




## Article

# Antibiotic Resistance of Bacteria in Two Marine Mammal Species, Harbor Seals and Harbor Porpoises, Living in an Urban Marine Ecosystem, the Salish Sea, Washington State, USA

Stephanie A. Norman <sup>1,2,3,\*</sup> , Dyanna M. Lambourn <sup>4</sup>, Jessica L. Huggins <sup>5</sup>, Joseph K. Gaydos <sup>6</sup> , Sandra Dubpernell <sup>2</sup>, Susan Berta <sup>2</sup>, Jennifer K. Olson <sup>7</sup> , Victoria Souze <sup>8</sup>, Alysha Evans <sup>8</sup>, Betsy Carlson <sup>9</sup>, Mandi Johnson <sup>9</sup>, Rachel Mayer <sup>10</sup>, Cathy King <sup>3</sup> and Alyssa Scott <sup>7</sup>

- <sup>1</sup> Marine-Med: Marine Research, Epidemiology, and Veterinary Medicine, Bothell, WA 98021, USA
  - <sup>2</sup> Orca Network/Central Puget Sound Marine Mammal Stranding Network, Freeland, WA 98249, USA; dubpernell@yahoo.com (S.D.); susan@orcaneetwork.org (S.B.)
  - <sup>3</sup> World Vets, Gig Harbor, WA 98332, USA; drcathy@worldvets.org
  - <sup>4</sup> Marine Mammal Investigations, Washington Department of Fish and Wildlife, Lakewood, WA 98498, USA; Dyanna.Lambourn@dfw.wa.gov
  - <sup>5</sup> Cascadia Research Collective, Olympia, WA 98501, USA; JHuggins@cascadiaresearch.org
  - <sup>6</sup> SeaDoc Society, UC Davis Wildlife Health Center—Orcas Island Office, Eastsound, WA 98245, USA; jkgaydos@ucdavis.edu
  - <sup>7</sup> The Whale Museum, Friday Harbor, WA 98250, USA; jolson296@gmail.com (J.K.O.); alyssa@whalemuseum.org (A.S.)
  - <sup>8</sup> Whatcom County Marine Mammal Stranding Network, Lummi Island, WA 98262, USA; vsouze56@gmail.com (V.S.); adelsby@gmail.com (A.E.)
  - <sup>9</sup> Port Townsend Marine Science Center, Port Townsend, WA 98368, USA; bcarlson@ptmsc.org (B.C.); mjohnson@ptmsc.org (M.J.)
  - <sup>10</sup> Sno-King Marine Mammal Response, Seattle, WA 98117, USA; Rachel.Mayer@comcast.net
- \* Correspondence: stephanie@marine-med.com



**Citation:** Norman, S.A.; Lambourn, D.M.; Huggins, J.L.; Gaydos, J.K.; Dubpernell, S.; Berta, S.; Olson, J.K.; Souze, V.; Evans, A.; Carlson, B.; et al. Antibiotic Resistance of Bacteria in Two Marine Mammal Species, Harbor Seals and Harbor Porpoises, Living in an Urban Marine Ecosystem, the Salish Sea, Washington State, USA. *Oceans* **2021**, *2*, 86–104. <https://doi.org/10.3390/oceans2010006>

Academic Editor: Alexander Werth  
Received: 4 November 2020  
Accepted: 19 January 2021  
Published: 25 January 2021

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**Abstract:** The pervasive use of antibiotics in human medicine, veterinary medicine, and agriculture can result in a significant increase in the spread and environmental persistence of antibiotic resistance in marine ecosystems. This study describes the presence and distribution of antibiotic-resistant bacteria in Salish Sea harbor seals (*Phoca vitulina*) and harbor porpoises (*Phocoena phocoena*) and evaluates species, age class, and geographic differences in resistance patterns. Isolates from 95 dead-stranded animals (74 seals/21 porpoises) were tested for resistance to a suite of 15 antibiotics. Of the 95 sampled, 85 (89%) (67 seals/18 porpoises) successfully yielded 144 isolates, with 37% resistant to at least one antibiotic and 26% multi-drug resistant (24% and 39% of seal and porpoise isolates, respectively). Overall, and by study region, porpoises were significantly more likely to harbor resistant organisms compared to seals. Significant differences between age classes were noted for the antibiotics amoxicillin, cephalexin, and cefovecin. Overall isolate resistance was significantly greater in porpoises than seals for several individual antibiotics. Multiple antibiotic resistance (MAR) indices greater than 0.2 were observed in 55% of multi-drug resistant isolates, suggesting seal and porpoise exposure to anthropogenic pollution. The relatively high and disparate prevalence of antibiotic resistance in these common, but ecologically dissimilar, marine mammals reflects a potentially large environmental pool of antibiotic resistant organisms in the Salish Sea or inherently different resistance gene patterns between the two species.

**Keywords:** antibiotic resistance; antimicrobial; multi-drug resistance; MAR index; harbor porpoise; harbor seal; marine ecosystem; Salish Sea

## 1. Introduction

Normal bacterial flora of an animal can shape its growth, development, and behavior, as well as mate selection [1]. However, host flora may change when antibiotic-resistant

microbes are introduced into the organism [1]. Antibiotic resistance is a global concern [2] and has been characterized as the “quintessential One Health issue” [3,4]. The ubiquitous use of antibiotics in human and veterinary disease treatment and agriculture has resulted in a significant increase in the spread and environmental persistence of antibiotic resistance [5,6]. This includes the release of wastes that carry both antibiotics and antibiotic-resistant bacteria into the coastal marine environment [7]. Antibiotic-resistant microbes and genes within the aquatic environment have been documented in various marine species from cephalopods to marine mammals and elasmobranchs, such as sharks [6,8–13].

Although antibiotic-resistant bacteria have been documented in multiple marine species, most wild animals have never been directly exposed to antibiotics. When wastewater discharge, carrying antibiotics and resistant bacteria into terrestrial waterways, finds its way to marine coastlines, it may cause disease in marine organisms, contributing to antibiotic resistance [14]. This, and other anthropogenic contributions, may elevate natural background levels of antibiotic resistance genes in aquatic environments, encouraging their transfer into pathogens or serving as a means for antibiotic resistance propagation [15]. When resistant bacteria are introduced to animals or their environment, the animals may become sick, or resistance traits may be transferred to other bacterial species, or they may become a reservoir that transfers the bacteria and resistance back to humans and the environment [16].

Information on antibiotic resistance in marine species in Washington State’s inland marine waters, collectively referred to as the Salish Sea, is relatively limited. Preliminary work reported resistance in young stranded harbor seals (*Phoca vitulina*) in rehabilitation [17], harbor seal pups found dead during beach searches [18], and in local endangered southern resident killer whales (SRKW) (*Orcinus orca*) breath and feces [19,20]. To determine the types and degree of antibiotic resistance in local marine mammal populations and to begin to better understand how resistance moves through wild animals and ecosystems, further examination is needed in this region on a wider-breadth of species, age classes, and locations throughout the Salish Sea. Additionally, investigating marine mammal species that are sympatric with resident killer whales, especially within this urban marine ecosystem, would also give insight into the ways in which antibiotic-resistant organisms potentially threaten the health of this endangered population [19,20]. The objective of this study was to evaluate the presence and distribution of antibiotic-resistant bacteria in Salish Sea harbor seals and harbor porpoises (*Phocoena phocoena*), with specific goals to describe the presence of antibiotic-resistant bacteria, determine differences in resistance between and within the two species, and describe geographic patterns.

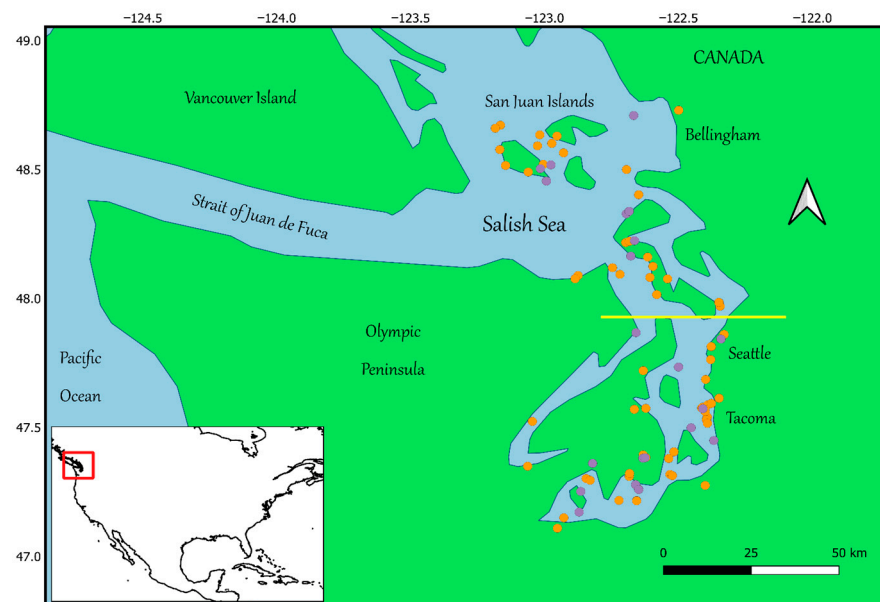
## 2. Materials and Methods

### 2.1. Study Population

A cross-sectional, opportunistic, sampling study was conducted from October 2018–May 2020 to determine the prevalence (%) of antibiotic-resistant bacteria from samples of fresh dead (Code 2) [21], stranded marine mammals in the inland waters of Washington State (Salish Sea). Specifically, two local species were targeted for sampling: harbor seals and harbor porpoises. These species were chosen for several reasons: they both occur in the Salish Sea in the greatest numbers compared to other marine mammal species; they are the most commonly stranded species within the Sea [22,23]; harbor seal and porpoise populations inhabiting the Salish Sea generally tend to stay more localized without traveling great distances, compared to their outer coast cohorts [24]; and these two species would be the most likely to carry antibiotic-resistant bacteria originating from terrestrial sources surrounding the Salish Sea, compared to more migratory or less common marine mammal species within the Sea. Lastly, they provide an opportunity to compare a completely aquatic species (porpoise) to one that is semi-aquatic (seals). Age class determination (adult, subadult/juvenile, pup/calf) for harbor seals was based on size and time of year [25] and on straight length for porpoises using ranges from known-age animals examined in Washington State [26].

## 2.2. Study Area

Two general sampling regions within Washington State's portion of the Salish Sea were investigated: the northern region encompassing the San Juan Island archipelago and waters north to the Canadian border, south to the southern tip of Whidbey Island, and west to the entrance of the Strait of Juan de Fuca; and the southern region, starting just south of Whidbey Island and encompassing Hood Canal, the Seattle/Tacoma Metropolitan, and continuing south to Olympia (Figure 1). The sampling regions represented areas with variable anthropogenic activities and human population density. Tourists heavily visit the northern portion during the summer months, but less so the rest of the year; however, currents may transport untreated wastewaters down from the cities of Vancouver and Victoria, British Columbia into this region [27]. The lower half of the northern region contains two military bases (Naval Air Station Whidbey Island and Naval Station Everett), heavily visited Whidbey and Camano Islands, as well as agricultural areas on these islands and the mainland at corresponding latitudes. The southern sampling region encompasses major areas of dense human population, Seattle and Tacoma, as well as many passages, inlets, and islands, with varying degrees of industrial development, agriculture, or population density.



**Figure 1.** Distribution of stranded harbor seals (orange dots) and harbor porpoises (purple dots) sampled for antibiotic resistant organisms in the Salish Sea, Washington State, USA. Yellow horizontal line delineates northern and southern portions of the study area.

## 2.3. Sample Collection

All animals were externally examined and, if feasible, a necropsy (or thorough internal examination) was performed within 24 h of being recovered from the beach (stored at 4 °C overnight if not done the same day). If a necropsy could not be performed during this time frame, a swab was collected rectally to sample the large intestine, and the carcass was frozen for examination at a later date or left at the stranding site. Samples for aerobic culture and sensitivity were collected postmortem from the large intestine/feces of each animal either by careful placement of a sterile swab rectally to prevent skin or fur contamination of the swab, or during examination of the large intestine at necropsy. Additional swabs were collected from any lesions noted on gross external and/or internal examination that were suspicious for bacterial involvement such as swelling, discharge, or redness [17]. Sampling of wounds or lesions was conducted using standard techniques to minimize environmental contamination [28]. All swabs were held in Amies transport medium without charcoal (BBL™ CultureSwab™ Plus Collection and Transport Swabs, Beckton Dickinson and Co.,

Sparks, MD, USA). Samples were refrigerated and shipped overnight to a local veterinary reference laboratory for processing (Phoenix Laboratory, Mukilteo, WA, USA).

#### 2.4. Bacterial Isolation and Antimicrobial Sensitivity Testing

Targeted bacteria included those that are of interest in marine mammal health, and overlap with health of terrestrial animals, humans, and other marine animals such as fish. They included *Streptococcus* spp. (alpha, beta, and gamma), *Staphylococcus aureus*, *Vibrio* spp., *Aeromonas hydrophila*, *Proteus vulgaris*, *Klebsiella* spp. (respiratory samples), and *Clostridium perfringens*. Given the extensive microbiome of the gastrointestinal tract, select organisms commonly considered enteric pathogens were targeted, and included *Salmonella*, *Clostridium* spp., *Campylobacter*, and *Escherichia coli*.

For each bacterial isolate, the following data were collected: animal and tissue of origin, stranding location coordinates of animal from which sample(s) were collected, taxonomic identification of isolate by the public veterinary laboratory, and sensitivity to each of the antibiotics tested. Standard methods were used to identify the bacteria, including growth on appropriate selective and differential media, gross colony appearance, morphology on gram stain, and biochemical reaction.

Conditions for aerobic bacterial culture were implemented, and bacterial isolates were identified based on colony morphology, growth characteristics, gram stain, and biochemical testing. For swabs of the large intestine (rectally or from descending colon at necropsy), a battery of primary plating media was used for routine bacterial fecal culture. Routine fecal culture setup was designed to optimize the recovery of *Salmonella*, *Shigella*, *Campylobacter*, and *E. coli*; thus, fecal specimens received for culture were plated onto at least four media: (i) MacConkey (MAC) agar, (ii) tryptic soy agar with 5% sheep blood, (iii) a selective/differential medium designed for the recovery of *Salmonella* and *Shigella*, and (iv) a medium designed for the recovery of *Campylobacter* [29]. In addition, blood agar plates were used to aid with the recovery of *Aeromonas* spp., *Plesiomonas* spp., and *Vibrio* spp., while Hektoen enteric agar was used to isolate and differentiate members of the species *Salmonella* and *Shigella*. Phenyl ethyl alcohol agar with 5% sheep blood was used to cultivate gram positive bacteria. The inoculated media was placed in a 37 °C incubator overnight. The following day, isolates were sub-cultured to isolate organisms. Identification of individual bacterial species was based on pH change indicators for substrate utilization, production of metabolic byproducts, and hydrolysis of substrates.

Swabs from tissues other than feces were streaked onto tryptic soy agar with 5% sheep blood, chocolate agar, Columbia CNA agar with 5% sheep blood, MacConkey agar, and thioglycolate agar as indicated for bacterial isolation. The inoculated media was placed in a 37 °C incubator overnight. The following day, isolates were subcultured to isolate out the targeted pathogenic organisms, with the number of isolates retrieved from a plate varying depending on the type of bacteria. Individual profiles of antimicrobial susceptibility testing and identification were developed using the same protocol, equipment, and antibiotic panel applied for the fecal cultures. Bacteria considered indicative of contamination, or those that were considered nonpathogenic, were presumptively identified and did not include susceptibility testing. A microbe was considered multi-drug resistant if an isolate was not considered susceptible to at least two or more bactericidal or bacteriostatic agents in at least two antimicrobial classes (adapted from Sweeney et al., 2018 [30]). *E. coli* isolates obtained from this study were archived by adding glycerol to a final concentration of 10–20%, freezing the culture, and storing it in an ultra-low-temperature freezer at –80 °C.

Further identification of Gram-negative bacteria was conducted using an Analytical Profile Index (API) 20E system (Biomérieux, Marcy-l'Étoile, France). Briefly, the bacterial suspensions were inoculated on a strip of 20 dehydrated reagents. These reagents are miniature versions of traditionally utilized biochemical assays for identifying bacterial species. The strip panel was incubated from 24 to 48 h at 35 °C. A numerical value, the API code, matched to the corresponding bacterial species in the API database, was assigned to the results. Bacterial isolates were not further confirmed through molecular testing.

Microaerophilic species such as *Campylobacter* were isolated using enriched media such as Skirrow agar and were grown in generator envelopes delivering 6% oxygen, 10% carbon dioxide, and 84% nitrogen for up to five days at 42 °C [29].

Individual profiles of antimicrobial susceptibility testing and identification were developed for each bacterial isolate of interest using an automated VITEK 2 instrument (Biomérieux, Marcy-l'Étoile, France). The antibiotic panel is a standard suite used by a local veterinary reference laboratory (Phoenix Laboratory, Mukilteo, WA, USA) and represents many of the most commonly used antibiotic classes in veterinary (and human) medicine in the United States. Antibiotics tested against isolates of clinical relevance included aminoglycosides (amikacin, gentamicin), carbapenems (imipenem), cephalosporins (cephalexin, cefovecin, cefpodoxime, ceftiofur), fluoroquinolones (enrofloxacin, marbofloxacin), penicillins (amoxicillin, amoxicillin/clavulanic acid), (sulfonamides) trimethoprim-sulfamethoxazole, tetracyclines (doxycycline), chloramphenicol, and florfenicol. The same set of antibiotics was used for a given species of bacteria, regardless of the location or timing of sampling, with results expressed as sensitive, intermediate, or resistant. For this study, isolates determined to be intermediate were not included in calculating resistance. Bacteria considered indicative of contamination or nonpathogenic were presumptively identified and were not subject to susceptibility testing.

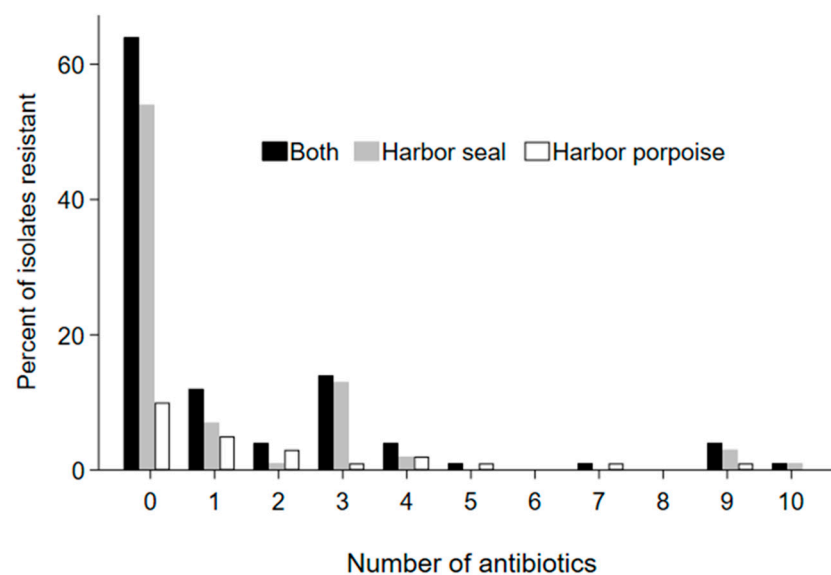
### 2.5. Analysis

Descriptive frequencies were calculated for each isolated bacterium from both collection regions (North and South Salish Sea) and for both species, including prevalence of single and multi-drug antibiotic resistance and occurrence of antibiotic resistance within taxonomic groups of bacterial isolates. Chi-square tests were used to compare resistance to each class of antibiotics by bacterial isolate and to analyze resistance patterns between the porpoises and seals and by age class. To avoid the chance of false discovery rate due to multiple simultaneous comparisons of antibiotics, a separate chi-square test was run for each drug. The proportions of resistance for each isolate and for all isolates pooled were compared between the two sampling regions using logistic regression. Odds ratios (OR) with their 95% confidence intervals (CI) were calculated to estimate risk of antibiotic resistance between the two species, two regions, and three age classes. For cells with expected counts of <5, Fisher's exact test was used. To analyze differences in the level of antibiotic resistance (defined as the mean number (count) of antibiotics to which a bacterial isolate was resistant) between seals and porpoises, we applied a generalized linear model with a negative binomial distribution to account for any over-dispersion of the data. Statistical significance was considered at  $p$ -value < 0.05. Analyses were performed using STATA 15.0 (STATA, College Station, TX, USA).

The proportion of antibiotics to which a particular isolate was resistant was used to generate a Multiple Antibiotic Resistance Index (MAR: range 0 to 1), which has been used to reflect potential anthropogenic impacts and degree of antibiotic exposure for an environmental isolate [31]. The index was calculated as the ratio of the number of resistant antibiotics to which an isolate is resistant to the total number of antibiotics to which it was tested. The resulting indices were further grouped based on whether their value was 0,  $\leq 0.2$  (amount of antibiotic resistance typical of nonpoint sources of anthropogenic pollution) and  $> 0.2$  (amount of antibiotic resistance considered characteristic of point-source pollution) [31–33]. Due to the inherent multi-drug resistance of many *Pseudomonas* spp. isolates, an additional calculation of MAR indices, without inclusion of this genus, was conducted. Water sources with a MAR index  $> 0.4$  are usually from human fecal origin and those  $< 0.4$  from nonhuman fecal contamination [34,35]. Lastly, spatial patterns of multi-drug resistance were determined by looking for clusters in the data using the program SaTScan. A Bernoulli model for spatial clusters was used, limiting cluster radius to 5 km and significant if  $p$ -value < 0.05 [36].

### 3. Results

A total of 95 animals were sampled (74 harbor seals [40 female:34 male], 21 harbor porpoises [11 female:10 male]), of which 85 (89%) animals (67 harbor seals/18 harbor porpoises; 24 adults/17 juveniles or subadult/44 pups or calves) successfully yielded 151 bacterial isolates that demonstrated identifiable bacterial growth, representing 26 genera and at least 31 confirmed individual species (Table S1). Eighty-six animals underwent full necropsies, and the remaining were only examined externally at time of sampling. Of the 95 sampled animals, 14 (10 seals, 4 porpoises) were frozen before any sampling took place. Out of these 14 that were frozen before sampling, only one did not yield any bacterial growth from the sampled tissue, in this case, the large intestine. In another animal, an adult harbor seal, the large intestine was sampled pre-freezing, while its other organs were sampled post-thaw at necropsy. For the remaining 13 that were necropsied post-freezing, sampling of organ lesions resulted in bacterial growth. From the 151 isolates, antibiotic resistance determination was performed on 144 (95%) (111 from seals/33 from porpoises). Seven isolates were not tested due to lab limitations (e.g., inability to regrow isolate) or testing was not applicable. Of the 144 isolates tested for resistance, 37% were resistant to at least one antibiotic, 26% were multi-drug resistant, while 61% were sensitive or intermediate to all antibiotics tested. Antibiotic resistant strains were isolated from both species of marine mammals, with 35% of the 74 seals and 52% of the 21 porpoises tested found to have a bacterial isolate resistant to at least one antibiotic (Figure 2). Multi-drug resistance was observed in 24% and 39% of tested seals and porpoises, respectively.



**Figure 2.** Percentage of bacterial isolates from dead stranded harbor seals and porpoises with antibiotic resistance based on the number of antibiotics to which the isolate was resistant.

Due to the predominance of samples originating from the large intestine, the most frequently cultured bacterium from both marine mammal species was *E. coli* (54%) (Table 1). The next most frequently cultured organisms were beta-hemolytic *Streptococcus* spp. (6%), *Pseudomonas aeruginosa* (3%), *Aeromonas hydrophila* (3%), *Edwardsiella tarda* (3%), *Shewanella algae* (3%), and *Salmonella* spp. (2%). The remaining genera were represented by single isolates. The other most commonly sampled anatomical sites were lung parenchymal lesions noted on gross examination at necropsy, followed by peritoneal fluid in harbor seals presenting with peritonitis (Table 2). There was a significant difference between seals and porpoises in the proportion of isolates that displayed resistance to at least one antibiotic ( $p$ -value = 0.004), as well as amongst age classes for both species combined (specifically juveniles compared to pups/calves,  $p$ -value = 0.025), but not between the two sampling regions ( $p$ -value = 0.248). A significant difference remained between the porpoises and

seals when accounting for sampling region ( $p$ -value = 0.006). Additional analyses targeting only *E. coli* isolates ( $n = 112$ ) and only intestinal isolates ( $n = 100$ ) resulted in similar significant differences. For isolates other than *E. coli* ( $n = 62$ ), significant differences were noted amongst age classes ( $p$ -value = 0.001) but not between species and regions, in proportion of isolates with any resistance.

**Table 1.** Bacterial species identified from various tissues collected from fresh, dead-stranded harbor seals (*Phoca vitulina*) and harbor porpoises (*Phocoena phocoena*) from the Salish Sea, Washington State, USA. Where applicable, the first number in parentheses refers to the number of isolates resistant to at least one antibiotic, followed by number of isolates resistant to more than one antibiotic. N/A = not tested for resistance.

Organism	Number of Isolates	
	<i>Phoca vitulina</i>	<i>Phocoena phocoena</i>
<i>Acinetobacter</i> spp.	0	1 (1/0)
<i>Actinomyces</i>	1 (N/A)	0
<i>Aeromonas hydrophila</i>	2 (2/1)	3 (3/2)
<i>Arcanobacterium haemolyticum</i>	1 (N/A)	0
<i>Arcanobacterium phocae</i>	2 (2/0)	0
$\beta$ -hemolytic <i>Streptococcus</i>	8 (8/8)	1 (1/1)
<i>Buttiauxella agrestis</i>	0	2 (0/0)
<i>Campylobacter</i> spp.	2 (N/A)	0
<i>Escherichia coli</i>	72 (8/4)	10 (1/1)
<i>Edwardsiella hoshinae</i>	1 (0/0)	0
<i>Edwardsiella tarda</i>	4 (0/0)	0
<i>Elizabethkingia meningoseptica</i>	1 (1/1)	0
<i>Enterobacter</i> spp.	1 (1/1)	0
<i>Escherichia fergusonii</i>	2 (2/2)	0
Gamma ( $\gamma$ ) hemolytic <i>Streptococcus</i>	1 (0/0)	0
<i>Gardnerella vaginalis</i>	1 (1/0)	0
<i>Granulicatella adiacens</i>	1 (N/A)	0
<i>Hafnia alvei</i>	1 (1/1)	0
<i>Moellerella wisconsensis</i>	0	1 (N/A)
<i>Pantoea agglomerans</i>	1 (1/1)	0
<i>Pasteurella</i> group	1 (0/0)	0
<i>Pasteurella multocida</i>	1 (N/A)	0
<i>Photobacterium damsela</i>	0	1 (0/0)
<i>Plesiomonas shigelloides</i>	1 (1/0)	1 (1/0)
<i>Proteus penneri</i>	0	1 (1/1)
<i>Proteus vulgaris</i>	1 (1/1)	0
<i>Providencia rettgeri</i>	0	1 (1/1)
<i>Pseudomonas aeruginosa</i>	5 (5/5)	0
<i>Pseudomonas fluorescens</i>	0	2 (2/2)
<i>Salmonella</i> spp.	3 (3/3)	0
<i>Serratia fonticola</i>	1 (0/0)	0
<i>Shewanella algae</i>	2 (1/0)	2 (2/2)
<i>Shewanella putrefaciens</i>	0	1 (1/1)
<i>Vibrio alginolyticus</i>	0	2 (2/1)
<i>Vibrio cholera</i>	0	1 (0/0)
<i>Vibrio fluvialis</i>	0	1 (1/0)
<i>Vibrio parahaemolyticus</i>	0	2 (2/0)
<i>Vibrio vulnificus</i>	1 (1/1)	0
Total	117	33

**Table 2.** Bacterial isolates by source and marine mammal species. Harbor seal = *Phoca vitulina*; harbor porpoise = *Phocoena phocoena*. In the case where more than one bacterial species was identified, the number of isolates was greater than the number of swabs taken. Where applicable, the first number in parentheses refers to the number of isolates resistant to at least one antibiotic, followed by number of isolates resistant to more than one antibiotic. N/A = not tested for resistance.

Source	Number of Isolates	
	<i>Phoca vitulina</i>	<i>Phocoena phocoena</i>
Abscess (brain)	1 (1/1)	0
Abscess (hind flipper)	2 (1/1)	0
Bronchiole	1 (N/A)	0
Colostrum	2 (1/1)	2 (2/2)
Kidney	1 (0/0)	2 (1/0)
Large intestine	81 (18/12)	22 (12/8)
Lung parenchyma	13 (7/7)	0
Lymph node (unspecified)	2 (2/2)	0
Lymph node (mediastinal)	0	2 (2/1)
Nares	1 (0/0)	0
Oral ulcers	1 (1/0)	0
Peritoneal fluid	9 (3/2)	0
Pleura	0	2 (1/1)
Scapular joint	1 (1/0)	0
Thoracic cavity	0	2 (1/1)
Tonsil	0	2 (1/0)
Uterus	2 (1/1)	0
Total	117	34

The most common species of organisms and patterns of antibiotic resistance obtained from seals and porpoises were markedly dissimilar (Table 3). Combining seals and porpoises together, gram-negative bacteria accounted for a majority (132/151, 87%) of the isolates that were identified, given the intestines were the most sampled source and were most susceptible to enrofloxacin (130/131 isolates, 99%) and marbofloxacin (129/131 isolates, 98%) and least susceptible to amoxicillin (30/130 isolates, 23%) and cephalexin (26/130 isolates, 20%). Only one *E. coli* isolate exhibiting resistance to any antibiotic was recovered from porpoises though all had swabs from the large intestine submitted. Out of the large intestinal swabs submitted from harbor seals that grew *E. coli* colonies ( $n = 70$ ), only nine (13%) produced isolates that were resistant to at least one antibiotic. Of all the bacteria tested for their antimicrobial susceptibility pattern, the *Pseudomonas* spp. were the most resistant (range: 9–10/15 antibiotics). *Pseudomonas aeruginosa* was isolated from five harbor seals (lung = 3; large intestine = 1; uterus = 1) and *P. fluorescens* from two harbor porpoises (large intestine = 2) (Table 1). They were most susceptible to the aminoglycosides and fluoroquinolones tested, and all but one isolate were also susceptible to doxycycline. One isolate each of *Pseudomonas fluorescens*, *Shewanella algae*, and *Proteus vulgaris* were resistant to imipenem, a member of the carbapenem class of antibiotics.



**Table 3.** Antibiotic resistance patterns of the most commonly ( $\geq 2$  isolates) identified bacteria in dead stranded harbor seals (*Phoca vitulina*) and porpoises (*Phocoena phocoena*) from the Salish Sea, Washington, USA. Number at the bottom of each column is the percent resistant out of the total. Gram stain = negative (N) or positive (P).

Bacteria	Gram Stain	Number Isolates	AM	AC	AX	CP	CF	CV	CR	CH	DX	EN	FL	GE	IM	MA	TMS
<b><i>Phoca vitulina</i></b>																	
<i>Escherichia coli</i>	N	72			4					1	5	1	1	1		1	3
Beta-hemolytic <i>Streptococcus</i>	P	8											8			8	
<i>Pseudomonas aeruginosa</i>	N	5			5	5	5	5	5	5	1		5				5
<i>Salmonella</i> spp.	N	3	3			3								3			
<i>Aeromonas hydrophila</i>	N	2			2	2			1								
<i>Shewanella algae</i>	N	2				1											
<b><i>Phocoena phocoena</i></b>																	
<i>Aeromonas hydrophila</i>	N	3			3	2					1						
<i>Pseudomonas fluorescens</i>	N	2		2	2	2	2	2	2	2			2		1		1
<i>Shewanella algae</i>	N	2			1	2					2				1		
<i>Vibrio alginolyticus</i>	N	2		1	1				1								
<i>Vibrio parahaemolyticus</i>	N	2			2												
<b>Total</b>		103	3	3	20	17	7	7	9	8	9	1	16	4	2	9	9

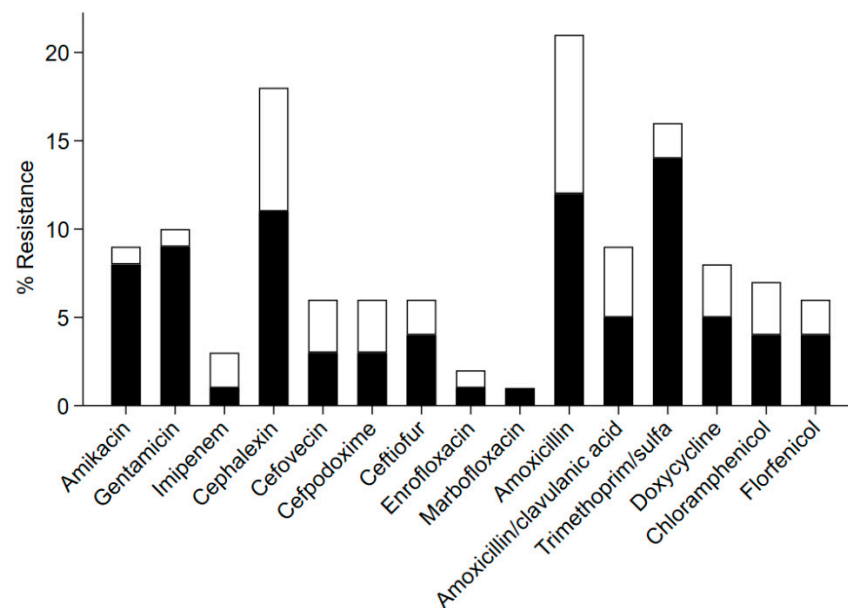
AM = Amikacin, AC = Amoxicillin/clavulanic acid, AX = Amoxicillin, CP = Cephalexin, CF = Cefpodoxime, CV = Cefovecin, CR = Ceftiofur, CH = Chloramphenicol, DX = Doxycycline, EN = Enrofloxacin, GE = Gentamicin, FL = Florfenicol, IM = Imipemem, MA = Marbofloxacin, TMS = Trimethoprim/sulfa.

Beta-hemolytic *Streptococcus* spp. made up more than half (9/13, 56%) of the gram-positive isolates tested for their antibiotic susceptibility. Other gram-positive organisms isolated and tested were *Arcanobacterium phocae* (15%), *Acinetobacter* spp. (8%), and gamma-hemolytic *Streptococcus* (8%). The beta-hemolytic *Streptococcus* spp. isolates were primarily cultured from the lungs (4/9) and were susceptible to most of the antibiotics tested; however, they were all resistant to gentamicin, amikacin, and trimethoprim/sulfamethoxazole. The *Arcanobacterium* isolates were resistant to trimethoprim/sulfamethoxazole and susceptible to the remaining antibiotics.

There was a significant difference in resistance to at least one antibiotic between marine mammal species ( $p$ -value = 0.004). Harbor porpoises were at significantly greater (more than three times) risk of having an organism resistant to any antibiotic compared to seals (OR = 3.25; 95% CI: 1.44–7.16). Likewise, a similarly significant difference between the two species was noted when simultaneously accounting for region ( $p$ -value = 0.006), but not by region alone, as noted earlier. Similar results were noted when intestinal isolates alone were evaluated. Examining only *E. coli* isolates ( $n = 82$ ), juvenile harbor porpoises were at greatest risk of resistance compared to calves or adults (OR = 3.34; CI = 1.16–9.65). Marine mammal species and region did not influence *E. coli* isolate resistance significantly. However, in porpoises, *E. coli* isolates were more resistant to the penicillin and cephalosporin antibiotic classes than in seals. Significant differences between age classes, for seals and porpoises combined, were noted for the antibiotics amoxicillin ( $p$ -value = 0.023), cephalexin ( $p$ -value = 0.019), and cefovecin ( $p$ -value = 0.043), specifically for juveniles compared to pups/calves. Pups/calves and juveniles were the source for a majority of the isolates resistant to amoxicillin (19/30, 63%) and cephalexin (15/26, 58%), heavily represented by *Pseudomonas* spp., *Vibrio* spp., and *E. coli* for both antibiotics. No significant differences between the northern and southern study regions by individual antibiotic were observed.

Significant differences in number of isolates (i.e., counts) resistant to antibiotics were not observed between seals and porpoises, study region or age class, individually, nor when all three variables were included in the negative binomial model.

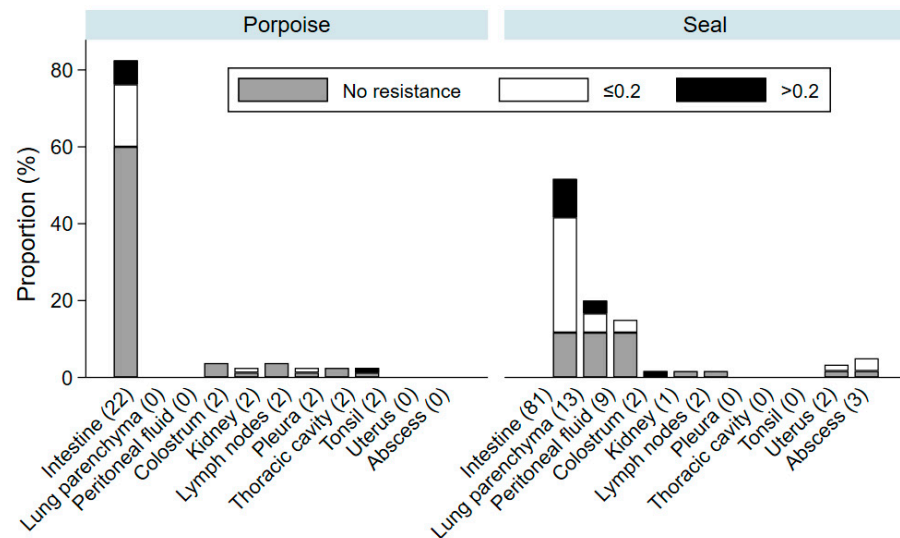
The percentage of total isolates demonstrating resistance to each of the 15 tested antibiotics is shown in Figure 3. Resistance of bacterial isolates to individual antibiotics was significantly greater for porpoises compared to seals for amoxicillin/clavulanic acid ( $p$ -value = 0.039; OR = 3.43, 95% CI: 1.06–11.07), amoxicillin ( $p$ -value = 0.003; OR = 3.78, CI: 1.58–9.07), cephalixin ( $p$ -value = 0.027; OR = 2.83, CI: 1.13–7.10), and cefovecin ( $p$ -value = 0.041; OR = 3.93, CI: 1.06–14.54). When analyzing only intestinal isolates, resistance patterns were similar, with the exception there was also significantly greater resistance to cefpodoxime in porpoises ( $p$ -value = 0.028). For non-*E. coli* isolates only, significant differences in resistance between species were noted for gentamicin ( $p$ -value = 0.039; OR = 9.33, CI: 1.12–77.62) and for trimethoprim/sulfamethoxazole ( $p$ -value = 0.011; OR = 7.73, CI: 1.58–37.72), and when adjusted for age class, with no documented resistance to either of the two fluoroquinolones for any of these isolates. When adjusting for study region (north versus south), significant differences between seals and porpoises were again observed for the same four antibiotics. Greater than half (54%) of the 14 isolates that were resistant to amoxicillin/clavulanic acid was attributed to *Pseudomonas* spp., with five in seals and two in porpoises; approximately half of the isolates resistant to amoxicillin and cephalixin were sampled from the large intestine of harbor seals and consisted of a variety of organisms. Four isolates were resistant to imipenem, three of which originated from porpoises.



**Figure 3.** Percentage of bacterial isolates from harbor seals (black) and harbor porpoises (white) displaying resistance to individual antibiotics, grouped by class.

Of the 144 isolates tested for antibiotic sensitivity, 40 (28%) exhibited resistance to multiple antibiotics, with 10% having a multiple antibiotic resistance (MAR) index value > 0.2. An additional calculation of the MAR index value, excluding *Pseudomonas* spp. isolates ( $n = 7$ ), demonstrated MAR in 23% of 137 isolates, with 20% having an MAR index > 0.2. Of the species-specific isolates tested for antibiotic resistance, 24% (27/111) and 39% (13/33) of seal and porpoise isolates, respectively, were multi-drug resistant (21%; 22/106 and 35%; 11/31, respectively, when *Pseudomonas* excluded). The MAR indices ranged from 0.06 in harbor seal *A. phocae* and *Gardnerella vaginalis* isolates to 0.67 in a *P. aeruginosa* isolate from a seal. The percentage of bacterial isolates within each MAR classification (0,  $\leq 0.2$ , >2.0) are illustrated by tissue for each marine mammal species (Figure 4). Only tissues represented

by at least two isolates were included in the analysis of the MAR index, thus including large intestine, lung parenchyma, peritoneal fluid, colostrum, kidney, lymph node, pleura, thorax, tonsil, uterus, and abscess samples. Isolates with MAR indices  $> 0.2$ , as well as no multi-drug resistance, were represented by tissues within the respiratory, reproductive, and lower gastrointestinal systems (lung and pleura, colostrum and uterus, large intestine).



**Figure 4.** Proportion of bacterial isolates from harbor seals and porpoises with multiple antibiotic resistance originating from individual tissue sources. Proportions are categorized based on their Multiple Antibiotic Resistance Index (MAR): MAR = 0 (no resistance), MAR =  $0 \leq 0.2$ , or MAR  $> 0.2$ . Numbers in parentheses represent number of bacterial isolates from each tissue source.

Though not significant at the alpha level of 0.05, two spatial clusters of multi-drug resistant isolates were observed. One cluster ( $p$ -value = 0.081) was located in the central portion of the Salish Sea on Whidbey Island consisting of three harbor seals, two with *P. aeruginosa* and one with *Serratia fonticola*. The other cluster consisted of two harbor porpoises found stranded near the far southern portion of the Salish Sea, from which *Shewanella* spp. were isolated.

#### 4. Discussion

The results of this study demonstrated differences in antibiotic resistance between harbor seals and porpoises inhabiting an urban marine ecosystem in the inland waters of Washington State, near coastlines associated with anthropogenic impacts. Antibiotic resistance was demonstrated in animals stranded throughout the Salish Sea, suggestive of a baseline level of resistance throughout the region. Prior to this study, bacteriologic cultures and antimicrobial susceptibilities were performed on cases submitted as part of necropsy-related sampling, but not consistently throughout the Salish Sea due to variations in stranding response capabilities, logistics, or funding. The results reported here indicate a relatively high level (37%) of antimicrobial resistance in bacteria isolated from stranded marine mammals.

Significant differences were again seen between the two species when the geographic sampling area (north vs. south) was taken into account. Additionally, there were differences in the patterns of antibiotic resistance, or the antibiotic classes to which bacteria demonstrated resistance, between seals and porpoises. Both of these marine mammal species live and forage in nearshore coastal environments, resulting in exposure to sites highly impacted by humans (agricultural runoff, sewage treatment effluent, aquaculture) and the land-to-sea transfer of pathogens [14,37–39]. However, harbor porpoises may differ sufficiently from harbor seals in their habitat use resulting in a greater exposure to anthropogenic pollution. Specifically, porpoises are obligate water dwellers whereas

harbor seals spend a portion of their life hauled out of the water to rest and nurse their young. Further work involving molecular techniques will be needed to work out specific mechanisms of resistance between the species but is beyond the scope of this study.

More widespread antibiotic resistance has been reported in stranded marine mammals compared to free-ranging populations, likely due to a bias toward more diseased animals representing in stranded cases [40]. The extent of resistance observed here is consistent with other studies of marine mammals in coastal oceans, noting a relatively high prevalence and similar patterns of antibiotic resistance as presently observed [8,11,13,40]. For example, Lockwood et al. [17] observed that only one antibiotic was observed capable of killing or inhibiting growth of all the isolates tested from harbor seals in the northern Salish Sea, while in bacterial isolates from vertebrates off the northeastern USA coast, 58% of isolates were resistant to at least one antibiotic and 43% to more than one [40]. Isolates ( $n = 733$ ) originating from bottlenose dolphins (*Tursiops truncatus*) on the east coast of Florida, USA over the periods 2003–2007 and 2010–2015 had an overall 88.2% prevalence of resistance to at least one antibiotic [41].

Organisms such as *Acinetobacter* spp., *A. hydrophila*, *Plesiomonas shigelloides*, *P. fluorescens*, and *Serratia fonticola* are opportunistic in nature and may cause secondary infections in humans and immunocompromised marine mammals such as SRKWs [19]. *E. coli* was the most common isolate from the intestinal tract ( $n = 69$ ), followed by *Vibrio* spp. ( $n = 7$ ), consistent with the findings of Stewart et al. (2014) where *Vibrio* spp. (15%) and *E. coli* (6%) were the two most commonly isolated fecal organisms in dolphins. In previous Salish Sea harbor seal studies, *E. coli* was the most consistently isolated organism from sources other than the intestine such as wounds, umbilici, ears, and nares of animals admitted to rehabilitation [17]. Additionally, it was the second most frequently isolated microbe from brain, liver, respiratory tracts, and kidney of free-ranging pups [18], behind *Proteus* spp., which represented only 2/150 (1.3%) isolates in the present study. Enterobacteriaceae are not typically considered primary pathogens but may be secondary opportunistic invaders of preexisting wounds [42]. They were the most frequently isolated gram-negative organisms (56%) in rehabilitating harbor seals in the Salish Sea [17] and were found in 60% of harbor seals live-stranded in California admitted to rehabilitation [8]. While frequently isolated in free-ranging bottlenose dolphins along the east coast of Florida [11,13], Enterobacteriaceae were not the most commonly identified gram-negative bacteria of the present study. They represented only 38% of gram-negative isolates. Ongoing studies are planned to investigate the pathogenic impact of different types of *E. coli* through molecular studies of isolates recovered in this study and to help discover the role of this genus in causing morbidity and mortality in harbor seals, porpoises, and SRKWs. All three *Salmonella* isolates were notably resistant to the aminoglycosides, amikacin and gentamicin, as well as the cephalosporin, cephalexin. This latter drug is of note due to increasing prevalence of multi-drug resistance in *Salmonella*, with special concern to related cephalosporins such as ceftiofur and ceftriaxone [43]. Carbapenem class resistance, seen in isolates of *P. fluorescens*, *S. algae*, and *P. vulgaris*, was accompanied by multi-drug resistance in each of the isolates. Though not treated by carbapenems, marine mammals represent potential reservoirs of multi-drug resistant bacterial strains potentially able to infect humans or other animals [44]. Resistance to carbapenems is an ongoing global public-health problem. This type of antimicrobial resistance, especially when mediated by gene transfer, is spreading rapidly causing serious outbreaks and dramatically limiting treatment options in humans and domestic animals [45,46].

In this study, beta-hemolytic *Streptococcus* was the most common gram-positive isolate and was most frequently recovered from lung tissue (4/9, 44% of beta-hemolytic *Streptococcus* isolates). In rehabilitated Salish Sea harbor seals, it was also the most common gram-positive isolate, frequently found in wounds [17]. Additionally, this organism was isolated from 16% of wounds in live-stranded California harbor seals [8], 21% (4/19 brain and liver isolates) of dead stranded Salish Sea harbor seals [18], and 13 stranded and 3 net caught harbor porpoises (primarily liver and kidney) from the Baltic and North Seas [47].

This contrasts with a study of resistance in seals from the northwest United States Atlantic coast in which *Enterococcus* spp. were the most common gram-positive isolate with 25% (8/32) of isolates originating from the integumentary system [48].

Among groups of bacterial isolates commonly sampled, occurrence of any antibiotic resistance ranged from 10% of isolates (*E. coli*) to 100% (*Pseudomonas* spp., beta-hemolytic *Streptococcus* spp., *Shewanella algae*, *Aeromonas hydrophila*, and *Vibrio parahaemolyticus*) (Table 1), comprising a range of resistance across varying taxonomic groups. It was surprising to observe such low antibiotic resistance in *E. coli* compared to other taxonomic groups, but this was also noted in a large study of antibiotic resistance in seabirds, marine mammals, and fish along the northeast USA [40]. Furthermore, *E. coli* isolated from environmental aquatic samples, that included treated sewage water, rivers, and drinking water, also demonstrated antibiotic resistance [49–51]. Other studies examining resistance in *E. coli* reported higher incidences, ranging from 46% in Tanzanian drinking water [52] to 100% in India [53].

Antibiotic resistance has been reported in aquatic ecosystems contaminated with heavy metals, especially zinc and cadmium, which are thought to contribute to selection of antibiotic-resistant strains such as *E. coli* and *P. aeruginosa* [54]. Heavy metals in the environment may serve as co-selecting agents for antibiotic resistance in human pathogens in reservoirs such as aquatic ecosystems. Heavy metals are not yet quantified in Salish Sea harbor porpoises, but recent studies in regional harbor seals detected significant differences in trace element concentrations among age classes, regions within the Salish Sea, and years sampled [55], and elevated cadmium, copper, and zinc in non-pup seals from the northern vs. southern Salish Sea [56]. Efforts are ongoing to characterize metals in Salish Sea porpoises to inform future antibiotic resistance studies in porpoises.

The greatest proportion of resistant isolates were recovered from juvenile animals, though they were the least represented in number. The absolute counts of resistant isolates amongst the age classes were not significant and may reflect differences in bacterial species within each age group. Most antibiotic resistance studies do not examine differences in susceptibility patterns amongst age classes. Some studies have noted increased antibiotic resistance by methicillin-resistant *Staphylococcus aureus* in older human populations [57]), as well as differences in bacterial species and susceptibility patterns between foals and adult horses [58]. Collecting appropriate proportions of varying age classes for valid comparisons in wildlife studies presents challenges when relying on passive collection of stranded animals. Although not well studied, differences in age class-related resistance between harbor seals and porpoises may reflect differences in microbiome composition such as the nascent fecal biome of pups and calves compared to older animals as has been described in spotted hyenas (*Crocutta crocutta*) [59].

Harbor porpoises, which are more phylogenetically related to the endangered SRKWs, presented with more widespread antibiotic resistance than harbor seals and may represent sentinels for SRKW health. There is overlap in resistant bacteria between harbor porpoises and SRKWs with resistant isolates of *P. fluorescens* and *V. alginolyticus* (from feces in porpoise and breath in SRKW) [19]. Although only eight *E. coli* isolates were obtained from harbor porpoise, they were all sensitive to all antibiotics. They may not have been the same extraintestinal strains documented in SRKWs that were the same clonal lineages (ST73 and ST127) that are often associated with human community-acquired urinary tract disease [20]. Planned genotyping of the present study's isolates will further define their clonal lineages and relationship to SRKW and human isolates.

Multi-drug resistance is an increasingly common problem in human and veterinary medicine and requires a One Health approach to address the multiple facets of health that interplay in forming and promoting resistance [60,61]. For this to happen, we need a deeper, or more complete "One Health" understanding of antimicrobial resistance in free-ranging wildlife, especially regarding the land-sea transfer to marine mammals. Indices of multiple antibiotic resistance (MAR) of  $\geq 0.2$  were observed in 25/54 (46%) of the resistant bacterial isolates, suggesting seal or porpoise exposure to bacteria from significantly polluted sites.

The influence of anthropogenic activities in aquatic environments, and on their nearshore sediments and soils, may amplify the generation of new antibiotic resistance genes and the spread of resistant bacteria and have serious ramifications on environmental and public health [62,63]. This highlights the importance of the environment as a reservoir of resistance genes and dispersal vectors. The resistance patterns observed in this study suggest that some components of resistance are likely related to environmental origins and may spread without the selective pressure of antibiotic use.

It is unclear how the high levels of antibiotic resistance observed in some of the animals sampled relates to the larger coastal environment since environmental samples were not evaluated or whether the resistance is acquired from other aquatic species. The lack of spatial trends in resistance may reflect the level of sampling, spatial use by the animals sampled, or the complex state of ecosystem connectivity and estuarine exchange flow within the Salish Sea and its coastal shoreline. Connectivity is especially vital in coastal systems where energy and biota are constantly moving and exchanging among ecosystem components [64,65]. Systematic environmental sampling for antibiotic resistance in bacteria throughout the Salish Sea would provide greater insight into determining if antibiotic resistance patterns in marine wildlife are representative of their habitat. Additionally, it might show if human alteration of habitat is fragmenting the region's connectivity or changing normal ecosystem processes that help limit transfer of antibiotic resistant bacteria. For example, Lamb et al. [66] demonstrated that healthy seagrass meadows can reduce the land-sea transfer of harmful bacteria. Seagrass meadows can naturally remove or reduce pathogens in terrestrially-sourced wastewater by up to 50% and reduce coral reef disease, yet in Washington's half of the Salish Sea, localized small-scale eelgrass (*Zostera marina*) declines have been significant [67].

The findings from this study are significant for marine mammal and human health. Since marine mammals are apex predators, with their health reflective of their shared marine ecosystem with humans, they serve as sentinels of ecosystem health [68]. This is particularly key in a region such as the Salish Sea where much of the human population lives along the coastline. The patterns of antibiotic resistance described in two Salish Sea marine mammal populations may indicate a potential public health risk since disease related to these bacteria may coincide with emerging infectious diseases in these mammals resulting from environmental perturbations or increasing resistance in aquatic species in general. Study results will help inform local human and veterinary health officials, as well as raise public awareness of drug resistance in the marine environment.

Though zoonotic pathogens from marine mammals are not widely distributed in human populations, aquatic mammals are known to carry pathogens that pose a risk to human health [69,70]. Importantly, several bacteria were identified in this study that can cause infections of public health significance and are considered reportable to the Centers for Disease Control and Prevention (CDC) [71], including *Vibrio cholerae*, *V. parahaemolyticus*, and *V. vulnificus*. *Vibrio* species are abundant and ubiquitous in the marine environment and are a frequent cause of gastrointestinal illness in humans associated with seafood consumption (*V. parahaemolyticus*) in the Pacific Northwest [72] and wound infections resulting in high morbidity and mortality (*V. vulnificus*) [73]. An isolate of *V. cholerae* was identified from the large intestine of a harbor porpoise calf found in the northern portion of the study area near the San Juan Islands, but characterized as non-toxigenic O1, not O139, the cause of reportable cholera [71]. A survey of estuarine waters of Washington, Oregon, and California detected non-O1 strains of *V. cholerae* in Washington [74]. That study posited that the low incidence of non-O1 *Vibrio* strains detected suggested a potential for human, and thus marine mammal, infection; however, the low frequency of toxigenic strains indicates a lower threat to animals in the Pacific Northwest compared to other regions such as the Gulf of Mexico and Atlantic coast. Other potential sources of exposure to zoonotic bacteria from marine mammals include occupational exposure [69,75], touching live or dead animals on the beach, and consumption of marine mammals [76].

A confounding factor of this study is that most of the samples were fecal swabs obtained via rectal sampling or at necropsy due to ease of procurement, and as previously described, most of the bacteria isolated from the fecal swabs were *E. coli*, which showed relatively low incidence of antibiotic resistance compared to other tissues and other bacterial groups. Thus, it is possible that the differences between the seals and porpoises and age groups may have been due to the sample types collected; however, these were controlled for in the analyses and thus do not exert an undue influence on the analytical results. Sampling of previously frozen carcasses (14 in total) might have hindered the ability to isolate bacteria (and thus resulting resistance patterns). Effort was made to minimize sampling post-thaw, but was not always avoidable. The greatest impact on the analyses was a reduction in the number and types of bacterial isolates that would have been obtained if the post-thaw animals had been sampled prior to freezing. The methods used in this study were limited to aerobic culture of organisms using commercial instruments and databases, and a limited number of antibiotics, and was not intended to investigate the complete microbiomes of either harbor seals or porpoises. Rather, it was designed to evaluate samples of convenience for bacterial prevalence and sensitivities that are of SRKW and public health relevance, although tested samples likely contained additional bacterial species that were present but not speciated due to lack of growth from suboptimal storage or other extrinsic factors, resulting in underreporting of prevalence. High-throughput genome sequencing methods, and more recently DNA-based metagenomics, have revolutionized the ability to quickly look at dominant bacteria and antibiotic resistance genes (resistomes) to determine the relative contribution and importance of a bacterial species to an animal's microbial community [19,20,77,78]. Though resistomes were not examined in this study, likely resulting in antibiotic resistance genes being missed, future studies should include genome sequencing in addition to traditional culture methods.

## 5. Conclusions

In this study, we document widespread antibiotic resistance in bacterial isolates from two species of marine mammals, with more marked multi-drug resistance in harbor porpoises compared to harbor seals. The high incidence of single and multi-drug resistance was consistent with other studies in marine species. Results of this study also confirm previous work suggesting multi-drug resistance may be common in bacteria originating in marine mammals. The novelty of this study is its focus on two species with overlapping ranges but dissimilar ecological niches (completely aquatic versus semi-aquatic). We provided an initial simultaneous glimpse at resistance patterns in multiple regions and age classes within an urbanized marine ecosystem that may serve as a reservoir for antibiotic resistance. These two marine mammal species live in nearshore waters of the Salish Sea and likely come into regular contact with humans and their associated activities. Thus, the relatively high occurrence of antibiotic resistance may reflect a large environmental reservoir of antibiotic resistant organisms occurring in this body of water, or inherent differences in resistance patterns or susceptibility to resistance genes between the two species. Due to the large geographic area of the Salish Sea and the variety of its anthropogenic activities, further investigation of temporal and spatial resistance patterns, as well as environmental sampling, will better inform natural resource managers working to recover endangered SRKWs as well as public health officials in the region. By engaging the human, animal, and environmental health sectors together to monitor antibiotic resistance patterns in marine species, efforts to address antibiotic resistance in a collaborative One Health approach will benefit this urban ecosystem and its inhabitants.

**Supplementary Materials:** The following is available online at <http://www.mdpi.com/xxx/s1>. Table S1: Raw data results from sampling of harbor seals and harbor porpoises for aerobic bacterial culture and sensitivity.

**Author Contributions:** Conceptualization, S.A.N.; methodology, S.A.N., D.M.L., and J.L.H.; software, S.A.N.; validation, S.A.N., D.M.L., J.L.H., and J.K.G.; formal analysis, S.A.N.; investigation, S.A.N.,

J.K.G., J.L.H., D.M.L., J.K.O., V.S., A.E., A.S., S.B., S.D., C.K., B.C., M.J., and R.M.; resources, S.A.N., J.K.G., J.L.H., D.M.L., J.K.O., V.S., A.E., A.S., S.B., S.D., C.K., B.C., M.J., and R.M.; data curation, S.A.N.; writing—original draft preparation, S.A.N.; writing—review and editing, S.A.N., J.K.G., J.L.H., D.M.L., J.K.O., V.S., A.E., A.S., S.B., S.D., C.K., B.C., M.J., and R.M.; visualization, S.A.N. and J.K.G.; supervision, S.A.N.; project administration, S.A.N. and S.B.; funding acquisition, S.A.N., S.B., S.D., J.L.H., V.S., C.K., J.K.O., and B.C. All authors have read and agreed to the published version of the manuscript.

**Funding:** Funds to support this project were provided by the generous donations of more than 148 public citizens submitted through Experiment ([www.experiment.com](http://www.experiment.com)), a web-based source for crowdfunding science-based research projects, and from the Wildlife Disease Association. Efforts to support collection of stranded marine mammals were partially funded through the John H. Prescott Marine Mammal Rescue Assistance Grant Program.

**Institutional Review Board Statement:** IACUC approval was not required by any institutions or organizations for this work, as all carcasses used in this study were found dead prior to inclusion in the study. Authorization to collect dead, beach-cast marine mammals is given to each collaborative organization in this study through a permit from the United States National Marine Fisheries Service Marine Mammal Health and Stranding Response Program (#18786-04).

**Data Availability Statement:** The data generated in this study are available in Table S1.

**Acknowledgments:** Study animals were collected with the assistance of volunteers and staff from Orca Network/Central Puget Sound Marine Mammal Stranding Network (specifically Garry Heinrich, Bob and Kelly Post, Mary Jo Adams, Jill Hein, and Matt Klope), Washington Department of Fish and Wildlife, Cascadia Research Collective (specifically Erin Keene), San Juan County Marine Mammal Stranding Network (The Whale Museum), Seal Sitters Marine Mammal Stranding Network (especially Lynn Shimamoto), Whatcom County Marine Mammal Stranding Network, Port Townsend Marine Science Center/East Jefferson County Marine Mammal Stranding Network, Sno-King Marine Mammal Rescue, MaST Center Marine Mammal Stranding Team at Highline College, and World Vets. Meridith Grogan, Meghan Riddell, and Ronda Reisdorph (Phoenix Laboratory, Mukilteo, Washington), and Linda Rhodes (NOAA Fisheries, Northwest Fisheries Science Center, Seattle, Washington) for help handling isolates, and Cindy Wu (Experiment.com) for technical help with project crowdsourcing. We appreciate the suggestions of four anonymous reviewers that improved the manuscript.

**Conflicts of Interest:** The authors declare no conflict of interest.

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