# *Ichthyophonus* in Pacific herring: Investigating a transmission hotspot

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## Abstract

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*Chapter 1.* Sample collection and processing methods were optimized and validated for the detection of *Ichthyophonus* DNA in environmental samples using both spiked and field samples. Methods performance testing revealed low efficiency in sediment samples, suggesting the extraction techniques were not yet effective for detecting *Ichthyophonus* DNA in spiked sediment samples. The optimized techniques worked well for spiked water samples, and in field application of the method, revealed presence of *Ichthyophonus* DNA in natural seawater samples collected from a known *Ichthyophonus* transmission hot spot in Cordova Harbor, AK.

*Chapter 2.* A geographic hot spot of high *Ichthyophonus* infection prevalence occurred in juvenile Pacific herring from Cordova Harbor, AK, USA during 2015 and 2019. Over-winter infection prevalence (0-5%) was similar to that of juvenile herring in other locations; however, prevalence in Cordova Harbor increased rapidly during the spring, peaking at 64% (2015) and 40% (2019) in June and July. Reasons for the geographical hot spot remain undetermined, but seasonal shifts in herring diet were coincident with the changing infection prevalence in Cordova Harbor.

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# ACKNOWLEDGEMENTS

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# DEDICATION

For my family who push me to succeed and are always there for me, through fires and pandemics. Y también por la raza. ¡Sí se puede!

# ASSESSMENT OF MARINE WATER AND SEDIMENT SAMPLES FOR *ICHTHYOPHONUS* DNA

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## ABSTRACT

Sample collection and processing methods were optimized and validated for the detection of *Ichthyophonus* DNA in environmental samples using both spiked and field samples. Methods performance testing revealed low efficiency in sediment samples, suggesting the extraction techniques were not yet effective for detecting *Ichthyophonus* DNA in spiked sediment samples. The optimized techniques worked well for spiked water samples, and in field application of the method, revealed presence of *Ichthyophonus* DNA in natural seawater samples collected from a known *Ichthyophonus* transmission hot spot in Cordova Harbor, AK.

## INTRODUCTION

*Ichthyophonus* is a genus of mesomycetozoan parasite that infects more than 80 marine and freshwater fishes in cold and temperate waters of the northern hemisphere (Gregg et al. 2016). Infections are typically chronic with few apparent effects on hosts; however heavy infections can lead to host mortality and population-level impacts (reviewed in McVicar 2011 and Burge et al. 2014). Heavy infections can also result in product marketability issues stemming from unsightly lesions in infected host skeletal muscle (Noga 1993; Kocan et al. 2010; McVicar 2011).

Although a life cycle for *Ichthyophonus* has been proposed based on observations of parasite stages in vitro (Okamoto et al. 1985), the complete parasite life history has not been confirmed in natural marine or freshwater environments. Perhaps the most obvious information gap involves undocumented routes of parasite transmission to clupeids and other planktivorous fishes, which periodically experience epizootics of ichthyophoniasis. In piscivorous fishes, transmission occurs via ingestion of infected fish tissues (Kocan et al. 2010; LaPatra and Kocan 2016); the route of transmission among non-piscivorous fishes, however, remains unknown. Transmission through cohabitation can occur in the freshwater *Ichthyophonus* genotype (Sindermann and Scattergood 1954; Torgersen et al. 2002; Kocan 2018), but has not yet been demonstrated with the ubiquitous marine genotype in planktiviorous fishes (Gregg et al. 2016). The possibility of an intermediate or paratenic host has been proposed (Sindermann and Chenoweth 1993; Gregg et al. 2012), but the existence of a non-piscine host has not yet been confirmed. Unfortunately, any effort to mitigate the impacts of *Ichthyophonus* on populations of planktivorous fishes must be predicated on an understanding of these unknown exposure and transmission mechanisms.

It is possible that planktivorous fishes become infected after consuming free-living stages of the *Ichthyophonus* parasite directly from water. *Ichthyophonus* is a resilient parasite that can survive in seawater for extended periods (Spanggaard and Huss 1996; Hershberger, Pacheco, and Gregg 2008; Hershberger, Pacheco, Gregg, et al. 2008) and germinate in infected tissues for several days after host death (Spanggaard and Huss 1996; Kocan et al. 2014). If a free-living parasite stage occurs in the environment, it may be consumed directly by planktivorous fishes through particle or filter feeding. These observations support the possibility of a direct parasite life cycle that does not involve an unidentified intermediate host.

Environmental DNA (eDNA) sampling methods are an emerging tool, which can be used to elucidate cryptic parasite life cycles and transmission strategies and can take multiple forms. eDNA is any form of DNA collected from non-tissue samples in the form of cellular or free DNA. It can be used as a tool to study targeted species or broader species diversity, also known as DNA barcoding (Taberlet et al. 2012; Ruppert et al. 2019). As previous *Ichthyophonus* studies are limited to physical collection of the host fish species, eDNA sampling offers a novel approach to better understanding *Ichthyophonus* in wild populations.

To evaluate the possibility that free-living *Ichthyophonus* stages occur in the environment, a molecular sampling technique was optimized to quantify *Ichthyophonus* DNA in water and sediment samples. Using the optimized methods, water samples were analyzed from an *Ichthyophonus* transmission hotspot.

## METHODS

### *Sample Preparation*

Two sets of samples (seawater and sediment) were prepared to optimize sample processing, preservation and DNA extraction techniques and to test a previously validated qPCR assay with environmental samples as follows. To test water filtration and preservation methods, six *Ichthyophonus*-spiked samples and three control samples were created using artificial seawater at a volume of 500mL each. *Ichthyophonus*-positive samples were created by spiking samples with an *Ichthyophonus* suspension made from an isolate of confirmed-infected *Clupea pallasii* heart-explant cultures (that contained multiple life stages of *Ichthyophonus*) in artificial seawater (Instant Ocean, Blacksburg, VA). Dilutions of the stock *Ichthyophonus* isolate (considered 100%) were made to test extraction and qPCR amplification of varying amounts of *Ichthyophonus* cells at 1:2 and 1:10. To test sediment extraction methods, sediment was collected from both Port Angeles and Port Ludlow, Washington to create 12 *Ichthyophonus*-spiked samples and 4 negative control samples. Sediment samples were immediately preserved in either Longmire's solution (n=8; Longmire et al. 1997) or 75.5 or 95% grain alcohol (n=8; Everclear 190-proof). To evaluate the presence of inhibitors in field samples and test the efficiency of chosen sample preservation and extraction methods, another set of water and sediment samples was created as follows. Natural seawater and sediment were collected from a targeted field site, Cordova, Alaska. *Ichthyophonus* isolates of differing cell concentrations were made using both natural and artificial seawater and were spiked with the stock (100%) and two dilutions (50% and 10%) of the heart *Ichthyophonus* isolate described above. Control sediment lacked the addition of *Ichthyophonus*. For each of the 4 *Ichthyophonus* concentrations (0, 10, 50 and 100%), 3 of the sediment samples were spiked with *Ichthyophonus* isolates made with artificial seawater and 2 were made using Cordova seawater for a total of $n_{Ichthyophonus} = 15$; $n_{\text{control}} = 5$. The negative control sample consisted of 3.5g sediment with no added *Ichthyophonus*. The spiked samples (n=3) each contained 2.8 g of sediment and 0.7 mL of an *Ichthyophonus* isolate (one per concentration). All samples were mixed with a spatula and three subsamples (0.5 g) were removed for extractions and qPCR analysis a total of containing ($n_{Ichthyophonus} = 9$, three per spiked concertation; $n_{\text{control}} = 3$). All samples were preserved and processed as described below.

### *Processing and Extraction Methods*

*Seawater.*

Serial filtration of duplicate 500-mL artificial seawater samples through 1.5-µm (VWR Glass Fiber Grade 691), 0.45-µm (Millipore HA), and 0.22-µm (Millipore GS) filters was used to identify the filter pore size that retained the most *Ichythyophonus* cells. After filtration, each filter was cut in half. One half of the filter was stored in long-term storage at -80°C for later extraction if needed and the other half was used to compare preservation and DNA extraction method (*Figure 1.1*A). The serial filtering process was repeated in duplicate for each preservative to identify a method to be used in remote locations: Longmire's solution, 75.5% grain alcohol, or TE Buffer with immediate storage at -80°C. DNA was extracted from the remaining half filters using the Qiagen DNeasy Blood and Tissue kit with the following modifications to the standard protocol: ATL Buffer and Proteinase K volumes were doubled and filters were incubated at 56°C for one hour. DNA was then eluted in 200-µL of Buffer AE and stored at -20°C until used.

*Sediment.*

Three commercially available DNA extraction kits were tested on prepared sediment samples: Qiagen QIAamp FastDNA™ Stool Mini Kit (Quick-Start pathogen-detection protocol), MP Biomedicals FastDNA™ Spin Kit for Soil (standard protocol), or DNeasy® Blood & Tissue Kit, following the manufacturer's protocol with minor modifications: (1) ATL Buffer and Proteinase K volumes were doubled, (2) digestion of samples overnight at room temperature followed by vortex and incubation at 56°C for 15 minutes, (3) centrifugation at 11,000 x g for two minutes to pellet the sediment, (4) transfer of 600µL of the lysate to the spin column (no sediment), and (5) elution n 150 µL Buffer AE.

### *Real-time quantitative PCR*

*Ichthyophonus* DNA was quantified using a previously validated qPCR assay (White et al. 2013). The qPCR reactions were conducted in a final volume of 25-µm consisting of 12.5-µL 2x GoTaq qPCR Master Mix (Promega Corp., Madison, WI, US), 400nM of each *Ichthyophonus*-

specific primers (vc7F and vc5R; *Table 1.1*), 300nM of an *Ichthyophonus*-specific probe (6-FAM-labeled probe ICH21; *Table 1.1*), and 15µg bovine serum albumin. Internal amplification control (IAC) primers, HEX-labeled probe, and template were included in the reaction to test for inhibition (Nolan et al. 2016). Reactions were run in duplicate and included negative template controls and an oligo-based standard curve (*Table 1.1*). All qPCR assays were carried out on a CFX-96 C1000 (Bio-Rad) using the manufacturer's software with the following conditions: 10 min at 95°C, followed by 40 amplification cycles at 95°C for 30 s, 60°C for 1 min, and 72°C for 1 min. *Ichthyophonus* copy number for each sample was determined via regression analysis of the standard curve (*Figure 1.2)* and calculated as copies per reaction (for water filter and preservative samples), copies per gram (for sediment extraction and preservative samples), or copies per µL *Ichthyophonus* isolate (for method efficiency and inhibition tests). The limit of detection was 3 copies per reaction, and an amplification below three copies was considered a negative result.

### *Method Application in Natural Samples*

Water and sediment samples from Cordova Harbor, AK, USA, an *Ichthyophonus* transmission hot spot, were collected and processed using the optimized methods described herein. Seawater samples were collected at the surface and from 3m depth using a Niskin bottle (*Figure 1.3*). Positive controls consisted of water samples from *Ichthyophonus* exposure studies at USGS Marrowstone Marine Field Station, where herring (*C. pallasii*) were experimentally exposed to waterborne fish tissues infected with *Ichthyophonus*. Each day, a negative control was created by filtering deionized water to measure possible contamination during sample collection and processing. All water samples were filtered to 1.5-µm; the filter with retentate was then stored in Longmire's solution and frozen at -80°C. DNA was extracted from the filter residues of all water samples, including a no-sample control, and DNA was quantified by qPCR.

Sequence analysis was conducted on selected natural water samples, which yielded qPCR reaction copy values < 3 (n=3) and representative positive (n=3) and a negative control (n=1). Samples were considered positive if both technical replicates per sample amplified target DNA and had 100% identity with *Ichthyophonus* sequences in GenBank. Samples were considered presumptive positive if only one technical replicate amplified target DNA that also shared 100% identity with *Ichthyophonus*. Those that amplified target DNA but failed to share identity with *Ichthyophonus* were considered negative for *Ichthyophonus*. Samples were prepared for sequencing using a nested amplification process with *Ichthyophonus*-specific M13-tailed primers (Integrated DNA Technologies, Coralville, IA; *Table 1.1*). A nested PCR with target-specific primers was conducted because amplified samples had low quantities of *Ichthyophonus* DNA. M13-tailed primers elongated the DNA product for sequencing, and has been used successfully in other eDNA method studies (Williams-Woods et al. 2011; Stoeckle et al. 2018). The first round of amplification reactions was conducted with the *Ichythyophonus*-specific qPCR as described above, with template DNA increased from 2 µL/reaction to 4 µL/reaction. The second round conventional PCR were prepared in 50 µL total volume consisting of 10 µL 1st round sample template,1x Apex Taq RED Master Mix, 1.5 µL 25mM $MgCl_2$, 1 µL 10x M13-vc7F primer, 1 µL 10x M13-vc5R primer. Thermocycling conditions were the same as the first amplification round. Unpurified PCR products were sent to Genewiz® for Sanger Sequencing. Following sequencing, sequences were aligned and assembled in Sequencher, and consensus sequences were aligned with likely matches using the NCBI GenBank BLASTn program (Zhang et al. 2000; Morgulis et al. 2008).

### *Statistical Analyses*

*Water Processing Optimization*: The mean proportion of copies per reaction captured by each filter was calculated for each preservative to identify the filter size that captured the majority of *Ichthyophonus* DNA. Statistical significance was not calculated because serial dilution results in non-independent samples.

*Sediment Extraction Optimization.*

The most effective extraction method and preservative combination was identified using a Generalized Linear Mixed Effect Model (GLMM). The model was generated with R version 3.6.3 with the *lmer()* function in the "lme4" package, and p-values were calculated with the "lmerTest" package (Bates et al. 2015; Kuznetsova et al. 2017). The data were transformed with a 4th root transformation and analyzed with the following model with a Gaussian distribution:

$$(\text{Copies per gram})^{1/4} \sim \text{Preservative} + \text{Extraction Method} + \text{Sediment Type} + (1|\text{Extract ID})$$

*Method Efficiency.*

Method efficiency was analyzed with a Generalized Linear Model. The model was generated with the *glm()* function in the "lmer" package (Bates et al. 2015). The data were transformed with a log base-10 transformation and analyzed with the following model on a Gaussian distribution:

$$\log(\text{Copies per } \mu\text{L Ichthyophonus Isolate}) \sim \text{Ichthyophonus Concentration} * \text{Sample Type} + (1|\text{Extract ID})$$

*Inhibition.*

Inhibition was analyzed with a Linear Mixed Effects Model. Water samples and sediment samples were analyzed separately by comparing IAC template cycle quantity values (Cq) of samples extracted from water filters or sediment with non-water filter or sediment samples (including standards, reaction controls, and *Ichthyophonus isolate* DNA samples). The following model was generated with the *lmer()* function in the "lme4" package (Bates et al. 2015):

$$Cq \sim (\text{Filter or Sediment Presence/Absence}) + (1|\text{Sample Type})$$

## RESULTS

The highest proportion of *Ichthyophonus* DNA was recovered from water samples using the largest filter pore size. Among spiked water samples, the 1.5-µm filter captured 81.4%, 99.9%, and 97.8% of *Ichthyophonus* DNA for samples preserved in grain alcohol, Longmire's solution, and TE buffer, respectively (*Figure 1.4*). TE Buffer with immediate storage at -80°C produced the highest yield, followed by Longmire's solution (*Figure 1.4*). As the target site did not have access to -80°C storage, Longmire's solution was identified as the best performing preservative for the application of the method.

For sediment samples, the highest yield was obtained in samples preserved in Longmire's solution and extracted with the MP Bio FastSpin Soil Kit. In spiked DNA from sediment samples, use of Longmire's solution as a preservative resulted in greater concentration of DNA than use of grain alcohol ($\bar{x}_{\text{Longmire's}} = 4.84\text{x}10^6 \pm 1.0\text{x}10^6$, $\bar{x}_{\text{grain\_alcohol}} = 3.14\text{x}10^6 \pm 6.7\text{x}10^5$, estimate = +4.28 ± 1.49, $p = 0.00745$; *Figure 1.5*). The MP Bio FastSpin Soil Kit produced higher DNA yields than the Qiagen DNeasy Blood & Tissue Kit (estimate = +20.42 ± 1.84, $t = 11.1$, $p < 0.0001$) and the Qiagen QIAamp Fast DNA Stool Kit (estimate = +30.70 ± 0.193, $t = 16.7$, $p < 0.0001$; *Figure 1.5*).

Extraction method efficiency using the best performing preservative type and extraction method was further analyzed for a control (artificial seawater), environmental water sample (collected in Cordova), and environmental sediment sample (collected in Cordova) by adding various dilutions of *Ichthyophonus* to each treatment before extraction. Sediment sample extraction was inefficient when compared to direct extraction of *Ichthyophonus* isolate (estimate = -0.951 ± 0.178, $t = -5.35$, $p < 0.0001$; *Figure 1.6*). The water processing and extraction method was more efficient than direct extraction of *Ichthyophonus* isolate ($\text{estimate}_{\text{Artificial Seawater}} = 0.753 \pm 0.145$, $t_{\text{Artificial Seawater}} = 5.04$, $p_{\text{Artificial Seawater}} < 0.0001$; $\text{estimate}_{\text{Cordova Seawater}} = 0.577 \pm 0.205$, $t_{\text{Cordova Seawater}} = 2.81$, $p_{\text{Cordova Seawater}} = 0.0063$; *Figure 1.6*).

Inhibition during qPCR was not a factor in sediment or water samples. There was no significant difference in Cq of IAC template DNA between non-environmental samples and environmental samples for water (estimate = -0.035 ± 0.039, $t = -8.93$, $p = 0.37$) or sediment (estimate = -0.299 ± 0.14, $t = - 2.03$, $p = 0.22$; *Figure 1.6*).

Sequence analysis of selected samples from Cordova harbor demonstrated presence of *Ichthyophonus* DNA in two of the three samples examined. One sample obtained from a processing plant outflow pipe (on 4/21/18), amplified by qPCR and shared 100% identity with *Ichthyophonus* sequences in deposited in GenBank (e.g. accession number MT160366.1). The second sample, collected from within the marina in Cordova harbor was considered a presumptive positive, as only one of the two technical replicates amplified, but that replicate shared 100% identity with *Ichthyophonus* (*Table 1.2; Table 1.3)*. No BLAST matches were obtained from the third sample, in which one of two technical replicates amplified *Ichthyophonus* DNA by qPCR.

## DISCUSSION

This study demonstrated the utility of qPCR to assess *Ichthyophonus* DNA in seawater samples from both laboratory and field settings. While the *Ichthyophonus* qPCR assay used has previously been developed and validated for use in fish tissues (White et al. 2014), this is the first to test and apply *Ichthyophonus*-specific methods in environmental seawater and sediment samples. Similar methods using eDNA to investigate possible parasite transmission strategies and life cycles have been tested and applied for other parasites and pathogens (Bass et al. 2015; Huver et al. 2015). Many of these studies relied on traditional preservatives, which may be inaccessible in remote sites such as molecular-grade ethanol, highlighting the need to test alternative preservatives for eDNA.

Although preservation and filtration techniques were optimized for water samples, DNA extraction methods from sediment samples should be further optimized for application to the field. The extraction process warrants further adjustment to increase DNA yields or concentrations according to the needs of downstream applications. Increasing the elution volume generally results in increased yield, but decreased concentration (Xue et al. 2009). Moreover, increasing cell lysing either chemically (by increasing incubation time or buffer volume) or mechanically (by implementation of cell disruption with beads, vertexing, or benchtop homogenization) can increase DNA concentration and should be investigated to improve sediment extraction methods (Van Burik et al. 1998; Karakousis et al. 2006; Leuko et al. 2008). Unexpectedly, water eDNA methods produced higher yields than direct extraction of the *Ichthyophonus* isolate (*Figure 1.6*), potentially due to the use of Longmire's solution as a preservative. Longmire's solution is largely composed of sodium dodecyl sulfate, a detergent that may aid in breaking down of *Ichthyophonus* cell walls (Kirkpatrick et al. 1974).

While sequence results matched *Ichthyophonus* sequences deposited in GenBank, further method development is required to conclusively validate the samples. Sequences from two of three environmental samples generated matches in GenBank, all with E-values ≤ 0.0001. However, sequenced samples that failed to generate significant matches produced sequences longer than the expected fragment size (White et al. 2013) suggesting that either (1) the vc5F/vc7R primers were non-specific to *Ichthyophonus* when used for eDNA, or (2) nested amplification reactions caused products to self-adhere. Therefore, additional work is recommended to ensure the primers generate *Ichthyophonus*-specific PCR products that can be validated by sequencing.

Environmental monitoring can be used to improve disease models and predict future epizootics. For example, eDNA studies for the detection of *Hematodinium* spp. observed elevated DNA quantities prior to outbreaks of infection in blue crabs (Hanif et al. 2013). Disease models have also used data from metagenomic eDNA surveys to find associations between pathogenic parasites and other associated taxa (McCarthy et al. 2011). eDNA surveys can also sample over spatial or temporal gradients to link pathogen presence to abiotic factors, such as temperature, salinity, or seasonality, all of which could be used to inform disease and host population models, define current parasite ecological ranges, predict changes to parasite ecology in a rapidly warming climates (Zamora-Vilchis et al. 2012; Lutz et al. 2013; Chestnut et al. 2014; Dupont et al. 2016; Richey et al. 2020).

Future studies to monitor *Ichthyophonus* DNA in environmental samples will facilitate a better understanding of the natural life cycle of *Ichthyophonus*. In other parasite species, researchers have used eDNA studies to identify previously unknown intermediate or paratenic hosts, such as in *Marteilia sydneyi*, the source of QX disease in the Sydney rock oyster (*Saccostrea glomerata*), and *Marteilia refringens*, which causes merteiliosis in multiple bivalve hosts (Boyer et al. 2013; Adlard and Nolan 2015). Likewise, eDNA can be used to identify environmental reservoirs, a part of the life cycle that can be particularly difficult to identify in microparasites, as traditional sampling methods such as live culture are designed to sample prevalence in host tissues (Urich et al. 2008; Geisen et al. 2015; Brannock et al. 2016; Dupont et al. 2016).

Although eDNA has many applications, it is best used in conjunction with more conventional sampling techniques. While eDNA can expand sampling efforts and help narrow the search for an intermediate host, presence of parasite or pathogen DNA in a sample cannot serve as a substitute for disease sampling, which requires conventional confirmatory methods such as histology or live culture.

# FIGURES AND TABLES

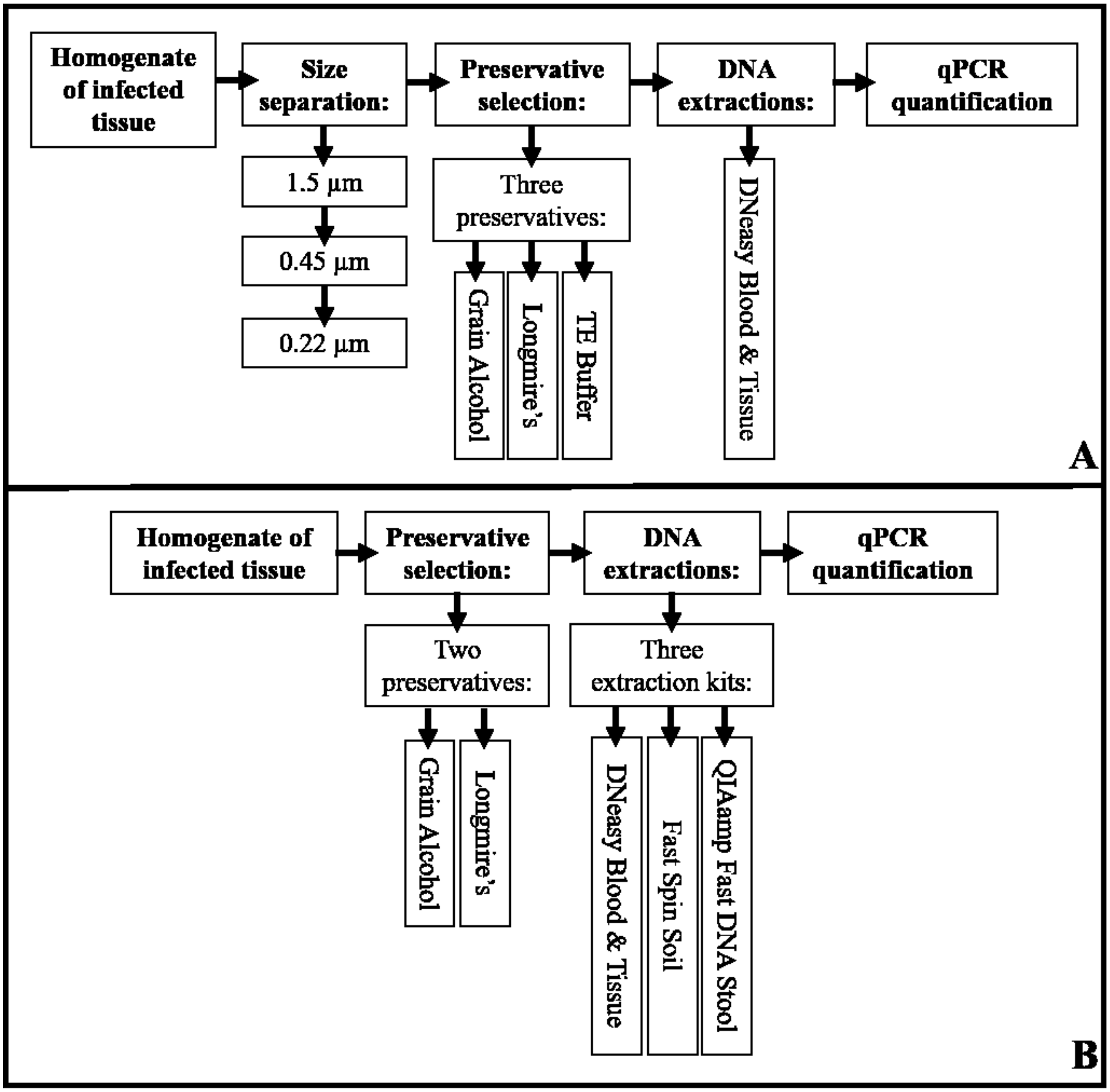

*Figure 1.1.* Processing, preservation, and extraction stages to optimize quantification of *Ichthyophonus* DNA in seawater and sediment samples.
(A) Processing stages evaluated to optimize quantification of *Ichthyophonus* DNA in seawater samples. (B) Preservative and extraction methods evaluated to optimize quantification of *Ichthyophonus* DNA in sediment samples. Note: all phases were conducted using a full factorial design so that each size fractionated sample was assessed with all three preservatives in duplicate for spiked samples and one sample for negative controls (not spiked).

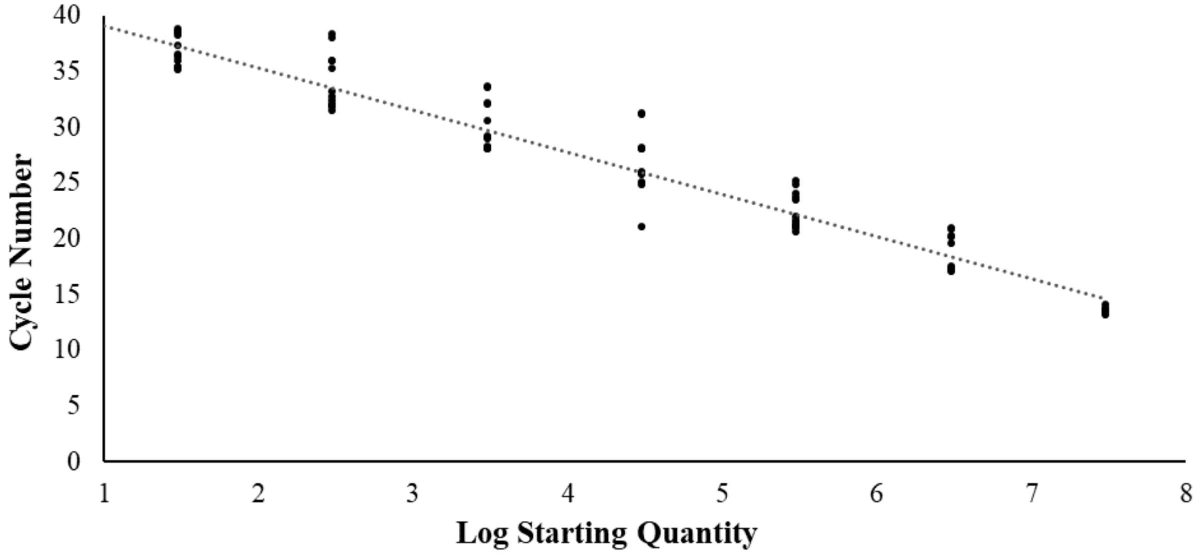

*Figure 1.2.* Standard curve for all qPCR runs.

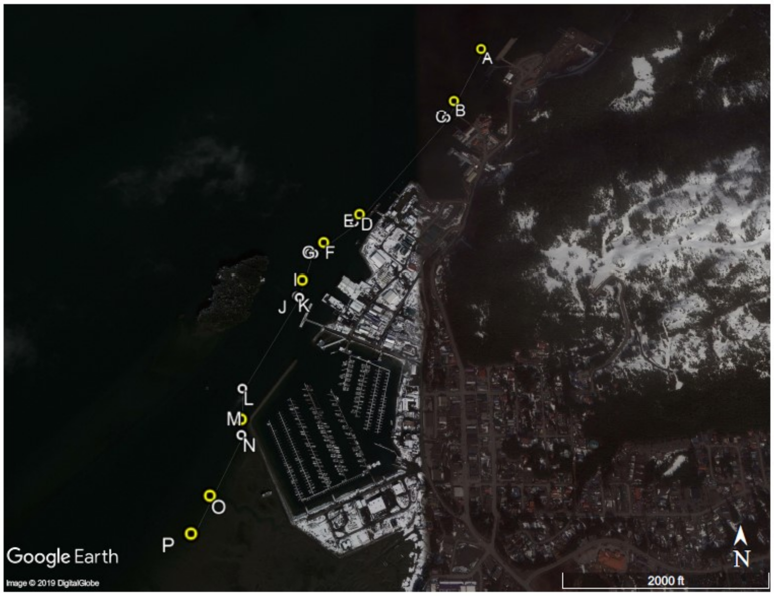

*Figure 1.3.* Water sampling sites in Cordova, Alaska.
Yellow targets are offal discharge sites. White targets are randomly assigned along the transect.

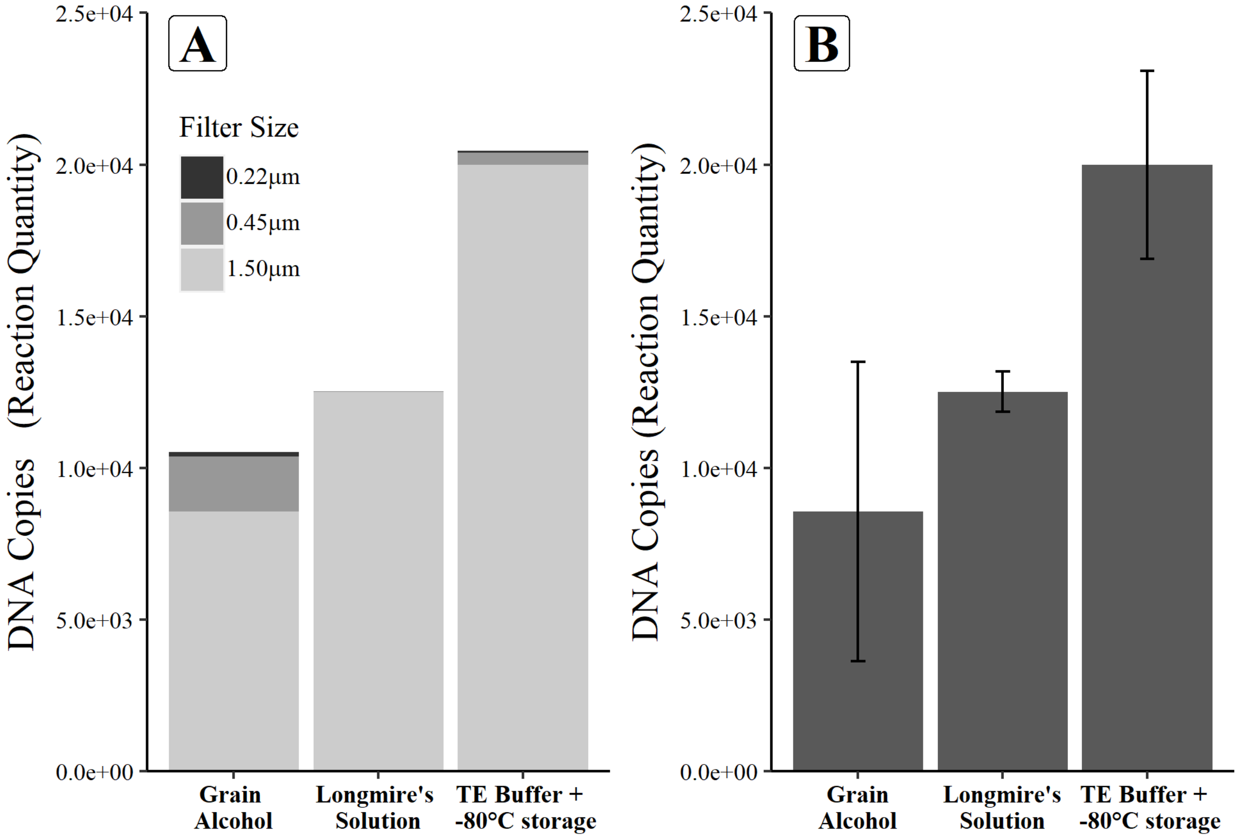

*Figure 1.4.* Comparisons of preservative types and filter pore sizes for water samples. (A) Comparison of preservative types and filter pore sizes. Amount of *Ichthyophonus* sp. DNA captured by each preservative type and filter size. (B) Amount of DNA captured in only 1.5-µm filters by preservative type. Error bars indicate standard error of DNA copies for 1.5um filters.

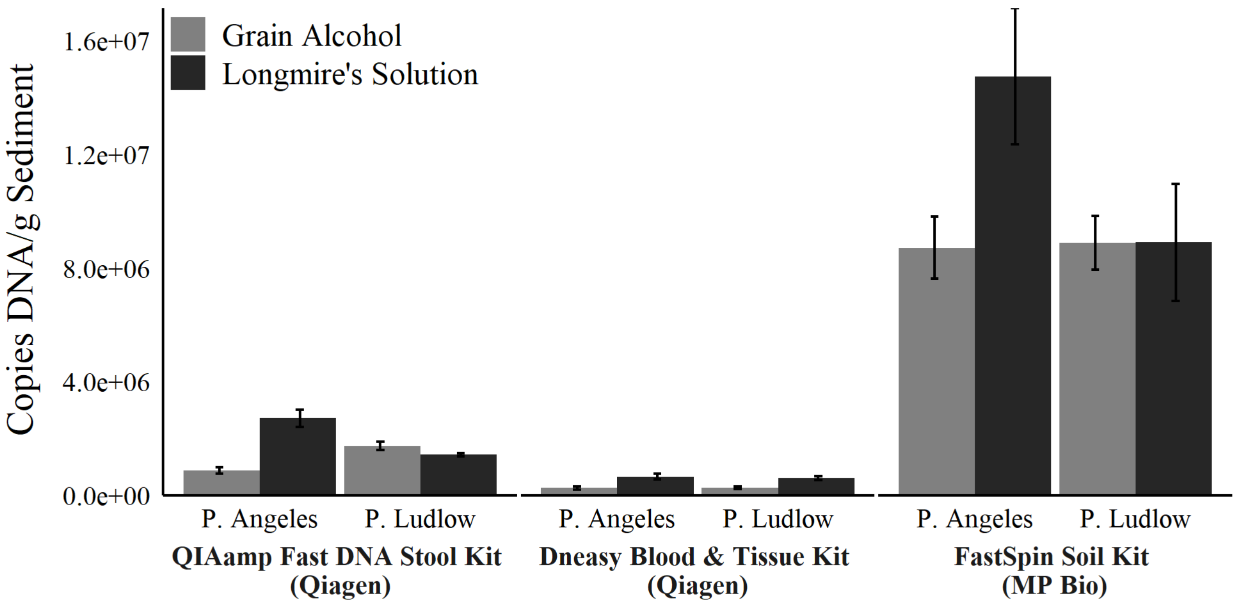

*Figure 1.5* Sediment extraction kit and preservative comparison.
Mean DNA copies per gram of sediment sample are plotted for each combination of sediment source, extraction method, and preservative. Error bars indicate the standard error above and below the mean. *Ichthyophonus*-negative control samples did not amplify target DNA and are not shown in the figure above.

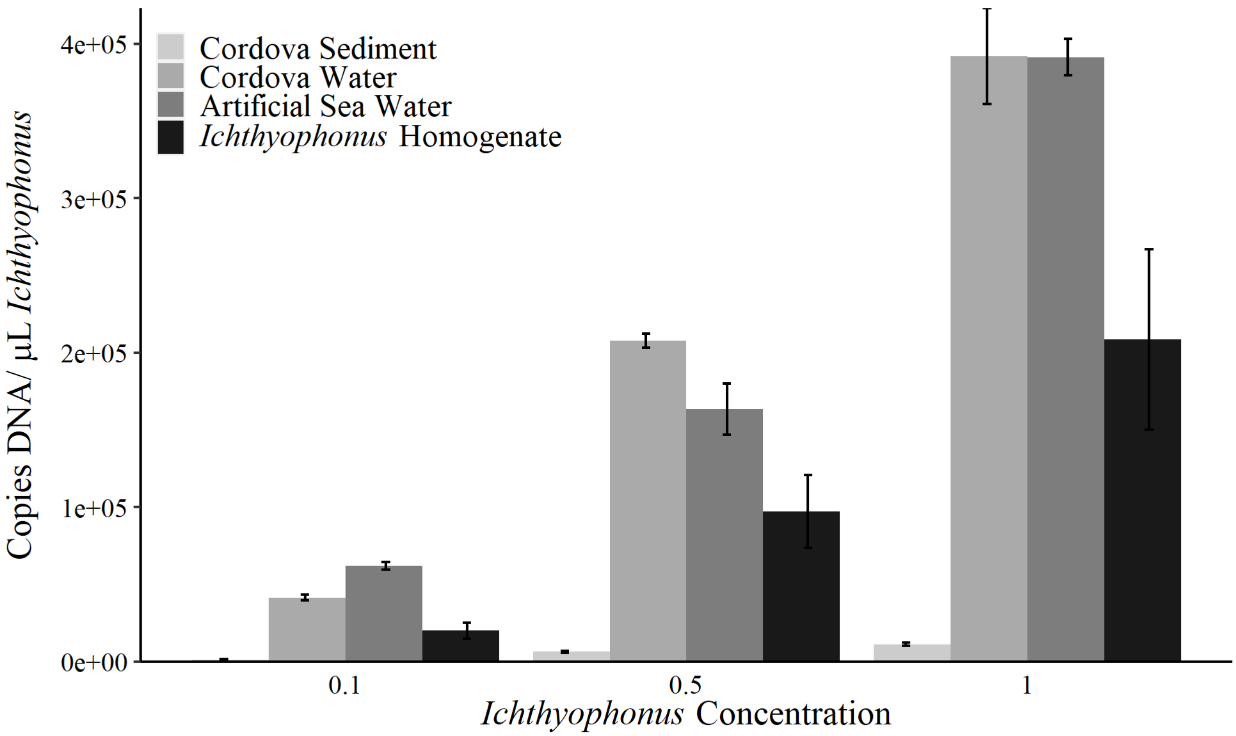

*Figure 1.6* Method efficiency.
Mean DNA copies per µL of added *Ichthyophonus* isolate are plotted for each combination of *Ichthyophonus* concentration and sample type. Error bars indicate the standard error in combined biological and technical replicates.

*Table 1.1*. Primers used in qPCR and sample preparation for sequencing.

[redacted]

*Table 1.2.* Outcomes of samples screened for *Ichthyophonus* presence by qPCR and sequencing testing.

| Sample Type | Coordinate | Depth (m) | Sample Date | Copies per reaction | qPCR Outcome | Sequencing Outcome | Final Outcome |
|---|---|---|---|---|---|---|---|
| Cordova Harbor | (60.545, -145.765) | Surface | 3/28/2019 | - | - | - | - |
| | (60.545, -145.765) | Surface | 3/28/2019 | - | - | - | - |
| | (60.545, -145.765) | Surface | 3/28/2019 | - | - | - | - |
| | (60.545, -145.765) | Surface | 3/28/2019 | - | - | - | - |
| | (60.545, -145.765) | Surface | 5/1/2019 | 10.66 | Suspected Positive | No Match | Suspect |
| | (60.545, -145.765) | Surface | 5/1/2019 | - | - | - | - |
| | (60.545, -145.765) | Surface | 5/1/2019 | - | - | - | - |
| | (60.545, -145.765) | Surface | 5/1/2019 | - | - | - | - |
| Offal Discharge Site | (60.546, -145.772) | Surface | 4/21/2018 | 39.68 | Presumptive Positive | 100% Identity Match | Confirmed Positive |
| | (60.544, -145.772) | 3 | 3/29/2019 | - | - | - | - |
| | (60.544, -145.772) | 3 | 3/29/2019 | - | - | - | - |
| | (60.549, -145.768) | 3 | 3/29/2019 | - | - | - | - |
| | (60.549, -145.768) | 3 | 3/29/2019 | 7.57 | Suspected Positive | 100% Identity Match | Presumptive Positive |
| | (60.549, -145.769) | 3 | 3/29/2019 | - | - | - | - |
| | (60.549, -145.769) | 3 | 3/29/2019 | - | - | - | - |
| | (60.551, -145.765) | 3 | 3/29/2019 | - | - | - | - |
| | (60.551, -145.765) | 3 | 3/29/2019 | - | - | - | - |
| | (60.544, -145.772) | 3 | 4/26/2019 | - | - | - | - |
| | (60.544, -145.772) | 3 | 4/26/2019 | - | - | - | - |
| | (60.544, -145.772) | Surface | 4/26/2019 | - | - | - | - |
| | (60.549, -145.768) | 3 | 4/26/2019 | - | - | - | - |
| | (60.549, -145.768) | 3 | 4/26/2019 | - | - | - | - |
| | (60.549, -145.769) | 3 | 4/26/2019 | - | - | - | - |
| | (60.549, -145.769) | 3 | 4/26/2019 | - | - | - | - |
| | (60.549, -145.769) | Surface | 4/26/2019 | - | - | - | - |
| | (60.551, -145.765) | 3 | 4/27/2019 | - | - | - | - |
| | (60.551, -145.765) | 3 | 4/27/2019 | - | - | - | - |
| *Ichthyophonus* Lab Exposure | - | Surface | 10/10/2018 | 2.68E+04 | Presumptive Positive | 100% Identity Match | Confirmed Positive |
| | - | Surface | 10/10/2018 | 1.90E+04 | Presumptive Positive | 100% Identity Match | Confirmed Positive |
| No Template Con | - | - | - | - | - | No Match | Negative |

*Table 1.3.* Results from samples indicated for sequencing in *Table 1.2*.
Bolded samples indicate matches were found in GenBank. “Sequences Aligned” indicates the number of sequences successfully assembled. “Query Length” is the final product length (bp) post-processing with Sequencher. “Max Score” is the maximum alignment score generated by GenBank. “Query Cover” is the percent of query length included in matched alignments. “E-value” is calculated by GenBank and represents the number of alignments expected with a similar score by chance.

| Sample | Sequences Aligned | Query Length | Max Score | Query Cover | E-value | % Identity | Genus Matched |
|---|---|---|---|---|---|---|---|
| [1] No Template Control | 2 | 7 | - | - | - | - | - |
| | 2 | 1 | - | - | - | - | - |
| [2] **Offal Discharge Site** | **4** | **29** | **54.7** | **100%** | **3.0E-05** | **100.00%** | ***Ichthyophonus*** |
| | 2 | 26 | - | - | - | - | - |
| | 2 | 25 | - | - | - | - | - |
| [3] Offal Discharge Site | 4 | 235 | - | - | - | - | - |
| | 4 | 1291 | - | - | - | - | - |
| [4] **Cordova Harbor** | **4** | **29** | **54.7** | **100%** | **3.0E-05** | **100.00%** | ***Ichthyophonus*** |
| [5] **Lab Exposure** | **3** | **29** | **54.7** | **100%** | **3.0E-05** | **100.00%** | ***Ichthyophonus*** |
| [6] **Lab Exposure** | **4** | **29** | **54.7** | **100%** | **3.0E-05** | **100.00%** | ***Ichthyophonus*** |

# *ICHTHYOPHONUS* IN JUVENILE HERRING: AN INVESTIGATION OF A TRANSMISSION HOTSPOT

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## ABSTRACT

A geographic hot spot of high *Ichthyophonus* infection prevalence occurred in juvenile Pacific herring from Cordova Harbor, AK, USA during 2015 and 2019. Over-winter infection prevalence (0-5%) was similar to that of juvenile herring in other locations; however, prevalence in Cordova Harbor increased rapidly during the spring, peaking at 64% (2015) and 40% (2019) in June and July. Reasons for the geographical hot spot remain undetermined, but seasonal shifts in herring diet were coincident with the changing infection prevalence in Cordova Harbor.

## INTRODUCTION

In 1989, the *Exxon Valdez* oil spill released 260,000 barrels of crude oil into the Prince William Sound, Alaska, USA (Piatt et al. 1990). The spill devastated marine life and many economically important fish populations were directly or indirectly affected. Notably, the local Pacific herring (*Clupea pallasii*) population crashed shortly following the spill. In 1994, herring numbers fell from a predicted 134,000 tons to an observed 30,000 tons (Marty et al. 1995). The definitive cause of this crash remain unresolved, but researchers have focused on three hypotheses: (1) oil contamination caused developmental defects in herring, leading to delayed mortality (Carls et al. 1999; Incardona et al. 2013; Incardona et al. 2015), (2) high predation combined with poor nutrition led to high mortality (Pearson et al. 1999; Deriso et al. 2008; Pearson et al. 2012), and (3) recurrent disease outbreaks caused the initial decline and prevented recovery (Marty et al. 1998; Pearson et al. 1999; Marty et al. 2003). Others suggest that the crash was caused by the cumulative impacts of multiple mechanisms (Carls et al. 2002; Hulson et al. 2007; Rice and Carls 2007; Elston and Meyers 2009). In recent years, research effort shifted towards identifying factors that are influencing the failed population recovery; mortality from viral hemorrhagic septicemia and *Ichthyophonus* disease represents a leading hypothesis (Pearson et al. 1999; Marty et al. 2010).

*Ichthyophonus* disease is caused by a mesomycetozoean parasite, *Ichthyophonus hoferi* or *I. irregularis* (Gregg et al. 2016; hereafter referred to as *Ichthyophonus*). A generalist parasite, *Ichthyophonus* infects over 80 fish species, including 35 marine and 45 freshwater fish hosts

(Gregg et al. 2016). While *Ichthyophonus* is common and ubiquitous across northern temperate waters, the life cycle and mode of transmission are still poorly understood. In piscivorous fishes, *Ichthyophonus* is transmitted by ingestion of infected fish tissues (Gregg et al. 2012; Kocan 2018). How the parasite is transmitted to planktivores, including clupeids, is unknown. Currently, two leading hypotheses exist to explain transmission in clupeids including: (1) opportunistic ingestion of infected fish tissues and (2) transmission via ingestion of a reservoir host (Gregg et al. 2012; Hershberger et al. 2015). While no such reservoir host has been identified, successful transmission in the laboratory has been achieved by ingestion of infected fish tissues (Hershberger et al. 2015).

Previous work has documented unusually high rates of *Ichthyophonus* infection in juvenile herring from Cordova Harbor, Prince William Sound, Alaska, with the highest infection prevalence reported at 35% (Hershberger et al. 2016). The study found that Cordova Harbor had the highest infection prevalence of any site sampled in the Prince William Sound, and was the only harbor. To further understand factors associated with increased *Ichthyophonus* infection prevalence, the infection status of herring was surveyed in Cordova Harbor and several harbor and non-harbor sites within the Gulf of Alaska and Puget Sound (Washington). These sites were used to estimate the pattern of infection prevalence inside and outside of harbors and to investigate whether diet might influence infection prevalence.

## METHODS

### *Field Sampling Methods*

Between the months of April 2018 and May 2019, *C. pallasii* juveniles (year class 2018, <150 mm) were collected by cast net, dip net, purse seine, or trawl from 18 sites across Alaska and Washington, USA (*Figure 2.1*). Of these, four sites included similarly sized harbors: Cordova, AK (60.545°N, 145.765°W), Homer, AK (59.642°N, 151.548°W), Port Angeles, WA (48.126°N, 123.453°W), and Port Ludlow, WA (47.922°N, 122.686°W). An additional 12 sites, representing non-harbor habitats of Prince William Sound and Puget Sound, were sampled opportunistically. Fork length (mm), wet weight (g), and clinical signs of *Ichthyophonus* infection were recorded for each fish. *Ichthyophonus* infection was confirmed by microscopic analysis of heart-explant cultures grown in a broth medium (Hershberger et al. 2019). Age was confirmed on a subsample of individuals, based on scale or otolith annuli (Watson 1964; Chilton and Stocker 1987; *Table 2.1*).

### *Stomach Contents*

Due to logistical constraints, the preservation of fish stomachs differed slightly among sites. For fish collected in Cordova, AK, digestive tracts were dissected immediately following capture and euthanasia, preserved in 75.5% grain alcohol, and frozen at –20°C. Upon returning to the laboratory, the preservative was replaced with 95% ethanol and returned to –20°C. For fish collected in Homer Harbor, Alaska and the non-harbor site in the Prince William Sound, whole fish samples were immediately placed on ice, transported to the laboratory, and stored at –20°C within 48 hours of initial capture. Stomachs were thawed and placed in 95% ethanol prior to stomach content analysis. Stomach contents were analyzed under a dissecting microscope to quantify the following digestion metrics: 1) stomach fullness, 2) digestion level, estimated according to the state of prey exoskeletons, and 3) prey items identified to the lowest taxonomic identity, which were counted and blot weighed. Stomach content analysis was only conducted for fish from Alaska sites.

### *Statistical Analysis*

Differences in size of captured fish were analyzed with a Generalized Linear Model. The model was generated with R version 3.6.3 with the *lmer()* function in the "lme4" package, and p-values were calculated with the "lmerTest" package (Bates et al. 2015; Kuznetsova et al. 2017). The following generalized linear model was run on a Gaussian distribution:

*Fork Length ~ Habitat Type*Time*

A generalized additive model with a binomial distribution and a logit link (Guisan et al. 2002) was used to compare *Ichthyophonus* infection prevalence among sites. The response variable was *Ichthyophonus* infection status, and the fixed effects included (1) whether the collection site was located in Cordova Harbor (1 = yes, 0 = no), (2) whether the collection site was located in a harbor or non-harbor (1 = yes, 0 = no), and (3) host fork length. Two smoothers accounted for (1) the date of sampling (i.e., with day 1 = first day of sampling in 2018, year removed to account for the effect of seasonality; # knots = 10) and (2) year of sampling (i.e., to account for interannual variation in prevalence between 2015, 2018, and 2019; # knots = 3; (Hastie and Tibshirani 1986; Guisan et al. 2002). These analyses were conducted using the *gam()* function in the "mgcv" package in R (Wood 2017).

Differences in herring diet were compared using a permutational multivariate analysis of variance (PERMANOVA) test assessing Bray-Curtis distances in the composition of stomach contents. These analyses were done for both prey item counts and mass. Diet composition was calculated as percent mass (grams of diet item *X*/grams of all diet items) and percent count (number of diet item *X*/number of all diet items) for each fish examined. Taxonomic categories for diet items were summarized to the following levels: harpacticoid copepods, non-harpacticoid copepods, barnacle larvae, non-copepod and non-barnacle crustaceans, molluscs, annelid worms, foreign materials (including fibers), unidentified eggs, and diatoms. Copepod taxa could not be differentiated in larval stages and were therefore lumped with "non-harpacticoid copepods". The *adonis()* function in the "vegan" package in R (Oksanen et al. 2019) was used to perform a permutational multivariate analysis of variance (PERMANOVA) test, assessing Bray-Curtis distances in the composition of gut contents. To test for differences in stomach content composition among sites, two models were run (with response variables mass and counts, respectively) with the following fixed effects: (1) whether the site was Cordova or a non-Cordova site, (2) fish fork length, (3) sample site (i.e., to account for multiple fish individuals evaluated at each site), and (4) digestion grade. To test the difference between harbor and non-harbor sites, a model was run each for measurements of percent mass and abundance with the following fixed effects: (1) whether the collection site was located in a harbor (1 = yes, 0 = no), (2) fish fork length, (3) sample site (i.e., to account for multiple fish individuals evaluated at each site), and (4) digestion grade. P-values were obtained from 5,000 permutations of the data. A SIMPER analysis was then performed to identify the diet items associated with the differences (identified by the PERMANOVA analysis) between all sites and between harbor versus non-harbor sites, using the *simper()* function in the "vegan" package in R.

Tests for seasonal shifts in stomach contents were conducted using samples collected in Cordova Harbor from late-February through early June 2019. The same taxonomic grouping and

PERMANOVA analytical approach were used, and two models were generated (one for counts, one for mass) with the following fixed factors: (1) sampling date, (2) fish fork length, and (3) digestion grade.

## RESULTS

Herring captured at non-harbor sites grew at a faster rate than herring within harbors (interaction term estimate ± standard error = +0.038 ± 0.0005, t=7.03, $p<0.0001$). Overall, all herring increased in size with time (time main effect estimate = +0.015 ±0.003, t=4.73, $p<0.0001$, *Figure 2.2*). When fork lengths of all herring were pooled across all time points, those at non-harbor sites were larger than those collected within harbors (99.61 mm ± 0.74 mm and 70.57 mm ± 0.28 mm, respectively; habitat main effect estimate = -674 ± 100, t=-6.73, $p < 0.0001$).

Juvenile herring from Cordova Harbor had the highest measured infection prevalence of any site (63.75% in 2015, 40.00% in 2019; *Table 2.2*) and were more likely to be infected with *Ichthyophonus* than were those from other sites (estimate ± SE = +1.52 ± 0.374, z = +4.08, n = 2205, $p < 0.0001$; *Figure 2.3*). Fish collected from harbor sites were more likely to be infected with *Ichthyophonus* than were fish collected at non-harbor sites (effect of non-harbor, estimate ± SE = –1.56 ± 0.470, z = –3.39, n = 2205, p = 0.0007). The smoother explained a significant proportion of variance in the data, suggesting that there was significant temporal variability in *Ichthyophonus* prevalence ($\chi^2 = 114.9$, $p < 0.0001$). The data show a marked pattern in Cordova where infection prevalence begins to rise in April (*Figure 2.3*).

Herring stomach contents differed among the three sites (Cordova Harbor, Homer Harbor, and a non-harbor site within the Prince William Sound) for both measures of *relative abundance* ($F_{1,156} = 14.64$, p = 0.0002) and *percent mass* ($F_{1,157} = 3.47$, p = 0.0262; *Figure 2.4*). However, when comparing Cordova as a unique site against non-Cordova sites, diet compositions of herring from Cordova Harbor were only unique from the remaining sites in the measurement of diet item *relative abundance* ($F_{1,156} = 25.31$, p = 0.0002), but not *percent mass* ($F_{1,157} = 1.94$, p = 0.121; *Figure 2.4*C). Results of the SIMPER post-hoc analysis show that diet compositions by measures of diet item *relative abundance* were most similar between Homer Harbor and the non-harbor site in the Prince William Sound (34.29% similar) while diet compositions from Cordova Harbor and the on-harbor site in the Prince William Sound were the least (25.90% similar). Similarities between diet compositions were different when measured by *percent mass*, with herring diet compositions from Cordova Harbor and the non-harbor site in the Prince William Sound being the most similar (68.29% similar) and diet compositions from Cordova Harbor and Homer Harbor being the least similar (60.51% similar; *Figure 2.4*; *Table 2.3*).

Diet compositions were significantly different between harbors and non-harbors for both measurements of *relative abundance* and *percent mass* ($F_{1,156} = 18.96$, $p < 0.0002$; $F_{1,157} = 4.06$, p = 0.015; respectively). A SIMPER post-hoc test shows that diet compositions were 28.11% similar between herring caught in harbors and non-harbors when measured by *relative abundance*, while measures by *percent mass* show diet compositions in herring from harbors and non-harbor sites were 68.17% similar (*Figure 2.4*; *Table 2.3*).

# DISCUSSION

Factors unique to Cordova Harbor likely accounted for the high *Ichthyophonus* prevalence observed. The elevated prevalence of *Ichthyophonus* in harbors does not fully explain the unusually high prevalence in herring from Cordova, because peak infection prevalence (40% in June 2019 and 74% in June 2015) far exceeded peak prevalence in any other harbor (Homer, 11% in May 2019). Factors unique to Cordova such as seasonality in diet composition, plankton assemblages, or exposure to fish tissues from nearby fish processing plants may be linked with high infection prevalence.

The seasonality of *Ichthyophonus* prevalence and in diet composition might provide a clue as to why herring in Cordova harbor experience exceptionally high infection rates. Non-harpacticoid copepods and barnacles were the main drivers of the difference in stomach contents (for both count and weight data) between March 31, 2019 and May 1, 2019. Herring collected on March 31, 2019 had eaten few barnacle cyprinus or barnacle nauplii and their diet was composed mostly of non-harpactoid copepods. By May 1, 2019, herring diet had shifted to include mostly barnacle larval stages (*Figure 2.4*; *Table 2.3*). This diet shift coincides temporally with a spike in infection prevalence. Furthermore, barnacles were only seen as a major component of diet in herring captured in harbors (Cordova and Homer), but not of herring from non-harbors (non-harbor site in the Prince William Sound), aligning with the findings that herring from harbors are more infected with *Ichthyophonus* than herring from non-harbors. While this correlation suggests barnacles as a potential reservoir of *Ichthyophonus*, it is important to note that we only have one site and one time-point for non-harbor fish, and this correlation is therefore tenuous. Previous studies have used molecular methods to screen copepods as potential reservoir hosts of *Ichthyophonus* but have been unable to confirm the presence of *Ichthyophonus* in histology samples (Lowe 2011). It would be worthwhile to screen barnacle larval stages as a potential reservoir host of *Ichthyophonus* using the same protocols.

Other seasonal conditions in Cordova may also inform wild *Ichthyophonus* transmission in herring. Changes in temperature have not been linked to increases in infection prevalence (Gregg et al. 2011), so other patterns might instead be a driving factor. The late winter / early spring increase in *Ichthyophonus* prevalence in Cordova also corresponds with the opening of the halibut fishery and halibut processing in Cordova. Three offal discharge pipes begin emitting processed halibut as early as April just outside the harbor, and there are six total processors operating in Cordova. While infection prevalence in Cordova Harbor was measured in fish caught within the harbor, spatially separated from the offal discharge pipes by a breakwater, it is possible that the herring were exposed to offal discharge. As the leading *Ichthyophonus* transmission hypothesis points to infection by ingestion (Kocan et al. 2010; Hershberger et al. 2015; LaPatra and Kocan 2016; Kocan 2018), and Cordova Harbor fish processing plants process *Ichthyophonus* hosts such as Pacific halibut (*Hippoglossus stenolepis)* (Alaska Department of Environmental Conservation 2015; Gregg et al. 2016), elevated infection prevalence in Cordova Harbor could result from herring feeding on infected offal. Nearly 70 years ago, it was hypothesized that fish processing plant waste can aggregate parasites and disease-causing agents, thereby increasing infection prevalence in receiving waters (Dogiel 1958). To date, no studies exist to explicitly measure the impact of offal on disease prevalence in a wild population of fish, yet this pattern is well documented in other systems and species

(Ludlam 1954; Grainger and Jenkins 1996; Ballachey et al. 2002; Fatma and Mahdey 2010). In terrestrial systems, offal and other animal waste is often regulated, as it is known to spread disease to both humans and wildlife (Ludlam 1954; Ballachey et al. 2002; Fatma and Mahdey 2010). As Cordovan fish processing plants are classified as remote sites, they are excused from the regulation requiring screening of offal to meet limitations for total suspended solids, oil and grease, and allowable pH levels and are only required to grind offal to ½-inch pieces (Federal Water Pollution Control Act 2007; Stoner 2013). While fish tissues are not an expected component of herring diet, it is possible that herring may inadvertently ingest offal during filter feeding or opportunistically feed on the available tissues. Due to the grinding treatment and small sample volume of herring stomachs, potential offal material was not differentiated from "unidentified materials" via microscopic analysis, future studies might revisit this question by analyzing stomach contents with molecular methods to detect offal, as in White et al. 2014.

Increased *Ichthyophonus* transmission within harbors in general could be due to several other factors that increase host susceptibility or *Ichthyophonus* transmissibility for harbor-bound herring relative to their non-harbor cohorts. While harbors and other enclosed habitats may increase transmission of directly transmitted parasites by increasing the opportunities for infected individuals to interact with and infect non-infected individuals, (Anderson and May 1978; Burdon and Chilvers 1982; Greer et al. 2008), direct transmission of *Ichthyophonus* in clupeids is not supported by laboratory-controlled studies (Gregg et al. 2012).

There are many potential mechanisms driving the difference in herring body size between harbor and non-harbor sites, and whether this relates to *Ichthyophonus* prevalence within and outside of harbors is unknown. The difference in body size might be caused by size-dependent triggers of migration patterns of juvenile herring, where the migration from shallow rearing waters to deeper offshore waters is driven by maturity to a specific size mark, rather than by age of the herring. Alternatively, harbor-bound herring may have smaller body sizes because of lower food availability within the harbor. Why the herring remain in the harbor despite reduced food resources is undetermined, but it is possible that there is a survival trade-off, with the harbor offering protection from predators or that the shape of the harbor effectively traps schooling herring. As herring within all harbors were much smaller than those captured at non-harbor sites, this pattern is not specific to Cordova Harbor, suggesting the existence of a larger pattern driving size differences in herring.

Herring sampled within Cordova harbor may not accurately represent the larger population of Prince William Sound herring, and the heightened infection prevalence may not be of long-term significance to the larger population of Prince William Sound herring.
It is unknown how harbor-bound herring reconnect with the larger metapopulation, and as such, the impacts of high *Ichthyophonus* infection prevalence in juveniles within the harbor cannot be defined. As herring within the harbor represent a small fraction of the Prince William Sound population, it is likely that high infection prevalence in harbor-bound herring has minimal impact on the herring population as a whole. It is also possible that the Spring increase in infection prevalence is a result of healthy fish differentially migrating to deeper waters. While this could cause an increase in infection prevalence which does not accurately reflect increased *Ichthyophonus* transmission, this mechanism appears to be significant only within Cordova harbor, and not for herring in other harbors sampled.

# FIGURES AND TABLES

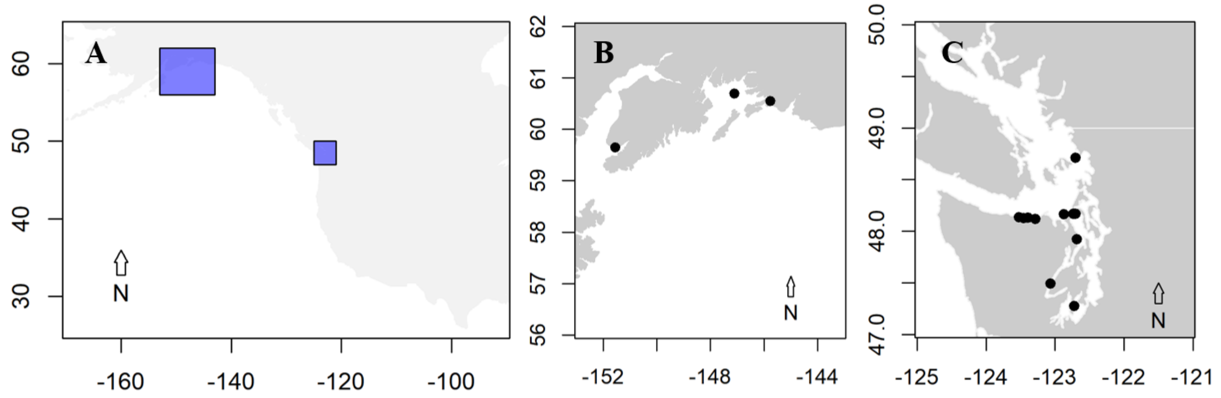

*Figure 2.1.* Map of sampling sites.
Squares in (A) displays the geographic locations of panels (B) and (C). (B) Sites in Alaska, including Cordova Harbor, Non-harbor site in the Prince William Sound, and Homer Harbor (C) Sites in Washington State, including all sites listed in *Table 2.2*.

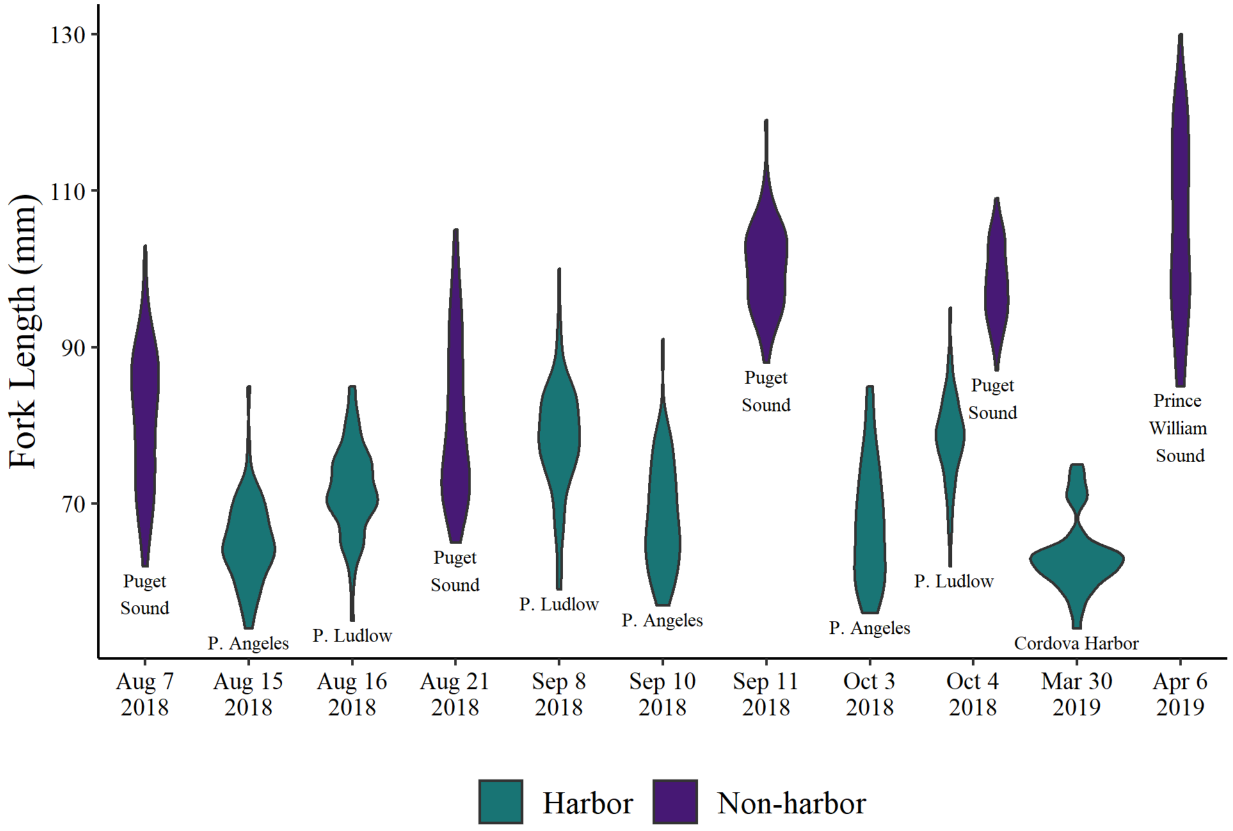

*Figure 2.2.* Fork lengths by habitat type through time.
A subset of fork length distributions is shown above where sample events for both habitat types overlap. Herring fork lengths of herring captured at harbor sites are shown in green; fork lengths of herring captured in non-harbor sites are shown in purple.

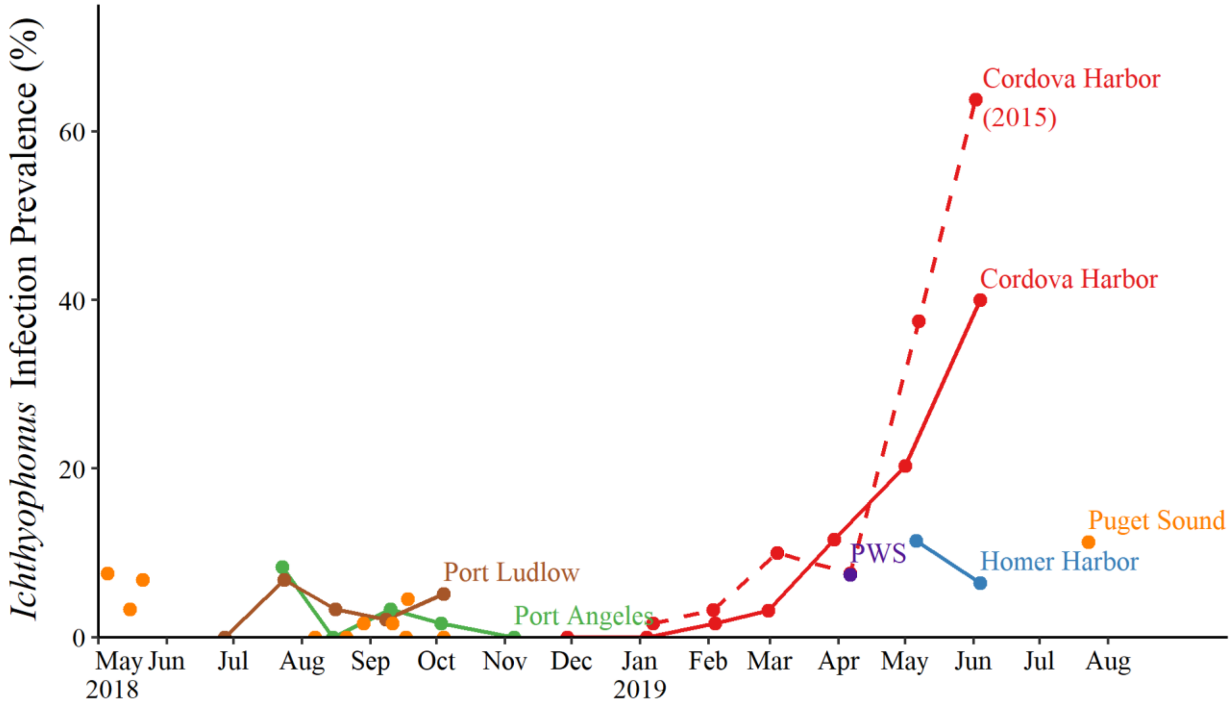

*Figure 2.3*. Prevalence of *Ichthyophonus* by site.
*Ichthyophonus* infection prevalence is shown by sampling date May 2018 – August 2019, including 2015 Cordova harbor infection prevalence (red dotted line).

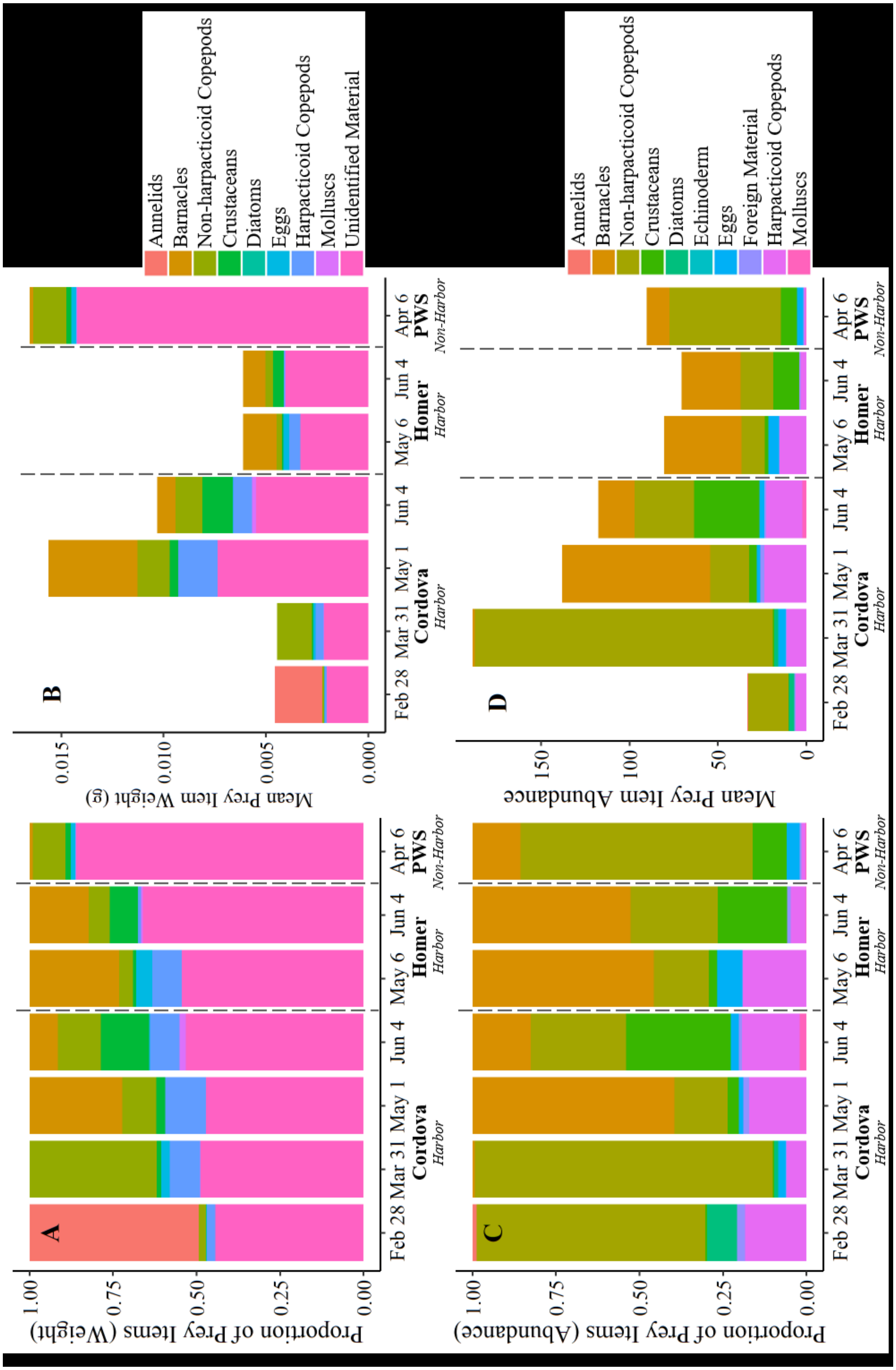

*Figure 2.4.* Stomach contents for each sampling event by mass (A, B) and counts (C, D). Prince William Sound is abbreviated to (PWS) for all plots. (A) shows proportion of diet items by mass, (B) shows mean mass (g) of diet items, (C) shows proportion of diet items by counts, (D) shows mean count of diet items. These data were analyzed with PERMANOVA and SIMPER tests; statistical outcomes are reported in the results section of the text.

*Table 2.1.* Results of aging analysis from otoliths and scales.

| **Site** | **Habitat** | **Month Collected** | **Length Range** | **Length Mean (N)** | **Aging Method** | **Age Class** |
|---|---|---|---|---|---|---|
| Cordova, AK | Harbor | June 2019 | 70.67 mm | 70.67 (1) | Otolith | 2018 |
| Homer, AK | Harbor | May 2019 | 86-93 mm | 90.00 (3) | Otolith | 2018 |
| Mats Mats, WA | Non-harbor | July 2019 | 135-144 mm | 141.80 (5) | Scale | 2018 |
| Prince William Sound, AK | Non-harbor | June 2019 | 90-105 mm | 98.00 (3) | Scale & Otolith | 2018 |

*Table 2.2.* A summary of fork lengths and *Ichthyophonus* infection prevalence by site and date.

| Site | Habitat | Coordinates | Gear Type | Sample Date | Length (mm) ± SD | Mass (g) ± SD | Prevalence (n) |
|---|---|---|---|---|---|---|---|
| Cordova Harbor (AK) | Harbor | (60.545, -145.765) | Cast Net | 1/7/2015 | 71.02 ± 8.70 | 2.52 ± 0.94 | 1.67% (60) |
| | | | | 2/3/2015 | 67.38 ± 5.96 | 2.04 ± 0.62 | 3.28% (61) |
| | | | | 3/4/2015 | 81.41 ± 8.16 | 3.68 ± 1.05 | 10.00% (80) |
| | | | | 4/6/2015 | 69.64 ± 6.89 | 2.17 ± 0.70 | 7.59% (80) |
| | | | | 5/7/2015 | 75.41 ± 9.52 | 3.16 ± 1.19 | 37.50% (80) |
| | | | | 6/2/2015 | 84.53 ± 10.94 | 4.59 ± 1.92 | 63.75% (80) |
| Cordova Harbor (AK) | Harbor | (60.545, -145.765) | Cast Net | 11/29/2018 | 58.05 ± 3.68 | 1.57 ± 0.38 | 0.00% (60) |
| | | | | 1/4/2019 | 79.76 ± 12.14 | 4.28 ± 1.93 | 0.00% (60) |
| | | | | 2/4/2019 | 64.65 ± 5.84 | 2.04 ± 0.47 | 1.67% (60) |
| | | | | 2/28/2019 | 67.83 ± 4.99 | 2.27 ± 0.46 | 3.17% (64) |
| | | | | 3/30/2019 | 63.37 ± 4.70 | 1.91 ± 0.47 | 11.63% (43) |
| | | | | 5/1/2019 | 64.25 ± 6.03 | 2.65 ± 0.88 | 20.34% (59) |
| | | | | 6/4/2019 | 74.57 ± 13.25 | 4.12 ± 2.46 | 40.00% (35) |
| Homer Harbor (AK) | Harbor | (59.643, -151.548) | Cast Net | 5/6/2019 | 81.74 ± 6.42 | 4.22 ± 0.73 | 11.43% (35) |
| | | | | 6/4/2019 | 83.55 ± 7.24 | 5.43 ± 1.48 | 6.45% (31) |
| Prince William Sound (AK) | Non-harbor | | Purse Seine | 4/6/2019 | 105.23 ± 11.55 | 12.72 ± 4.33 | 7.41% (81) |
| Port Angeles (WA) | Harbor | (48.126, -123.453) | Cast Net | 7/23/2018 | 55.00 ± 6.15 | 1.86 ± 0.91 | 8.33% (60) |
| | | | | 8/15/2018 | 65.12 ± 5.43 | 2.70 ± 0.77 | 0.00% (60) |
| | | | | 9/10/2018 | 68.35 ± 6.86 | 3.20 ± 1.08 | 3.33% (60) |
| | | | | 10/3/2018 | 67.53 ± 7.55 | 2.87 ± 1.06 | 1.67% (60) |
| | | | | 11/5/2018 | 71.23 ± 9.21 | 3.52 ± 1.03 | 0.00% (60) |
| Port Ludlow (WA) | Harbor | (47.922, -122.686) | Cast Net | 6/27/2018 | 54.81 ± 5.56 | 1.93 ± 0.59 | 0.00% (60) |
| | | | | 7/24/2018 | 62.10 ± 6.67 | 2.61 ± 0.79 | 6.78% (60) |
| | | | | 8/16/2018 | 71.82 ± 5.80 | 3.79 ± 0.85 | 3.33% (60) |
| | | | | 9/8/2018 | 78.32 ± 7.03 | 4.65 ± 1.06 | 2.08% (96) |
| | | | | 10/4/2018 | 78.17 ± 5.73 | 4.28 ± 0.95 | 5.17% (60) |
| Puget Sound (WA) | Non-harbor | (47.274, -122.723) | Trawl | 5/5/2018 | 99.70 ± 6.06 | 8.06 ± 1.56 | 7.41% (54) |
| | | (48.132, -123.392) | Trawl | 5/15/2018 | 118.38 ± 6.62 | 16.08 ± 2.72 | 3.33% (60) |
| | | (48.710, -122.703) | Trawl | 5/21/2018 | 116.07 ± 7.59 | 14.65 ± 3.55 | 6.78% (60) |
| | | (48.169, -122.703) | Dip Net | 8/7/2018 | 80.71 ± 8.96 | 5.51 ± 1.72 | 0.00% (60) |
| | | (48.170, -122.735) | Dip Net | 8/21/2018 | 80.58 ± 10.15 | 5.79 ± 2.21 | 0.00% (60) |
| | | | Dip Net | 8/29/2018 | 97.33 ± 7.65 | 9.84 ± 2.06 | 1.67% (60) |
| | | | Dip Net | 9/11/2018 | 100.32 ± 5.67 | 10.34 ± 1.89 | 1.67% (60) |
| | | (48.119, -123.283) | Trawl | 9/17/2018 | 84.98 ± 7.66 | 6.14 ± 1.82 | 0.00% (60) |
| | | (48.134, -123.521) | Trawl | 9/18/2018 | 98.95 ± 21.03 | 11.01 ± 3.15 | 4.55% (22) |
| | | (48.164, -122.871) | Dip Net | 10/4/2018 | 98.17 ± 4.90 | 8.67 ± 1.17 | 0.00% (60) |
| | | | Trawl | 7/23/2019 | 141.36 ± 6.22 | 31.84 ± 4.06 | 11.32% (60) |

*Table 2.3.* Summary of most abundant prey items by factor for both abundance and mass measurements.

| **Factor** | **Mean Proportion of Diet by Abundance** | | **Mean Proportion of Diet by Mass** | |
|---|---|---|---|---|
| Harbor | Harpacticoid Copepods | 32.80% | Barnacles | 10.24% * |
| Offshore | Non-harpacticoid Copepods | 68.57% | Non-harpacticoid Copepods | 10.32% * |
| Cordova | Harpacticoid Copepods | 40.04% | Non-harpacticoid Copepods | 8.01% * |
| Combined Non-Cordova | Barnacles | 40.04% | Barnacles | 11.42% * |
| Homer | Barnacles | 52.13% | Crustaceans | 3.96% * |
| Prince William | Non-harpacticoid Copepods | 68.57% | Non-harpacticoid Copepods | 10.32% * |

*Unidentified material is the most abundant item by mass for all factors; the second-most abundant prey item is listed instead.

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# VITA

Catrin Wendt studies patterns of Ichthyophonus disease in juvenile herring, with an emphasis on the herring population affected by the 1989 Exxon Valdez oil spill in Alaska. As a collaboration with USGS and the Prince William Sound Science Center, her research uses both traditional parasite culture and genetic methods like environmental DNA (eDNA).

More broadly, Catrin Wendt's past and present work focuses on marine conservation and ecology covering a variety of topics in a variety of marine ecosystems, including monitoring invasive European green crabs in salt marshes and building underwater camera systems for coral reef community conservation.