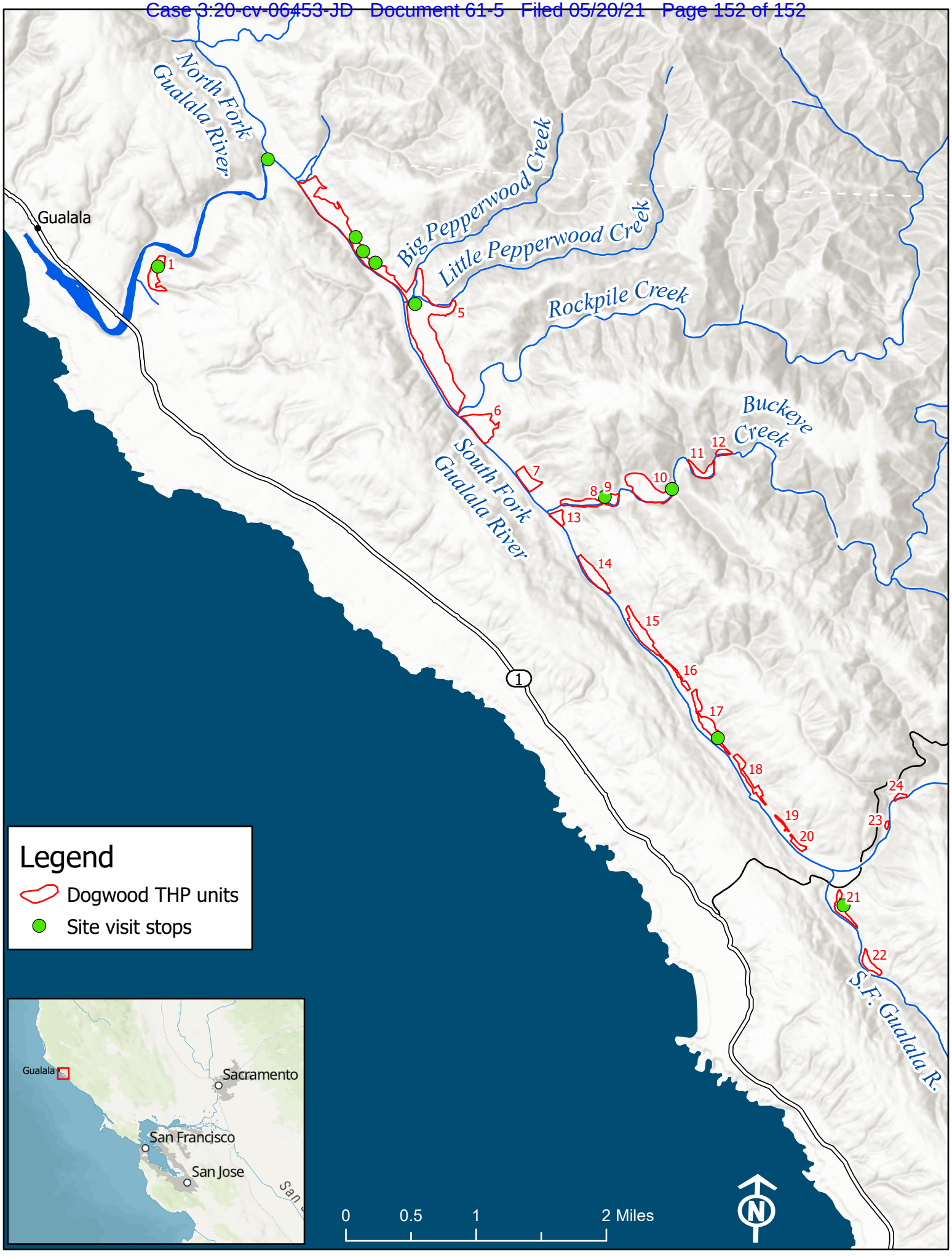
O'Neal, S. March 2011. Presentation to executive members of EPA Region 10 staff regarding existing natural resources of Bristol Bay. Seattle, WA.

O'Neal, S. January 2011. Presentation to executive members of the EPA Office of Wetlands, Oceans, and Watersheds regarding fish, wildlife, municipal water, and recreational resources. Washington, D.C.

O'Neal, S. August 2010. Community technical outreach regarding fisheries and freshwater resources to the communities of Koliganek and New Stuyahok, AK.

O'Neal, S. December 2009. Technical testimony to the Alaska Board of Fisheries regarding third party scientific review for large-scale mine permitting. Anchorage, AK.

Woody, C.A. and S. O'Neal (presenter). November 2008. Bristol Bay and Pebble Mine: Identified Risks. Presentations in the communities of Togiak and Dillingham, AK.


North Fork Gualala River
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Rockpile Creek
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