# Surveillance for antibiotic resistant *E. coli* in the Salish Sea ecosystem and surrounding regions

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## Abstract

Surveillance for antibiotic resistant *E. coli* in the Salish Sea ecosystem and surrounding regions

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Antimicrobial resistant bacteria (ARB) can be shared between humans and animals through a common environment. The surveillance of ARB in the environment can inform us about contamination of shared ecosystems, like the Salish Sea, and how that contamination affects both the animals that rely on the ecosystem and humans who also live within it. Researchers detect ARB and their antibiotic resistance genes (ARG) by sampling animals and their environment. With this information, researchers can draw conclusions on the routes of transmission and plan interventions to reduce the spread of ARB.

The objective of this study was to take a One Health approach to identifying *E. coli* in the Salish Sea assessing whether they were phenotypically and genotypically resistant to antibiotics and if so, which resistance genes they carried. The *E. coli* were isolated from samples of marine

water, marine animals from the Salish Sea, river otters in a freshwater river watershed, and freshwater sources. The isolates were analyzed using both antimicrobial susceptibility testing and whole-genome sequencing (WGS).

We collected marine water samples from North Puget Sound, Central Puget Sound, South Puget Sound, and the Strait of Juan de Fuca, freshwater from streams near marine beaches, and fecal samples from harbor porpoises (*Phocoena phocoena*), harbor seals (*Phoca vituline*), river otters (*Lontra canadensis*), and English sole (*Parophrys vetulus*). We isolated 551 *E. coli* colonies and characterized 305 isolates: 212 from marine water, 5 from freshwater, 3 from marine water by beaches, 52 from harbor seals, 7 from harbor porpoises, 24 from river otters, and 2 from English sole. Isolates were analyzed using minimum inhibitory concentrations (MIC): 20 (6.6%) were intermediate and 31 (10.2%) resistant to $\geq$ 1 class of antibiotics.

Whole genome sequence, sequence type (ST), resistance genes, and virulence factors were determined from sequence data. Using multilocus sequence typing (MLST), a total of 196 unique STs were identified. This included extra-intestinal pathogenic of *E. coli* (ExPEC)-associated STs (ST10, ST38, ST58, ST69, ST73, ST117, ST131, and ST405), which were identified in 37 isolates. The most commonly occurring ST was ExPEC-associated type, ST10 (n=12), and the least common ExPEC ST was ST405 (n=1). ResFinder was used to identify the genotypic antibiotic profile of resistant and intermediate *E. coli* isolates.

Correlation between resistance phenotype and genotype varied by specific antibiotic. Isolates that were intermediate and resistant to tetracyclines were found to have the best correlation, with all 16 phenotypically resistant/intermediate isolates carrying either *tet*(A) or *tet*(B) or both *tet* genes. Intermediate and resistant isolates (n=51) were subjected to

VirulenceFinder analysis for virulence factor characterization; *gad* (glutamate decarboxylase) was the most commonly identified virulence factor, appearing in 68% of isolates (n=35).

This study found that in marine water only 7% of the *E. coli* were non-susceptible (resistant or intermediate resistant) to antibiotics. In contrast, non-susceptible *E. coli* accounted for 26.9% of the isolates from marine mammals and 70% of the *E. coli* from river otter stools, making them potential sentinels for antibiotic-resistant *E. coli* in the Salish Sea and other ecosystems. Monitoring of the marine mammal microbiome may lead to information about how antimicrobial resistant genes (AMR) persist in the local ecosystems.

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**ABBREVIATIONS AND ACROYNYMS**

ARB: Antibiotic resistant bacteria

ARG: Antibiotic resistant gene

AMR: Antimicrobial resistance

CLSI: Clinical and Laboratory Standards Institute

*E. coli*: *Escherichia coli*

EMB: Eosin methylene blue

EPA: Environmental Protection Agency

ExPEC: Extraintestinal Pathogenic *E. coli*

SNP: Single nucleotide polymorphism

ST: Sequence type

MIC: Minimum inhibition concentration

MLST: Multilocus sequence type

MPN: Most probable number

NCBI: National Center for Biotechnology Information

PCR: Polymerase chain reaction

WA DOH: Washington Department of Health

WGS: Whole genome sequencing

WWT: Wastewater

WWTP: Wastewater Treatment Plant

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## INTRODUCTION

The anthropogenic use of antibiotics in clinical, agricultural, and community settings has contributed to the development of antibiotic resistance bacteria (ARB).[1] If left unaddressed, antimicrobial resistance has the potential to impact human, animal, and environmental health, making it a One Health issue for the world.[2] There are multiple routes of transmission of ARB and antibiotic resistant genes (ARG) including movement of contaminated wastewater and soil through an environment ecosystem such as the Salish Sea, exposing marine and land animals and humans.

The Salish Sea is shared between Washington State, USA and British Columbia, Canada. It is a marine inland sea with a complex estuarine system of interconnected marine waterways and basins, with one major (Strait of Juan de Fuca) and two minor connections (Deception Pass and Swinomish Channel) to the open Pacific Ocean. The Salish Sea is used for recreational purposes including swimming, fishing, and boating, as well as, for commercial aquaculture of fish and shellfish and is home to many animals and plants. It has major urban populations centers along its shores and is home to two contaminated Superfund sites and rivers drain from farmlands.

Over the past few decades there has been exponential growth and development around the Salish Sea especially within the State of Washington.[3] This has led to significant contamination, and threat to the health of the ecosystem including the marine waters, its flora and fauna, and the human population.[4] The Salish Sea receives treated wastewater (WWT) from wastewater treatment plants (WWTP) along the shoreline within the USA and Canada, as well as, partially treated wastewater released near the Strait of Juan de Fuca from Victoria, B.C., Canada.[5,6] Antibiotics used for human and agricultural purposes can be discharged directly into

rivers, irrigation, and marine water where ARB, ARGs and antibiotic residues may persist.[4] Previous reports have suggested that the Salish Sea has hot spots for high levels of antibiotic resistance genes, and antibiotic residues and have been identified in local salmon.[4,7] Previously we have cultured antibiotic resistant extra-intestinal pathogenic (ExPEC) *E. coli* from the feces of the endangered Southern Resident killer whale (*Orcinus orca*)[8] that bear similarities to human pathogenic strains. We conducted a larger study on presence of ARB throughout the Salish Sea environment and its animals using *E. coli* as a marker organism for antibiotic resistance in the environment and wildlife.

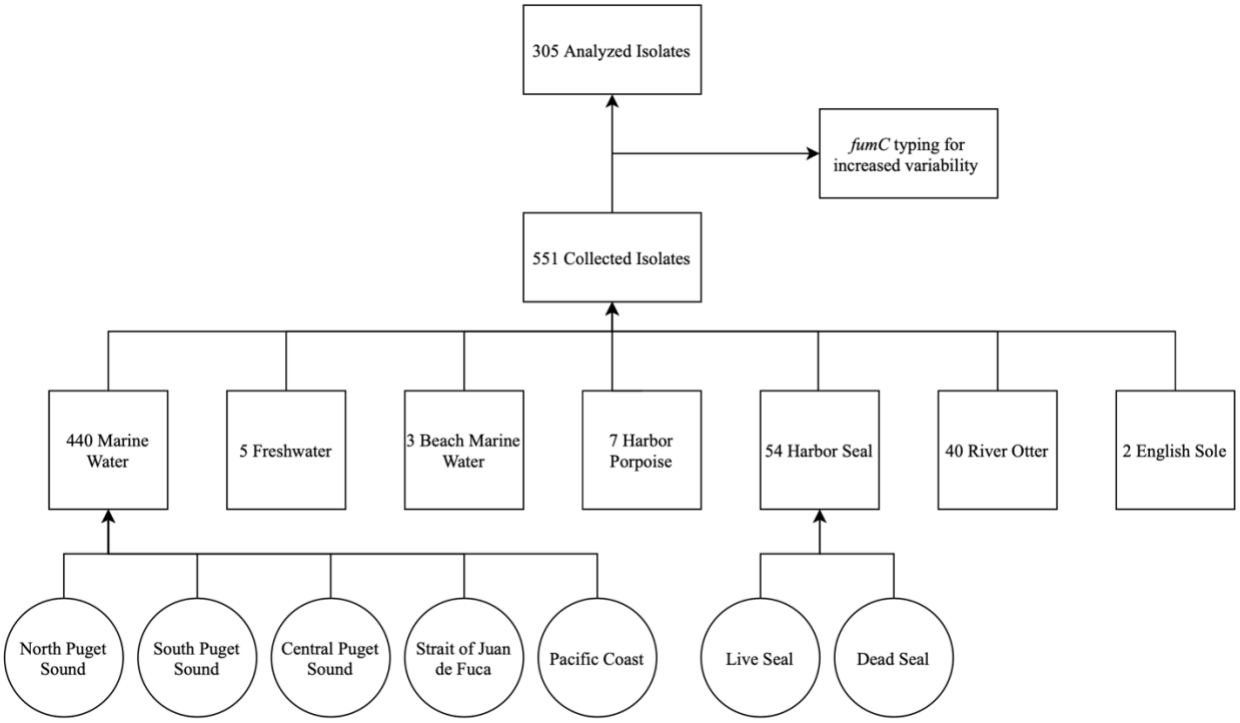
Antibiotic resistant *E. coli* have been previously identified in wildlife primarily from land animals and birds.[9] In contrast, most studies on marine animals have looked at bacteria that cause diseases or are relatively easy to isolate such as respiratory bacteria rather than normal flora intestinal bacteria such as *E. coli.*[10,11] Other studies have focused on ARB associated with fish in aquaculture setting especially in context of antibiotic treatment, but rarely do these studies include *E. coli.*[12,13]

Our pilot study focused on isolating and characterizing *E. coli* from otter latrines and cultured antibiotic resistant *E. coli* in most of the sites (data included in the current study). In previous studies elsewhere, *E. coli* has been isolated over a wide global area in diverse ecosystems, organisms, and locations, making it an ideal marker organism. *E. coli* has a large number of sequence types (ST) identified using Multi-Locus Sequence Type methods (MLST) that have been described in terms of pathogenesis. It also has a number of well characterized antimicrobial resistance genes[14] and virulence factors.[15]

## MATERIALS AND METHODS

The study included 551 isolates of *E. coli*, of which 305 were further characterized (**Figure 1**). These included isolates from marine waters associated with shellfish beds collected as part of routine public health monitoring, *E. coli* from marine water samples obtained from beach sites, *E. coli* from freshwater streams emptying into the Salish Sea, *E. coli* from river otter (*Lontra canadensis*) fecal collections (latrines), *E. coli* from rectal swabs and feces from live and dead harbor seals (*Phoca vituline*), dead porpoise (*Phocoena phocoena*) fecal samples, and *E. coli* isolated from English sole (*Parophrys vetulus*). Following isolation, the isolates were *fumC* typed using published methods to reduce clonality (**Figure 1**).[16] They were then tested for antibiotic susceptibility and submitted for whole genome sequencing.

**Figure 1.** Flowchart of sample collection source



## *E. coli* Collection and Processing

The aim of the study was to characterize the most diverse set of *E. coli* isolates from our sample collection. To do this, *fumC* typing was performed as previous described using *E. coli* MG1655 as a positive control.[16] Based on these results, we selected different *fumC* types from isolated collected in the same general location and time period, in order to characterize the most clonally diverse subset among the full collection.

Water Samples

Fresh water samples were randomly collected from Piper's Creek (Carkeek Park, Seattle, WA) and a beaver pond (Golden Gardens Park, Seattle, WA) during 2019 (**Figure 2A**). A total of 250 mL of freshwater were taken from each freshwater site one day for each site. Standard Quanti-Tray 2000 (IDEXX Laboratories, Westbrook, ME) protocol for *E. coli* was performed using 100 mL of water. For a subset of freshwater samples, a second IDEXX tray was set up with the addition of 1 mg/L cefotaxime (Thermo Fisher Scientific, Pittsburgh, PA) to water to screen for resistant isolates. We found both resistant and susceptible *E. coli*; one isolate with each *fumC* type was included in the study (n=5).

Marine water samples were taken at beaches close to shore at the same time as freshwater samples collection (n=3). A 100 ml sample of marine water was taken close to the shore of the beach at Carkeek Park and at the beach of Golden Gardens Park (**Figure 2A**). A 1:10 dilution of marine water was made using deionized sterile water. Another 1:10 dilution of marine water (10 ml seawater and 90 ml deionized sterile water) with an addition of 1 mg/L cefotaxime was made to select for resistant *E. coli*. There was no growth in the antibiotic containing trays.

Marine water samples from Washington Department of Health (WA DOH) were collected from GPS-located sites associated with shellfish beds in the Salish Sea; 212 isolates

were selected from a total of 440 *E. coli* from marine water. These water samples were taken as part of the WA DOH Shellfish Growing Program that monitors the Salish Sea. Marine water samples collected for the program were submitted to Public Health's Water Bacteriology Laboratory for fecal coliform analysis, using the Environmental Protection Agency (EPA) modified A-1 method.[17] We selected locations within four quadrants of the Salish Sea: North Puget Sound, Central Puget Sound, South Puget Sound, and Strait of Juan de Fuca (**Figure 2A**). The goal was to sample ≥ 50 *E. coli* isolates from each quadrant (**Table 1**).

The most probable number (MPN) of *E. coli* in the Salish Sea ranged from two to 13,000 colonies, with an average of 51 MPN in the WA DOH marine samples. Because of the way in which WA DOH isolates were collected we were unable to select directly for antibiotic-resistant *E. coli*.

English Sole samples

English sole were caught during summer 2019 by the WA Marine Resources Division (Washington Department of Fish and Wildlife) as part of annual studies. On the boat, crew removed the stomach and intestinal tract of the sole and squeezed contents of the tract directly into a 15 mL sterile conical tube, containing 3 mL of sterile saline to fully immerse the contents in the tubes; the tubes were placed on ice and transported to the laboratory within six hours of collection. In the laboratory, the samples were vortexed, and 1 mL of the sample was placed into 99 mL of sterile water and mixed with IDEXX Colilert powder. The IDEXX trays (IDEXX Laboratories, Westbrook, ME) were incubated at 37 °C for 18-24 h. *E. coli* were isolated from positive-growth wells and then further characterized. A total of 50 fish were cultured; two fish from two different Superfund sites within the Salish Sea, carried *E. coli*.

River otter samples

Samples of river otter feces were collected along the Duwamish River and Green River in Washington at several known otter latrine locations, from May 2018 to September 2018 (**Figure 2D**). Six locations were sampled from three zones defined by the proportion of impervious surface to distinguish urban areas; these zones were labeled as superfund zone, suburban zone, and rural zone.

Superfund zone samples included the Hamm Creek (7.2 km upriver from the Salish Sea). River otter samples from the suburban zone were taken at Black River (17.7 km upriver), Green River Natural Resources Area (29.8 km upriver), and Cottonwood Grove (32.2 km upriver). The rural zone included Green River Natural Area (61.2 km upriver) (unpublished information Dr. Michelle Wainstein). The other site Kenco (8.0 km upriver) did not have any *E. coli* isolated (**Figure 2D**).

There was no human contact with river otters during collection. Feces was scooped into 50 mL conical tubes. The tubes were cooled and transported to the laboratory for processing. Two mL centrifuge tubes were filled with otter feces to the 0.5 mL mark, then topped with 0.85% sterile saline to the 1.5 mL mark. Prior to plating, the tubes were vortexed, and a total of 0.1 mL of saline and feces mixture was plated onto eosin methylene blue (EMB) agar plates (Becton Dickinson, Franklin Lakes, NJ). Plates were incubated at 36.5°C overnight. Additional EMB plates were supplemented with one the following antibiotics per plate: ampicillin 25 mg/mL, kanamycin 25 mg/mL, sulfisoxazole 256 mg/mL, spectinomycin 100 mg/mL, streptomycin 100 mg/mL, tetracycline 25 mg/mL (Fisher Bioreagents, Pittsburgh, PA).

There was no difference in the level of antibiotic resistance between isolates removed from non-antibiotic compared to antibiotic supplemented plates when MICs were performed

(data not shown). Four *E. coli* colonies from each location and time point were re-streaked to the same-antibiotic EMB plates and incubated overnight. A total of 44 isolates were subjected to *fumC* typing. A total of 24 (54%) *fumC* distinct isolates from river otters were included in the study.

Dead Harbor seal and dead Harbor porpoise samples

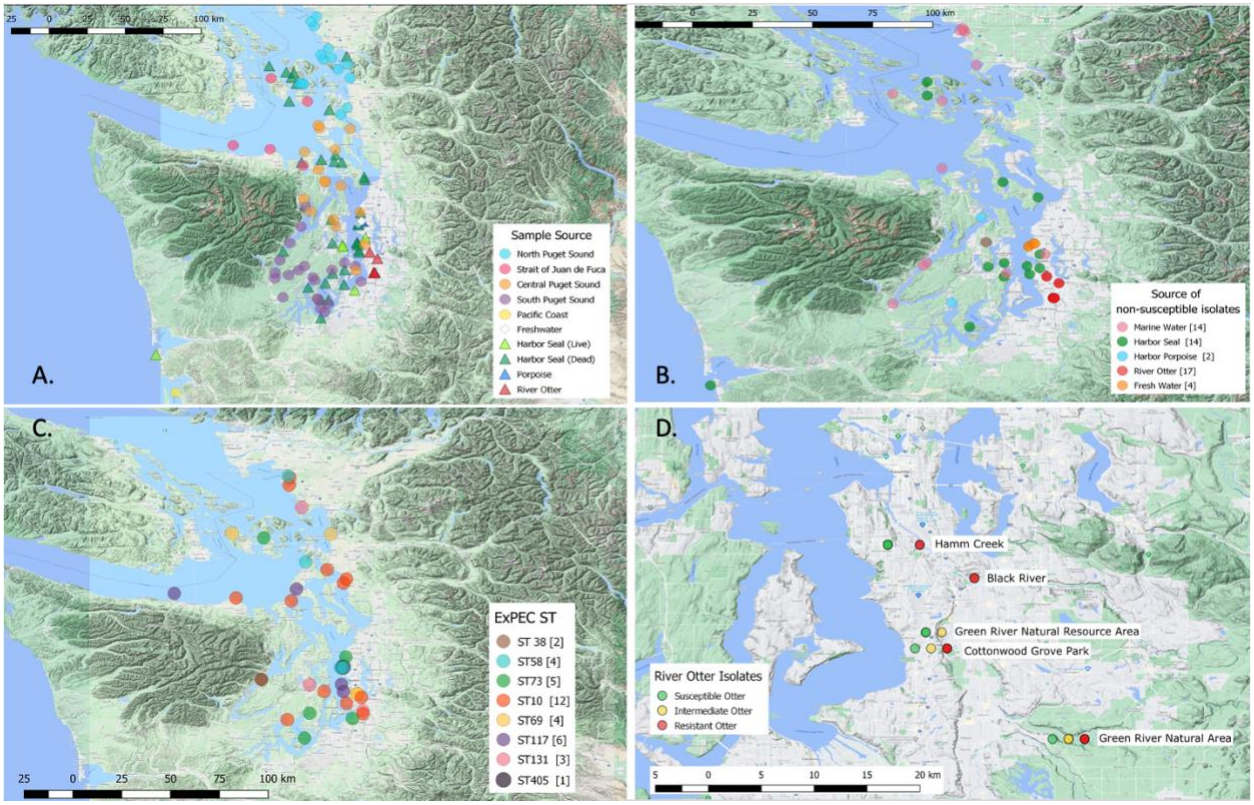
Fecal swabs were collected post-mortem from harbor seals and harbor porpoises and processed by Phoenix Laboratories Mukilteo, WA between fall of 2018 and fall 2019. Isolates were described previously.[18] Suspected *E. coli* isolates were sub-cultured and sent for species identification. Confirmed *E. coli* isolates were sub-cultured to a TSA slant and submitted to UW lab. Isolates were taken from the rectum of dead animals. A total of 35 dead seals and 7 porpoises were used in the study. These were subjected to *fumC* typing[16] and representative isolates were selected from each animal.

Live Harbor Seal samples

All but one of harbor seal samples were collected from docks where harbor seals haul out and defecate; a single sample was obtained from a stranded seal taken to Progressive Animal Welfare Society, Lynnwood, WA, after defecation (**Figure 2A, Figure S1**). The samples were collected by WA Department of Fish and Wildlife staff. There was no human contact with harbor seals during collection of fecal samples. The fecal samples were collected by staff wearing gloves and a clean wooden tongue depressor, then transferred to a Whirl-Pak bag. The collected fecal samples were submitted to the laboratory within six hours of collection. A pellet of the feces was taken using a sterile cotton swab, added to a Durham tube with Brilliant Green Broth (Fisher Bioreagents, Pittsburgh, PA), and incubated in an incubator at 37 °C overnight. Positive-growth tubes had 100 μL of the broth removed and streaked for isolation onto an EMB agar plate

(Fisher Bioreagents, Pittsburgh, PA). The plates are incubated overnight in the incubator at 37°C and *fumC* typed.[16] A total of 17 clonally distinct *E. coli* were selected. For non-DOH samples and dead animal fecal samples, *E. coli* were plated on EMB agar and biochemically tested with TSI and LIA slants according to manufacture instructions (Fisher Bioreagents, Pittsburgh, PA).

**Figure 2.** Map of *E. coli* isolate by location




A: Map of *E. coli* sample source; B: resistant and intermediate *E. coli* isolates; C: ExPEC ST of *E. coli* by location; D: river otter isolate results.

### *fumC* Typing PCR

The *fumC* PCR assay was performed as previously described.[16] Both strands of the DNA were sequenced at the Eurofins Genomics (Louisville, KY). Primers for *fumC* PCR were selected from a list by Wirth et al., 2006.[16] The sequences were edited, aligned and compared with

references set of *fumC* alleles and the Achtman MLST database (https://pubmlst.org/organisms/escherichia-spp, accessed multiple times). For the few that we could not assess in this manner, *fumC* was determined by WGS.

**Antimicrobial Susceptibility Testing**

Phenotypic characterization

The WA DOH marine, fish, and live seal samples were subjected to antibiotic susceptibility testing using Sensititre™ Nephelometer (Thermo Fisher Scientific, Waltham, MA). Cultures were removed from the -80°C freezer and grown on EMB plates for verification as *E. coli.* EMB plates were incubated overnight in an incubator at 37°C. EMB plates were examined for growth, colonies sub-cultured to sheep blood agar plates (Becton Dickinson, Franklin Lakes, NJ) and incubated overnight at 37°C.

Positive-growth plates were transported to WA DOH laboratory where minimum inhibitory concentration (MIC) testing was performed using broth dilution method using the Sensititre AIM procedures (Thermo Fisher Scientific). The isolates were prepared by swabbing 1-2 isolated colonies from the blood agar plates, transferring into a 5-mL sterile water tube, then vortexing. The suspension was adjusted to 0.5 McFarland dilution, as verified by the Sensititre Nephelometer. Then 50 µL of suspension were pipetted into an 11-mL Mueller Hinton Broth tube, and the cap replaced with a Sensititre AIM dosing head (Remel, San Diego, CA). The tube was seated into the AIM machine. The machine was set to a 50 µL inoculation volume and full tray dispensing option, then activated to aliquot HMB suspension into the panel wells. All wells were filled and panels were covered with self-adhering covers and incubated for 18-24 hours. Tested antibiotics included: amikacin, aztreonam, cefepime, cefotaxime, ceftazidime,

ciprofloxacin, doripenem, doxycycline, ertapenem, gentamicin, imipenem, levofloxacin, meropenem, minocycline, piperacillin/tazobactam, ticarcillin/clavulanic acid, tigecycline, tobramycin, and trimethoprim/sulfamethoxazole. Standard positive and negative controls for *E. coli* were used.

Following incubation, the panels were read using Sensititre SWIN software. To determine antibiotic susceptibility, each well was inspected for visual indication of microbial growth. Results were reported as the MIC for each antibiotic in μg/mL using the CLSI interpretive criteria (Clinical and Laboratory Standards Institute, 2021).[19]

*E. coli* from dead seals and porpoises were tested using the bioMérieux VIETK instrument (Durham, NC), while *E. coli* from river otters, fresh water, marine water by the shore, and rescued seal pups were tested using standard disk diffusion assay as previously according to CLSI guidelines (Clinical and Laboratory Standards Institute, 2021). Standard *E. coli* negative and positive controls were included.

Genotypic characterization

Whole genome sequencing (WGS) and multi-locus sequencing typing (MLST) were performed for all 305 *E. coli* isolates from marine water, live and dead seal, porpoises, river otter, and freshwater by partners at WA DOH. *E. coli* isolates were sequenced as part of the *Escherichia coli* GenomeTrakr Project of the Washington State Department of Health (ID 283914 - BioProject - NCBI).[20] The sequences are housed by the National Center for Biotechnology Information and are assigned an NCBI accession number, SRR ID, and MLST typed through Illumina (Illumina, San Diego, CA) sequencing. Isolates used for our study have an NCBI accession number starting with [SAMN]: 13337618, 13348248, 13352752, 13352855-13352864, 13392846, 13392848-13392863, 13392951-13392953, 13418005, 13429240,

13429289, 13482430, 13502693, 13502695, 13502889-13502891, 13513927-13513929, 13513928-13513930, 13513935-13513938, 13513942, 13513948, 13518346, 13518347, 13898866-13898880, 13911824, 13911825, 14057293, 14057294, 14080880-14080885, 14083856-14083863, 14083865, 14083866, 14083868-14083870, 14083873, 14084247, 14113834, 14113836-14113844, 14113847, 14113850, 14113860-14113863, 14137883-14137888, 14137890-14137892, 14137896-14137905, 14137905, 14137978, 14137979, 14138286, 14140185-14140189, 14140195-14140217, 14214490-14214498, 14270850-14270852, 14271025, 14271030-14271033, 14291765, 14316584-14316586, 14316588-14316590, 14316618, 14316619, 14316621, 14316622, 14316624, 14316625, 14316627, 14316629, 14316633, 14316684-14316687, 14593716-14593722, 14749987, 14749988, 14749995, 14750012, 14750852, 14750854-14750856, 15182299-15182304, 15182308, 15182310-15182316, 15182319, 15182320, 15182323, 15344667, 15344671, 15344672, 15344674, 15483654-15483656, 15777149, 15777151, 15777153-15777155, 15777158, 15777160, 15777162, 15777164, 15777165, 15777167, 16054328-16054337, 16054339-16054347, 16054538, 16056701-16056705, 16056743-16056748, 16136466, 16136468, 16136469, 16136474-16136479, 16136481, 16136482, 16136485, 16136487, 16136489, 16136490, 16202553-16202558, 16257942-16257946, 16377217-16377219, and 16439289.

**Statistical Analysis**

Statistical analysis was conducted with Fisher's Exact tests on the following null hypotheses: the proportion of resistance is equal among the four quadrants of the Puget Sound, the proportion of resistance is equal among marine water samples and mammal samples, and the proportion of resistance is equal among marine water samples and marine mammal samples. The

same tests were conducted to test for difference in non-susceptibility, resulting in a total of six hypothesis tests.

We compared antibiotic susceptibility rates within three *E. coli* isolate groupings, there were the comparisons: comparing isolates within the four quadrants of the Salish Sea; comparing marine water samples vs. animal samples (river otter, harbor porpoise, and harbor seal); marine water samples vs. marine mammal samples (excluding river otters). We performed each of the comparisons in parallel: susceptible vs. resistant, and susceptible vs. non-susceptible (resistant and intermediate). For each comparison, we used a Fisher's Exact Test with a confidence level of 0.008 using the Bonferroni adjustment for our six different hypothesis tests ($\alpha = 0.05/6 = 0.008$). All statistical analysis was conducted using R version 3.6.1.

**Phylogenetic Trees**

Phylogenetic trees were created in the Department of Environmental and Occupation Health Sciences' Linux Environment, Plasmid. Using the SRR ID generated by WGS, the raw sequencing files for the isolates are downloaded as FASTQ files into Plasmid. The program Trimmomatic cleaned the FASTQ files of our selected isolates by removing the Ilummina adapters.[21] Trimmomatic prepared our files for alignment against a reference genome and strains of the same ST type from our data using the program Snippy.[22]

Human reference genomes were selected based on the ST and CH type from GenBank.[23] Human reference for ST10 was RS218[24,25] and human reference for ST73 was CFT073.[26] Once our isolates were aligned with the reference strain, the program SNP-DISTS created the singly nucleotide polymorphism (SNP) difference matrix to analyze SNP differences of the isolates with the same ST type.[27] SNP-DISTS created an alignment file that was converted into a '.phy'

file by AliView.[28] The newly created file was then imported into Phylip[29] which converted our file into the appropriate format to use the program FigTree[30] to create the phylogenetic tree for ST type.

**Mapping**

All mapping was done using QGIS software, version 3.2.3. Several maps were created by sample location (**Figure 2A**) and determine if any spatial patterns existed based on non-susceptibility (**Figure 2B**), and ST (**Figure 2C**), and river otter source (**Figure 2D**). A figure of all locations of seal samples with MIC results was also created (**Figure S1**).

**AMR Genes and Virulence Factor Analysis**

For all 51 phenotypically resistant and intermediate isolates, we used ResFinder[31] to identify resistant genes. FASTQ files for each isolate were uploaded into ResFinder. We selected for the organism *E. coli*, choosing to show only known mutations and all acquired antimicrobial configuration, using a 90% threshold and 100% minimum for length for both selections.

VirulenceFinder[32] was used to identify virulence factors of our intermediate and resistant isolates (51). We selected for *E. coli*, using a 90% threshold and 100% minimum length, and used raw sequencing reads.

## RESULTS

Thirty-one (10.2%) of total isolates were resistant with 26 (84%) identified with known antibiotic resistant genes using WGS. An additional 20 (6.6%) of the isolates were phenotypically intermediate resistant with 2 (10%) carrying resistance genes by ResFinder. The remaining 254 *E. coli* (83.3%) were susceptible to the antibiotics tested (**Table S1)**. However, there was a significant difference in antibiotic resistant *E. coli* isolated from marine mammals or marine mammals and river otters versus antibiotic resistant *E. coli* isolated from marine water samples (p-value < 0.0001; p-value = 0.005).

### *E. coli Isolates and Antibiotic Resistance*

Our goal was to collect ~50 *E. coli* isolates from shellfish beds in each of the four quadrants of the Salish Sea within the State of WA (n=212 isolates). In contrast, we included all the *E. coli* isolates with different *fumC* types collected from the other water sites and animals for a total of 305 isolates. The distribution of the antibiotic susceptibility for *E. coli* are provided in **Table 1**.

The proportion of *E. coli* resistant to at least one tested antibiotic in the marine water samples ranged from 0%-8.2%, while intermediate susceptibility to at least one tested antibiotic ranged from 0%-6.1% (**Table 1**). In the freshwater samples, 60% of *E. coli* were resistant to at least one tested antibiotic and 20% showed intermediate susceptibility to at least one tested antibiotic (5 samples only). All *E. coli* taken from marine water near beaches were susceptible to all tested antibiotics (3 samples only). Among *E. coli* isolated from dead harbor seals, 17.1% were intermediate susceptibility to $\geq$ 1 antibiotic and 8.6% were resistant to $\geq$ 1 tested antibiotic, while among live seal isolates, 29.4% were resistant to $\geq$ 1 antibiotic and none were intermediate

susceptibility. Two *E. coli* from harbor porpoises were intermediate susceptibility to at least one tested antibiotic, and none were resistance to tested antibiotics.

Resistant and intermediate resistant (non-susceptible) *E. coli* around the Salish Sea were mapped to detect any clustering in location (**Figure 2B**). No non-susceptible samples were found in the Strait of Juan de Fuca (**Figure 2B**). There were correlations between location of the recovered non-susceptible *E. coli* and *E. coli* recovered from marine mammals and river otters (**Figure 2B, 2D**). The river otter samples were taken from the Duwamish River through the Green River sites with non-susceptible *E. coli* isolated along the entire length of the river (**Figure 2D**).

Among 31 phenotypically *E. coli* resistant, 25 (80.6%) carried between 1-4 genes coding for different classes of antibiotics, while six (19.4%) did not carry resistance genes[31] (**Table S1**); of these six, five were from river otters. Of the isolates with intermediate resistance, eighteen (90%) did not carry any known antibiotic resistance genes or mutations. One isolate, 343170-001-909 from marine water, carried a *bla*$_{\text{TEM}}$ gene and a *gyrA* mutation, and a second isolate carried the *qnrB19* gene (isolate GRNRA4A from river otter) (**Table S1**).

Fourteen isolates were resistant to tetracycline antibiotics and two showed intermediate susceptibility. All 16 isolates carried either the *tet*(A) gene (n=5), the *tet*(B) gene (n=10), or both *tet* genes (n=1; isolate 351565-001-1202 from marine water), providing 100% correlation between phenotype and genotype for this agent class (**Table 2**). Fifteen *E. coli* were phenotypically resistant to β-lactam antibiotics, of which 12 (80%) showed genotypic resistance by carrying *bla* genes (**Table 2**). Among fourteen isolates that were SXT resistant, seven (50%) carried a *sul* gene and six (42.9%) carried both *sul* and *dfr* genes, while two sulfonamide-resistant *E. coli* isolates did not carry a *sul* gene (**Table 2**). In contrast, fourteen isolates carried

aminoglycoside resistance genes but only four (28.6%) were phenotypically resistant, and one was intermediate resistant (**Table 2, Table S1**). We found four *E. coli* phenotypically resistant to fluoroquinolones and two phenotypically intermediate resistant; eight *E. coli* carried fluroquinolone resistant genes and of those, three (37.5%) had mutations (**Table 2**).

We did not test for macrolides or lincosamides though we had two river otter *E. coli* isolates which carried the *lnu*(F) gene, and one carrying the *mph*(A) (fresh water source). Similarly, most isolates were not tested for chloramphenicol or florfenicol, but one isolate carried the chloramphenicol *catA1* (fresh water source) or *floR* gene (live seal source) (**Table S1**).

Samples for river otters were taken along the Duwamish River. Results for river otter susceptibility testing were mapped based on location collected (**Figure 2D**). *E. coli* isolates from river otters included four (16.7%) intermediate and 13 (54.2%) resistant and the highest level of resistant isolates in the study included: two from Black River and Hamm Creek, four from Cottonwood Grove Park, and five from Green River Natural Area. There were four *E. coli* that were intermediate resistance from the river otter samples: one from Cottonwood Grove Park and Green River Natural Resources, and two from Green River Natural Area. No geographic pattern was apparent after mapping with either the resistant or intermediate *E. coli* (**Figure 2D**).

Of the 13 phenotypically resistant river otter isolates eight isolates carried 1-4 different classes of antibiotics (**Table 1**); however, five (38.5%) did not have any known antibiotic resistance genes (**Table S1**). Six isolates were tetracycline resistant with five carrying *tet*(B) and one carrying *tet*(A) and had 100% correlation between phenotype and genotype. In contrast, three of four phenotypically intermediately resistant *E. coli* from the river otters did not have any known resistance genes while the one isolated carried the *qnrB19* gene (**Table S1**).

***Virulence Factors in Non-Susceptible E. coli***

For all 51 resistant and intermediate isolates, we used VirulenceFinder to identify virulence factors.[32] Of these 51 isolates, there were only 3 that VirulenceFinder did not detect virulence factors for, all coming from a dead seal source (AN0041, AN0044, and AN0071). There were no identified virulence factors in two of the three ST372 isolates.

The virulence factor *gad*, which codes for enzyme glutamate decarboxylase, was found in 68% of resistant and intermediate *E. coli* isolates (n=35).[33] The complete list of virulence factors are found in **Table S1**. The virulence genes *gad* and *terC* were found in all 12 ST10 isolates. While the same four virulence genes were found in the two ST 58 isolates and the same 14 virulence factors were identified in the two ST117 isolates. The two ST131 isolates carried 10-12 virulence genes and the three ST362 isolates carried the same four virulence factors, with two ST1079 isolates also carrying the same three virulence genes (**Table S1**).

**Table 1.** Total number isolates characterized and antibiotic susceptibility testing results for each source.

| Sample Source (n=305) | Isolates Characterized | Intermediate | Resistant | Susceptible |
|---|---|---|---|---|
| Marine Water | 212 | 7 (3.3%) | 7 (3.3%) | 198 (93.4%) |
| North Puget Sound | 49 | 3 (6.1%) | 4 (8.2%) | 42 (85.7%) |
| Central Puget Sound | 55 | 0 (0%) | 2 (3.6%) | 53 (96.4%) |
| South Puget Sound | 56 | 3 (5.4%) | 0 (0%) | 53 (94.6) |
| Strait of Juan de Fuca | 52 | 1 (1.9%) | 1 (1.9%) | 50 (96.2%) |
| Freshwater | 5 | 1 (20%) | 3(60.0%) | 1 (20.0%) |
| Marine water by beaches | 3 | 0 (0%) | 0 (0%) | 3 (100%) |
| Harbor Seal | 52 | 6 (11.5%) | 8 (15.4%) | 38 (73.1%) |
| Dead Seal | 35 | 6 (17.1%) | 3 (8.6%) | 26 (74.3%) |
| Live Seal | 17 | 0 (0%) | 5 (29.4%) | 12 (70.6%) |
| Harbor Porpoise | 7 | 2 (28.6%) | 0 (0%) | 5 (71.4%) |
| River Otter | 24 | 4 (16.7%) | 13 (54.2%) | 7 (29.2%) |
| Sole | 2 | 0 (0%) | 0 (0%) | 2 (100%) |
| **Total** | **305** | **20 (6.6%)** | **31 (10.2%)** | **254 (83.3%)** |

**Table 2.** Resistant phenotype and genotype profiles by antibiotic class

| Antibiotic Class | Tetracycline | B-lactam | Aminoglycoside | Sulfonamide/ SXT | Fluoroquinolones |
|---|---|---|---|---|---|
| Resistant Phenotype | 16 | 15 | 5[a] | 14 | 6[b] |
| Resistant Genotype | 16 | 15 | 14 | 13 | 8[c] |
| Both Phenotype and Genotype | 16 | 12 | 4[a] | 8 | 6[b, c] |

[a] N=1 intermediate resistant; [b] N=2 intermediate resistant; [c] N=3 mutations

***MLST and ExPEC strains***

Among the study isolates, we identified 196 unique STs. Of these, 139 STs were represented by a single isolate, 28 STs were represented by two isolates (14.3%), 10 STs were represented by three isolates (5.1%), five STs were represented by four isolates (2.6%), seven Sts were represented by five isolates (3.57%), three STs were represented by six isolates (1.5%), two STs were represented by seven isolates (1.0%), and one ST was represented by eight isolates (0.5%). The most represented was ST10, with 12 isolates (0.5%) (**Table 3**).

Eight ExPEC STs: ST10[25], ST38, ST58, ST69, ST73, ST117, ST131, and ST405 (n=37) which have been previously been associated with human disease were further examined.[34,35] Twenty-one (56.7%) isolates were ExPEC *E. coli* isolated from marine water sample (**Table 3**). Other ExPEC isolates included: one isolate (3%) from freshwater, two isolates (5.4%) from marine water by beaches, eight (21.6%) isolates from dead harbor seals, five isolates (13.5%)

from harbor porpoises. However, there were no ExPEC *E. coli* came from a river otter source or sole sources.

The ST10 had 12 unique isolates (0.51% of total 305 isolates). ST38 had two isolates, ST58 and ST69 each had four isolates, ST73 (n=5), ST117 (n=6), ST131 (n=3), and ST405 (n=1) (**Table 3**). Based on maps created for ExPEC strains (**Figure 2C**), there was some clustering in the South Puget Sound area, where harbor porpoises were sampled, but ExPEC isolates appear in all sampled areas. Among the ST10 isolates, 25% of the isolates were drug resistant (n=3). ST58 *E. coli* isolates of which 50% were drug resistance (n=2). Among the ST69 isolates 25% drug resistance (n=1), ST405 isolates had 100% drug resistance (n=1), ST117 isolates of which 33% resistance (n=2), and ST131 isolates had 66% drug resistance *E. coli* (n=2) (**Table 3**).

We created phylogenetic trees for ST10 and ST73 (**Figure 3**). Among ST10 isolates, *fumC*:*fimH* types included C11:H23, C11:H27, C11:H43, and C11:H54. The single nucleotide polymorphism (SNP) matrix for ST10 showed that the two most closely related isolates -- two marine water samples (one from Central Puget Sound and the other from South Puget Sound) -- differed by 2933 SNPs. When comparing ST73 isolates, we also included characterized isolates from our previous study of Orca whales.[8] The closest ST73 isolates -- two seal samples taken near Whidbey Island -- had a SNP distance of 6 between the two isolates (**Figure 3**). The isolates from the current study and the previous study fell primarily into two distinct groups: one formed between the killer whale *E. coli* (which had the same *fumC:fimH* types) and a second formed by current study isolates (three different *fumC:fimH* types: C24:H9, C24:H10, and C24:H102; **Figure 3**).

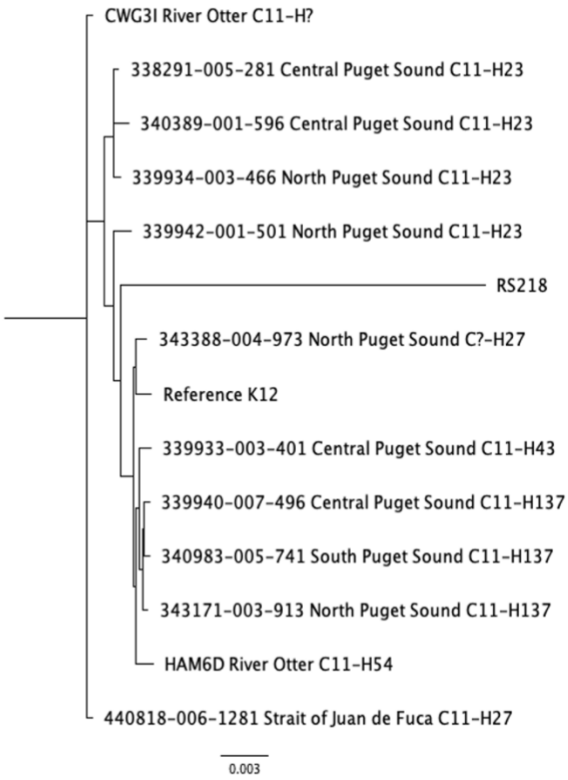
**Table 3.** Locations and count of resistant isolates for each ExPEC ST

| Sample Source (n=37) | ST10 | ST10 Resistant | ST38 | ST38 Resistant | ST58 | ST58 Resistant | ST69 | ST69 Resistant | ST73 | ST73 Resistant | ST117 | ST117 Resistant | ST131 | ST131 Resistant | ST405 | ST405 Resistant | Total |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Marine Water | 10 | 1 | 2 | 0 | 2 | 2 | 2 | 0 | 1 | 0 | 3 | 0 | 1 | 0 | 0 | 0 | **21** |
| North Puget Sound | 4 | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 7 |
| Central Puget Sound | 4 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 6 |
| South Puget Sound | 1 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 4 |
| Strait of Juan de Fuca | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 4 |
| Freshwater | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | **1** |
| Marine water by beaches | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | **2** |
| Harbor Seal | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 4 | 0 | 2 | 2 | 1 | 0 | 0 | 0 | **8** |
| Dead Seal | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 4 | 0 | 2 | 2 | 1 | 1 | 0 | 0 | **8** |
| Live Seal | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | **0** |
| Harbor Porpoise | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | **0** |
| River Otter | 2 | 2 | 0 | 0 | 0 | 0 | 2 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | **5** |
| Sole | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | **0** |
| **Total** | **12** | **3** | **2** | **0** | **4** | **2** | **4** | **1** | **5** | **0** | **6** | **2** | **3** | **2** | **1** | **1** | **37** |

**Figure 3.** ST10 and ST73 SNP Matrices and Phylogenetic Trees

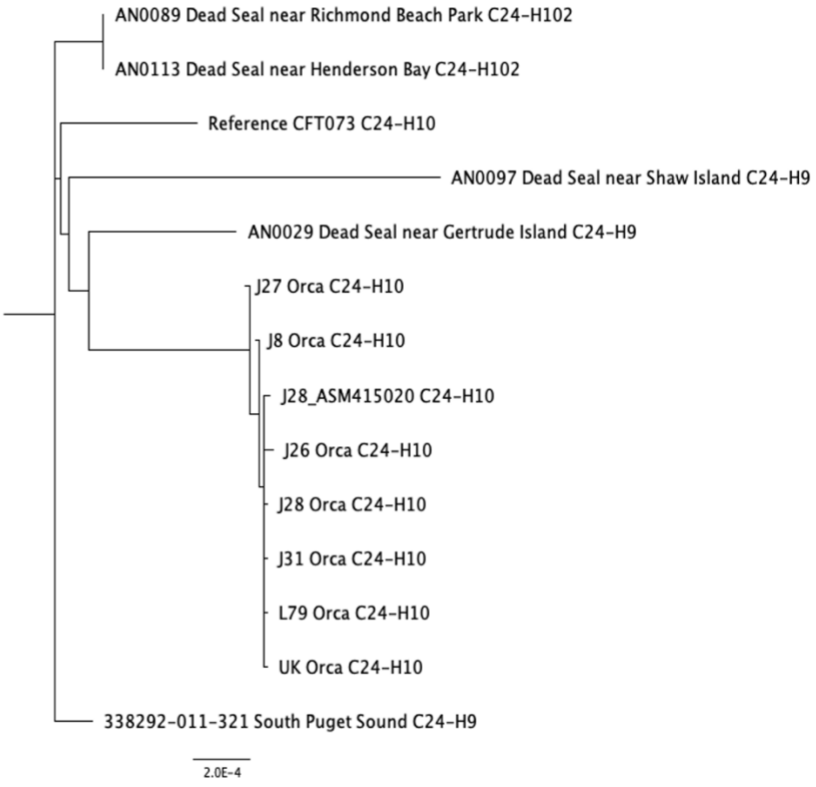
ST10

| SNP Distances | 440818-006-1281 | 338291-005-281 | 339933-003-401 | 339934-003-466 | 339940-007-496 | 339942-001-501 | 340389-001-596 | 340983-005-741 | 343171-003-913 | 343388-004-973 | CWG3I | HAM6D | RS218 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 440818-006-1281 | - | | | | | | | | | | | | |
| 338291-005-281 | 10015 | - | | | | | | | | | | | |
| 339933-003-401 | 20001 | 17283 | - | | | | | | | | | | |
| 339934-003-466 | 10865 | 3074 | 17608 | - | | | | | | | | | |
| 339940-007-496 | 19530 | 16448 | 5711 | 17361 | - | | | | | | | | |
| 339942-001-501 | 14189 | 9932 | 14630 | 10589 | 14636 | - | | | | | | | |
| 340389-001-596 | 12528 | 4954 | 19919 | 6661 | 19411 | 12708 | - | | | | | | |
| 340983-005-741 | 19769 | 16697 | 5755 | 17683 | 2933 | 14483 | 19574 | - | | | | | |
| 343171-003-913 | 19223 | 16145 | 5870 | 17242 | 3468 | 14262 | 18975 | 3461 | - | | | | |
| 343388-004-973 | 17423 | 14710 | 7329 | 15555 | 7061 | 12927 | 17510 | 7539 | 6758 | - | | | |
| CWG3I | 3042 | 9627 | 19858 | 10798 | 19679 | 14571 | 12723 | 19822 | 19211 | 17196 | - | | |
| HAM6D | 19909 | 17401 | 8876 | 18625 | 8371 | 15394 | 20369 | 8481 | 7755 | 9128 | 20441 | - | |
| RS218 | 95502 | 94257 | 93960 | 95140 | 95572 | 94700 | 95095 | 95072 | 94729 | 93846 | 95276 | 93414 | - |
| Reference K12 | 17155 | 16849 | 10208 | 17242 | 10041 | 14848 | 19807 | 9726 | 8993 | 7342 | 17338 | 9928 | 97196 |




ST73

| SNP Distances | 338292-011-321 | AN0029 | AN0089 | AN0097 | AN0113 | J26 | J27 | J28 | J28_ ASM415020 | J31 | J8 | L79 | Reference CFT073 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 338292-011-321 | - | | | | | | | | | | | | |
| AN0029 | 3377 | - | | | | | | | | | | | |
| AN0089 | 1394 | 3765 | - | | | | | | | | | | |
| AN0097 | 7162 | 8453 | 7251 | - | | | | | | | | | |
| AN0113 | 1384 | 3722 | 6 | 7233 | - | | | | | | | | |
| J26 | 4067 | 5248 | 4315 | 9179 | 4259 | - | | | | | | | |
| J27 | 3580 | 4652 | 3839 | 8653 | 3787 | 252 | - | | | | | | |
| J28 | 4029 | 5289 | 4342 | 9186 | 4288 | 187 | 161 | - | | | | | |
| J28_ ASM415020 | 3784 | 4845 | 4010 | 8606 | 3951 | 213 | 152 | 136 | - | | | | |
| J31 | 4094 | 5429 | 4429 | 9362 | 4376 | 175 | 137 | 82 | 114 | - | | | |
| J8 | 3656 | 4772 | 3881 | 8816 | 3827 | 214 | 176 | 129 | 132 | 101 | - | | |
| L79 | 4043 | 5220 | 4286 | 9197 | 4234 | 229 | 167 | 127 | 151 | 99 | 135 | - | |
| Reference CFT073 | 3099 | 5974 | 3213 | 8481 | 3130 | 5595 | 5168 | 5617 | 5316 | 5729 | 5222 | 5639 | - |
| UK | 4075 | 5218 | 4327 | 9257 | 4272 | 184 | 160 | 90 | 104 | 73 | 106 | 90 | 5670 |



***Statistical Analysis***

Our sample size was not large enough to detect statistical evidence that there were differences in proportions of resistance versus susceptibility between the four quadrants of Puget Sound ($p = 0.089$). Similarly, there was no statistical evidence of differences in proportions of non-susceptibility and susceptibility across the four quadrants of the Puget Sound ($p = 0.148$).

When comparing resistance of *E. coli* from animal sources and marine sources, there was statistical evidence of a difference in proportions of resistance versus susceptibility in isolates ($p < 0.0001$). The probability of obtaining a resistant *E. coli* from an animal source was 8.877 [99.2% CI: 2.67-35.29] times higher than the probability of obtaining a resistant *E. coli* from marine water. When non-susceptibility (resistant and intermediate) vs susceptibility for animal *E. coli* was compared to marine water *E. coli*, we found $p < 0.0001$. The probability of obtaining a non-susceptible *E. coli* from an animal source was 5.334 [99.2% CI: 2.21-13.40] times higher than the probability of obtaining a non-susceptible *E. coli* from marine water.

The final comparison was between *E. coli* from marine mammals, harbor seals and harbor porpoises vs. *E. coli* from marine water. First looking at the difference of proportion of resistance between marine mammals and marine water, the results of the test was p-value = 0.010; we determined that there was no statistical evidence of a relationship between marine mammal isolate source and the proportion of resistant *E. coli.* We ran a test to look at the proportion of non-susceptibility and susceptibility of marine mammals and marine water isolates. The results of the test were p-value = 0.005. The probability of obtaining a non-susceptible organism from a marine mammal was 3.014 [99.2% CI: 1.04-8.58] times higher than the probability of obtaining a non-susceptible organism from marine water.

Based on statistical analysis, we determined that there was statistical evidence that there is a difference proportion of resistance and non-susceptibility for mammals and the proportion of non-susceptibility in marine mammals when compared to marine water. With our sample size we did not find a difference in proportion of resistance or non-susceptibility among samples from the four quadrants of the Puget Sound.

## DISCUSSION

Our study of antimicrobial resistance in the Salish Sea ecosystem produced a number of important findings that deserve further investigation. We found that marine animals were more likely to carry resistant *E. coli* than marine water samples. Our few freshwater samples also had high proportion of resistant *E. coli,* but the numbers were too low for statistical analysis in the current study. The correlation between phenotypic resistance and genotypic carriage of genes conferring resistance varied with the antibiotic.

There are several reasons why marine mammals could be good sentinels of environmental AMR. River otters, harbor seals, and harbor porpoises share many of the same food sources. Understanding if there is any relationship with the marine mammal food chain and the proportion of resistant *E. coli* may shed light onto origin of resistant *E. coli* in these populations. Future research can look at different species involved in the food chain to better understand the exposure and carriage of ARB to marine mammals and river otters. The consumption of shellfish by river otters and other marine mammals can give us insight into what type of exposures come from the food chain. Pathogens that enter the marine environment accumulate in filter feeders who are preyed on by larger species. ARGs that come from livestock or human waste can contaminate the environment and lead to horizontal transfer of genes, risking transmission to human adapted pathogens. The exposure to pollutants from WWTP and agricultural run-off can have potential effects on the ecosystem level, thus sampling of animals that inhabit the marine environment can indicate health effects on humans.[36]

Our findings that freshwater resistant *E. coli* was greater than saltwater resistant *E. coli* was not surprising. Previous studies have shown that resistant *E. coli* are common in fresh water.[37,38] Possible reasons for this observation is that the survivability of *E. coli* in marine water

is dependent on many factors including light and salinity[39]; there is a more stable environment for *E. coli* in the intestinal tract of mammals.

Tetracycline resistant showed a 100% good correlation between phenotype and carriage of a *tet* gene, while aminoglycoside genes did not have a good correlation with phenotypic resistance (**Table 2**). We found a disconnect between the results of phenotypic susceptibility testing and the presence or absence of ARGs. This is a concern as more bacteria are simply being WGS and not examined for phenotypic function. What does it mean if clinical medicine if an organism is not phenotypically resistant but carries the gene by WGS? This is a question that has been hard to an answer.[40,41]

Spatial patterns of occurrence of resistant *E. coli* in seals is not available due to small sample size. There were more resistant and intermediate *E. coli* found from animal samples, which were mostly taken in the Central and South Salish Sea. There were no non-susceptible samples found in the Strait of Juan de Fuca. This was not expected due to the proximity to the WWTP in Victoria, BC.[5,6] The full susceptibility of bacteria recovered in the Strait of Juan de Fuca may not be fully representative of the bacterial ecology; there were no seal or porpoises found in the Strait of Juan de Fuca and more found in Central and South Puget Sound.

Among the resistant *E. coli* from river otters there was no clear pattern for resistance; there was no observed difference in resistance at the superfund site, the suburban area, and the rural area (**Figure 2D**). These results call for further research to determine if river otters can be sentinels for antimicrobial resistance in freshwater.

One weakness of this study is that sampling was conducted post-mortem for some harbor seals. Another weakness with the study is how the *E. coli* samples were selected. Our goal was to include as much variability as possible. We were limited in our marine mammal samples for any

spatial variety, since many of our marine mammal samples were found in the Central and South Puget Sound area. One limitation of the study was that the remaining 254 (83.3%) of the isolates were susceptible and their potential AMR genes were not examined because of the limited number of AMR genes and mutations found with the intermediate resistant isolates (**Table S1**).

There is more work to be done on research on marine mammal resistance. The long-term objective of the study is to understand the relationship between the ARB we see in the wild and ARB that exist in urban areas, and the possible impact it can have on human populations, wildlife, and surrounding ecosystems. The ocean environment is vastly diverse, and there are very little studies done on the presence of antibiotic resistant bacteria in marine species. Using this data and comparing with data of humans and domesticated animals on land, we can compare the amount of resistance, the types of strains present, and potentially trace the connection of repeated strains around the Pacific Northwest. The development of resistant genes has the potential to form anywhere and under many different circumstances but being able to trace where ARG appear and where they end up in the environment can shed light on where these genes travel from into the water.[11] Knowing the circumstances where ARBs are introduced to the environment and animals will help develop a holistic perspective on how to combat antibiotic resistance. Based on the results of this study, marine mammals and river otters have the potential to be sentinels for ARG in ecosystems that surround urban areas.

## CONCLUSION

There was no statistical difference in the proportion of resistance and non-susceptibility *E. coli* when comparing the four quadrants of the Puget Sound. This could be due to relatively low numbers taken at each quadrant. When comparing the proportion of resistance and non-susceptibility to susceptibility in our mammal samples and marine water samples, our analysis determined that there was a higher proportion of resistant and intermediate isolates taken from an animal sources with significant P-value ($p < 0.0001$). Looking solely at the different of proportion of resistant and intermediate isolates in marine mammals and marine water, our analysis revealed that there was a higher amount of non-susceptible isolates when the *E. coli* comes from a marine mammal source ($p = 0.005$). There was not an overwhelming spatial clustering of antibiotic resistant *E. coli* potentially due to the total distribution of marine mammals. We would have expected to see more in the Strait due to the secondary WWTP near Vancouver Island but there were few marine mammal samples found in those regions. We had the assumption that we would find a lot of resistant *E. coli* within more urban or agricultural areas, but we were limited due to our sampling methods. We observed clustering of resistant *E. coli* that correlated with where marine mammals and river otters were sampled. Our isolates from river otters were samples along 56-km river complex starting with the Lower Duwamish superfund site and ending with a rural area. We found resistant and intermediate isolates along the length of where we sampled. Using WGS to characterize our isolates, we found that there was a diverse number of STs found in our samples, and that ExPEC ST were present in the animal and water samples. There were very few clones which came from similar locations and sources, but none of the isolates were closely related to human related isolates. Future research

will look at human isolates in the region to better understand the flow of resistant *E. coli* in this ecosystem.

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## APPENDICES

**Figure S1.** Map of *E. coli* isolates from Harbor seals

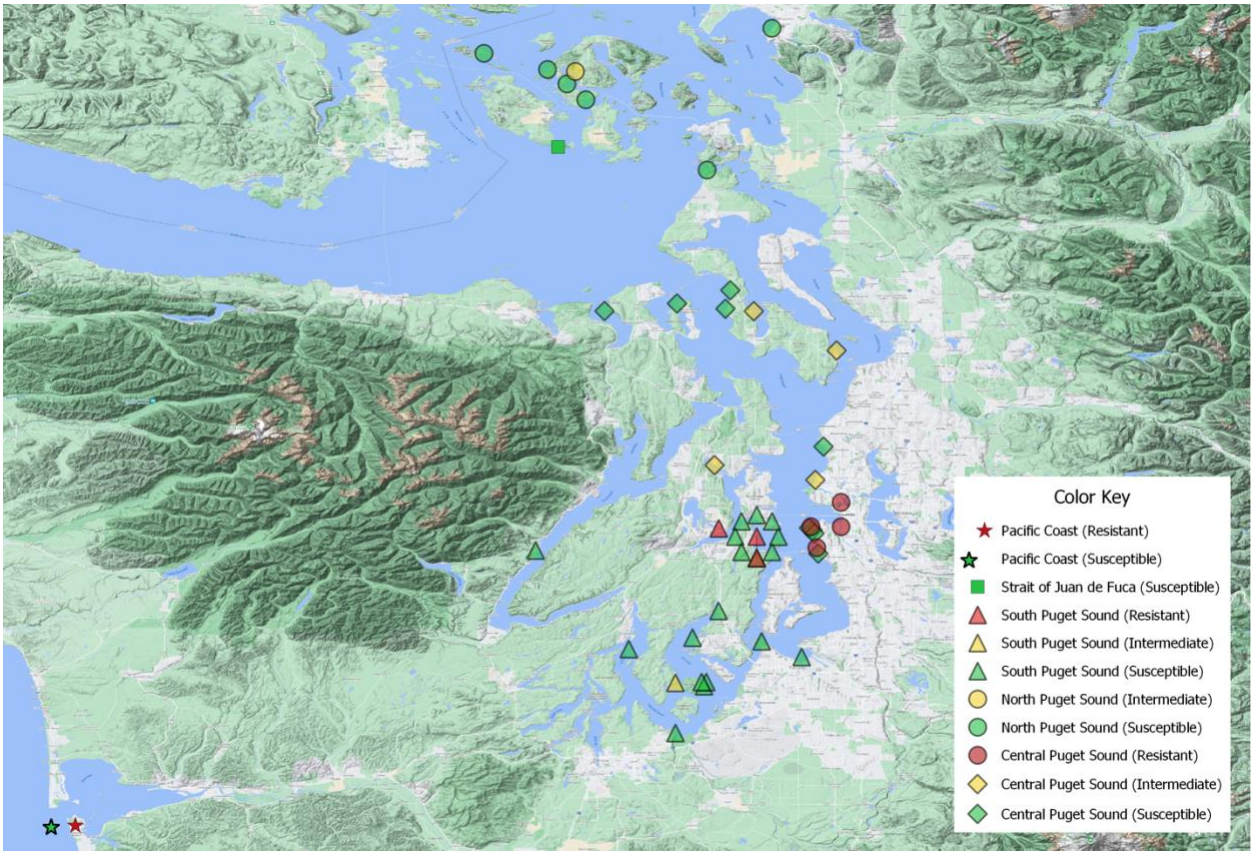

**Table S1.** Table of resistant genes and virulence factors [a]

| Isolate ID | Source | MLST | S/I/R | Resistance | Resistant Genes | Virulence Factor |
|---|---|---|---|---|---|---|
| 353985-001-1210 | South Puget Sound/Hood Canal | 2 | Intermediate | Imipenem (Intermediate) | None | *ast, chuA, lpfA* |
| 339942-001-501 | North Puget Sound | 10 | Resistant | Minocycline, SXT[b] | *qnrB19*, *sul*III, *dfrA12*, *floR*, *tet*(A) | *gad, terC* |
| HAM6D | River Otter | 10 | Resistant | Ampicillin, SXT, Tetracycline | *aph(6)-Id*, $bla_{\text{TEM-1B}}$, *tet*(B) | *astA, cia, gad, terC, traT* |
| CWG3I | River Otter | 10 | Resistant | Cefotaxime (Intermediate), Tetracycline, Minocycline (Intermediate), Sulfisoxazole (Intermediate) | *tet*(B) | *gad, kpsE, kpsM* II, *terC* |
| 344914-013-1036 | Central Puget Sound | 58 | Resistant | Doxycycline, Minocycline (Intermediate) | *tet*(B), *aph(3")-Ib*, *aph(6)-Id* | *cia, cvaC, etsC, fyuA, gad, hlyF, iroN, iss, iucC, iutA, lpfA, chF, ompT, terC, traT* |
| 339942-002-506 | North Puget Sound | 58 | Resistant | Aztreonam, Cefotaxime, Doxycycline, SXT, Ciprofloxacin (Intermediate) | *sul*III, *dfrA12*, *tet*(A), *floR*, $bla_{\text{CTX-M-15}}$, *qnrS1, qnrB19* | *gad, hlyF, lpfA, terC* |

| | | | | | | |
|---|---|---|---|---|---|---|
| HAM5E | River Otter | 69 | Resistant | Ampicillin, SXT, Tetracycline, Minocycline, Sulfisoxazole | *aadA5*, *aph(3'')-Ib*, *aph(6)-Id*, *bla*$_{\text{TEM-1B}}$, *catA1*, *qnrB19*, *qnrB82*, *sul*II, *tet*(B), *dfrA17* | *air*, *chuA*, *eilA*, *fyuA*, *gad*, *hra*, *iha*, *irp2*, *iucC*, *lutA*, *kpsE*, *kpsM* II K52, *lpfA*, *ompT*, *papA*, *fsiA*(F16), *papC*, *sat*, *senB*, *traT* |
| SSW080719 (AN0077) | Dead Seal | 117 | Resistant | Doxycycline | *tet*(B), *sul*II, *aph(6)-Id*, *aph(3'')-Ib*, *aph(3')-Ia* | *astA*, *chuA*, *etsC*, *fyuA*, *hlyF*, *hra*, *iroN*, *irp2*, *iss*, *lucC*, *ompT*, *pic*, *traT*, *vat* |
| SSW082919 (AN0092) | Dead Seal | 117 | Resistant | Doxycycline | *tet*(B), *sul*II, *aph(6)-Id*, *aph(3'')-Ib*, *aph(3')-Ia* | *astA*, *chuA*, *etsC*, *fyuA*, *hlyF*, *hra*, *iroN*, *irp2*, *iss*, *lucC*, *ompT*, *pic*, *traT*, *vat* |
| WDFW2019-154 (AN0107) | Dead Seal | 131 | Resistant | Amoxicillin, Gentamicin, Trimethoprim/ Sulfamethoxazole | *aac(3)-Iid*, *aadA2*, *dfrA12*, *sul*I, *mph*(A), *bla*$_{\text{TEM-1B}}$ | *afaA*, *afaC*, *afaD*, *afaE*, *chuA*, *fyuA*, *gad*, *iha*, *irp2*, *iss*, *iucC*, *iutA*, *kpsE*, *kpsM* II K5, *ompT*, *sat*, *senB*, *traT*, *yfcV* |
| 343170-001-909 | North Puget Sound | 131 | Intermediate | Ciprofloxacin (intermediate), Ticarcillin/Clavulanic Acid (Intermediate) | *bla*$_{\text{TEM-1B}}$, *gyrA* (S83L) | *afaA*, *afaD*, *chuA*, *fyuA*, *gad*, *kpsE*, *kpsM* II K5, *ompT*, *senB*, *traT*, *yfcV* |

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|---|---|---|---|---|---|---|
| GRNRA2B | River Otter | 131 | Resistant | Ampicillin, Imipenem (Intermediate), Kanamycin (Intermediate), Sulfisoxazole (Intermediate) | *bla*$_{\text{TEM-1C}}$ | *chuA, gad, ibeA, irp2, iss, kpsM* II, *papA(F48), sitA, yfcV* |
| WDFW2019-107 (AN0070) | Dead Seal | 162 | Intermediate | Florfenicol (Intermediate), Chloramphenicol (Intermediate) | None | *gad, lpfA, terC, traT* |
| CWG7G | River Otter | 162 | Resistant | Sulfisoxazole, Cefotaxime (Intermediate), Amikacin (Intermediate), Kanamycin (Intermediate) | None | *gad, hlyF, iss, iucC, iutA, lpfA, terC* |
| CWG7H | River Otter | 162 | Resistant | Ampicillin (Intermediate), Amikacin (Intermediate), Kanamycin (Intermediate), Sulfisoxazole | None | *gad,hlyF, lucC, lutA, lpfA, terC* |
| 342381-006-850 | Strait of Juan de Fuca | 206 | Resistant | Aztreonam, Cefotaxime, Ceftazidime | None | *astA, gad, traT* |
| PCB4Cef | Fresh Water | 297 | Resistant | Ampicillin, Amoxicillin/Clavulanic Acid, Ceftriaxone, Aztreonam, Ceftazidime, Ticarcillin/ Clavulanic Acid (Intermediate) | *bla*$_{\text{CMY-2}}$ | *cib, gad, lpfA, mchB* |

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|---|---|---|---|---|---|---|
| SKMMR2020-01-025 Gut #1 | Live Seal | 345 | Resistant | SXT | *dfrA5* | *cia, cvaC, etsC, gad, hlyF, iroN, iss, lpfA, ompT, sitA* |
| GRNRA3B | River Otter | 362 | Intermediate | Cefotaxime(Intermediate), Sulfisoxazole (Intermediate) | None | *chuA, iss, kpsE, kpsM* II K5 |
| GRNRA4A | River Otter | 362 | Intermediate | Cefotaxime (Intermediate), Imipenem (Intermediate), Meropenem (Intermediate), Amikacin (Intermediate), Kanamycin (Intermediate), Sulfisoxazole (Intermediate) | *qnrB19* | *chuA, iss, kpsE, kpsM* II K5 |
| GRNRA4B | River Otter | 362 | Resistant | Sulfisoxazole, Cefotaxime (Intermediate), Imipenem (Intermediate), Meropenem (Intermediate), Kanamycin (Intermediate), Ciprofloxacin (Intermediate) | None | *chuA, iss, kpsE, kpsM* II K5 |
| SKMMR2019-7-10PV (AN0044) | Dead Seal | 372 | Intermediate | Florfenicol (intermediate) | None | None |
| 19Pv16JulWI-07 Isolate #1 (AN0047) | Dead Seal | 372 | Intermediate | Florfenicol (intermediate) | None | *cea, focC, sfaE, focG, focI, fyuA, gad, hra, ibeA, iroN, irp2, iss kpsE, kpsM* II K24, *mchB, mchF, ompT, papA(F13), terC* |
| 19Pv29JulWI-09 Isolate #2 (AN0041) | Dead Seal | 372 | Intermediate | Florfenicol (intermediate), Amoxicillin (Intermediate) | None | None |

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|---|---|---|---|---|---|---|
| GG 14-6 Cef | Fresh Water | 405 | Resistant | Aztreonam, Cefepime, Cefotaxime, Ceftazidime, Ciprofloxacin, Doxycycline, Levofloxacin, Minocycline, Ticarcillin/ Clavulanic Acid, SXT | *sul*I, *mph*(A), $bla_{\text{CTX-M-15}}$, *aadA2*, *qepA4*, *dfrA12*, *catA1*, *tet*(B), *qepA*, *gyrA* S83L, *gyrA* D87N | *chuA, fyuA, irp2, kpsM* II K5, *sitA, traT* |
| GRNRA2E | River Otter | 538 | Resistant | Cefotaxime, Sulfisoxazole, Ampicillin (Intermediate), Imipenem (Intermediate), Meropenem (Intermediate), Amikacin(Intermediate) | *aac(2')-Iia* | *ibeA, neuC, ompT* |
| CRC-1702 (AN0006) | Porpoise | 569 | Intermediate | Florfenicol (Intermediate), Chloramphenicol (Intermediate) | None | *chuA, fyuA, ibeA, iss kpsE kpsM* II K1, *neuC, ompT, sitA, usp* |
| GG 14-5 Cef | Fresh Water | 616 | Resistant | Aztreonam, Cefotaxime, Ceftazidime (Intermediate), Cefepime | $bla_{\text{CTX-M-15}}$, *qnrS1*, *mph*(A) | *gad, terC, traT* |
| 343066-013-868 | South Puget Sound/Hood Canal | 641 | Intermediate | Aztreonam (Intermediate) | None | *gad, lpfA, ompT, traT* |
| PCO1 | Fresh Water | 681 | Intermediate | Ceftriaxone (Intermediate) | None | *chuA, cia, cibB, iss, ompT, traT* |

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|---|---|---|---|---|---|---|
| EPA Dock G Cip 1#5 | Live Seal | 744 | Resistant | Ciprofloxacin, Doxycycline (Intermediate), Levofloxacin | *aph(3")-Ib*, *aph(6)-Id*, *catA1*, *floR*, *sul*II, *tet*(A), *gyrA* S83L, *gyrA* D87N | *gad* |
| SKMMR2020-01-025 Fecal #1 | Live Seal | 744 | Resistant | Ciprofloxacin (Resistant), Levofloxacin (Resistant) | *aph(3")-Ib*, *aph(6)-Id*, *mdf*(A), *catA1*, *floR*, *sul*II, *tet*(A), *gyrA* S83L, *gyrA* D87N | *gad* |
| 351565-001-1202 | North Puget Sound | 744 | Resistant | Ciprofloxacin, Doxycycline, Levofloxacin, Minocycline, Trimethoprim/ Sulfamethoxazole | *sul*I, *dfrA17*, *tet*(A), *sul*II, *tet*(B), $bla_{\text{TEM-1B}}$, *aph(3")-Ib*, *mph*(A), *aadA5*, *catA1*, *aph(6)-Id*, *gyrA* S83L, *gyrA* D87N | *cvaC*, *etsC*, *gad*, *hlyF*, *iroN*, *iss*, *mchF*, *traT* |
| 339942-003-511 | North Puget Sound | 746 | Resistant | Cefotaxime, Doxycycline(intermediate), Gentamicin (Intermediate) | *aac(3)-Via*, *aph(3")-Ib*, *aadA1*, *aph(6)-Id*, *sul*I, $bla_{\text{SHV-12}}$, *tet*(A) | *cib*, *cma*, *fyuA*, *gad*, *hlyF*, *iroN*, *irp2*, *iss*, *neuC*, *terC*, *traT* |
| EPA Dock G#1 | Live Seal | 772 | Resistant | Doxycycline, SXT, Minocycline(intermediate) | *aadA5*, *sul*II, *tet*(B), *dfrA17* | *cma*, *gad*, *irp2*, *terC* |

| | | | | | | |
|---|---|---|---|---|---|---|
| 343389-008-981 | North Puget Sound | 942 | Intermediate | Amikacin (Intermediate), Ticarcillin/ Clavulanic Acid (Intermediate) | None | *lpfA, sitA, terC* |
| 354777-001-1214 | Strait of Juan de Fuca | 967 | Intermediate | Aztreonam (Intermediate) | None | *cba, chuA, cma, ibeA, kpsM* II_K5 |
| BR1F | River Otter | 1079 | Resistant | Ampicillin, Gentamicin, Tetracycline, Minocycline | *aac(3)-IV, aac(3)-Iva, aadA1, aph(4)-Ia, aph(6)-Id,* $bla_{\text{TEM-1B}}$, *lnu*(F), *tet*(B) | *gad, lpfA, terC* |
| BR1E | River Otter | 1079 | Resistant | Doxycycline, Gentamicin, Tobramycin, Minocycline (Intermediate) | *aac(3)-IV, aph(4)-Ia, aph(6)-Id,* $bla_{\text{TEM-1B}}$, *lnu*(F), *tet*(B) | *gad, lpfA, terC* |
| GRN1A | River Otter | 1246 | Intermediate | Ampicillin (Intermediate), Sulfisoxazole (Intermediate) | None | *gad, lpfA, terC* |
| 2019-SJ013 (AN0032) | Dead Seal | 1718 | Intermediate | Florfenicol (intermediate) | None | *gad, terC* |
| EJC-2019-03 (AN0009) | Porpoise | 1723 | Intermediate | Florfenicol (Intermediate), Amoxicillin (Intermediate), Chloramphenicol (Intermediate) | None | *cma, gad, ipfA, traT* |
| CWG3J | River Otter | 2144 | Resistant | Chloramphenicol, Tetracycline, Sulfisoxazole, Minocycline (Intermediate) | *aadA1, cmlA1, sul*III, *tet*(A) | *cib, gad, lpfA, ompT* |

| | | | | | | |
|---|---|---|---|---|---|---|
| GRNRA2F | River Otter | 2164 | Resistant | Cefotaxime, Imipenem, Meropenem (Intermediate), Kanamycin (Intermediate), Sulfisoxazole (Intermediate) | None | *gad, iss, lpfA, ompT, terC* |
| GRNRA4F | River Otter | 2521 | Resistant | Sulfisoxazole, Cefotaxime (Intermediate), Ampicillin (Intermediate), Imipenem (Intermediate), Meropenem (Intermediate), Kanamycin (Intermediate) | None | *gad, iss, lpfA, ompT, terC* |
| 345996-003-1186 | North Puget Sound | 2522 | Intermediate | Aztreonam (Intermediate) | None | *gad, lpfA* |
| CWG5A | River Otter | 2607 | Intermediate | Cefotaxime (Intermediate), Imipenem (Intermediate), Kanamycin (Intermediate) | None | *gad, lss, lpfA, ompT, terC* |
| WDFW2019-112 (AN0071) | Dead Seal | 3018 | Intermediate | Florfenicol (Intermediate) | None | None |
| 336039-006-31 | South Puget Sound/Hood Canal | 7706 | Intermediate | Ciprofloxacin (Intermediate) | None | *gad, iss* |
| HASE 6 CEF | Live Seal | 9001 | Resistant | Ampicillin, Amoxicillin/Clavulanic Acid, Ceftriaxone, Aztreonam, Cefotaxime, Ceftazidime, Ticarcillin/ Clavulanic Acid(Intermediate) | $bla_{CMY-2}$ | *astA, hlyF, hra, traT* |

| | | | | | | |
|---|---|---|---|---|---|---|
| 339940-002-477 | Central Puget Sound | 10718 | Resistant | Cefotaxime, Ceftazidime, Ticarcillin/Clavulanic Acid (Intermediate) | $bla_{CMY-2}$ | *gad, lpfA, ompT, terC* |

[a] As found by ResFinder 4.0

[b]SXT abbreviation for Trimethoprim/Sulfamethoxazole