ANTIMICROBIAL RESISTANCE IN *ORCINUS ORCA* SCAT: USING MARINE SENTINELS AS INDICATORS OF PHARMACEUTICAL POLLUTION IN THE SALISH SEA

by

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ABSTRACT

Antimicrobial resistance in *Orcinus orca* scat:
Marine sentinels as indicators of pharmaceutical pollution in the Salish Sea
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Antimicrobial drugs revolutionized health care, but drugs and antibiotic resistant bacteria (ARB) entering surface waters may increase resistance, alter bacterial populations, or create reservoirs of resistance genes transmittable to pathogens. This study assessed risk factors for colonization with ARB in the scat of *Orcinus orca* to determine if geographic, temporal, or animal traits related to resistance prevalence and/or patterns. Samples were collected June-October 2012 and August-October 2013, using a scat detection dog to locate feces. Eleven samples were plated on agar infused with ampicillin, chloramphenicol, erythromycin, and tetracycline for colony count and multidrug resistance assessment. PCR amplification of the resistance-conferring genes *erm*B, *mec*A, *tet*B and *tet*M was performed on 32 samples. The study area was divided into segments based on watershed traits, and distance from shore, number of septic tanks, wastewater treatment plants (WWTPs), land area and human population density were analyzed for each sample based on segment. Animal age, sex, and pod were studied as organism risk factors. Number of colonies, presence/absence of ARB gene, and multidrug resistance (MDR) rates were independent variables. A total of 1730 resistant colonies were cultured from 11 samples, with erythromycin ranking first in prevalence and total resistant colonies, followed by ampicillin, tetracycline, and chloramphenicol. The effect of sampling location on number of colonies was significant ($F_{2, 8}$= 6.78, p = 0.019), and a sample obtained from the Southern Gulf Island site had more ARB colonies and was significantly higher in bacteria resistant to ampicillin ($F_{2, 8}$ =19.75, p=8.05×10-4), erythromycin ($F_{2, 8}$ =5.36, p=0.03), and enteric bacteria resistant to ampicillin ($F_{2, 8}$ =37.07, p=8.99×10-5). This site has more WWTPs, including a plant that recently used only primary treatment, but no environmental factors were statistically related to colonies or MDR by regression analysis. Results showed 4 samples positive for the *tet*M gene, and although all 4 were from J-pod females in the same geographic segment, results were not significant. This study is the first to report positive identification of ARB in the feces of *Orcinus orca*, and though no specific environmental relationships were identified, the prevalence of ARB warrants further research, particularly into factors such as time and travel before sampling, to better understand the factors impacting ARB colonization in the SRKW and the use of marine mammals as sentinel species for pharmaceutical pollution in the Salish Sea.

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List of Abbreviations

Amp: Ampicillin

ARB: Antimicrobial resistant bacteria

Cm: Chloramphenicol

*erm*B: Erythromycin-ribosomal methylase B

Erm: Erythromycin

km: Kilometers

m: Meters

*mec*A: Methicillin-resistance gene A

MDR: Multidrug resistance

NaCl: Sodium chloride

ng/L: nanogram per liter

PCPP: Personal care product and pharmaceutical pollution

PCR: Polymerase chain reaction

SDR: Single drug resistance

SRKW: Southern Resident Killer Whale

*tet*B: Tetracycline-resistance gene B

*tet*M: Tetracycline-resistance gene M

Tc: Tetracycline

TSA: Tryptic soy agar

µg/L: Microgram per liter

WWTP: Waste water treatment plant

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Gabriel, you made me fierce. Thank you for the love and support, and living with whale poop in the freezer for much of my graduate school career. This book is for you.

"We have the ability to provide clean water for every man, woman and child on the Earth. What has been lacking is the collective will to accomplish this. What are we waiting for? This is the commitment we need to make to the world, now."

-Jean-Michel Cousteau

INTRODUCTION

Since the introduction of antimicrobials in the 1940's, bacteria have shown an increased response in resistance. Antimicrobials are the third-largest group of medicines prescribed for humans, and the largest category of medicines used in veterinary practices (Thiele-Bruhn, 2003). These antimicrobial drugs enter the environment directly through flushing unused drugs down the drain, and indirectly through unmetabolized compounds excreted by human and animal waste through wastewater effluent and leaking septic tanks,, and runoff and drainage from agricultural lands and aquaculture sites (Cabello, 2006; Kümmerer, 2004; Okeke and Edleman, 2001; Zhang et al., 2009). Increased input of antimicrobial drugs creates opportunities for environmental and pathogenic bacteria to develop selective resistance to pharmaceuticals due to the ease with which bacteria exchange genetic material and the speed at which they reproduce. More virulent and resistant bacteria are artificially selected by this increased exposure, and also threaten water quality through altered environmental bacteria populations. Given that increased levels of resistance in human gut bacteria can persist for up to two years after treatment has stopped (Jakobsson et al., 2010), the massive amount of antimicrobials that we are adding to our environment now may have long term consequences.

Increased human population and decreased wild habitat has intertwined the health of humans, wildlife, and the environment, breaking down divisions between these disciplines in the assessment of ecosystem health (King et al., 2008). This new paradigm, exemplified by the growing One Health movement,

has created a more collaborative and holistic approach to monitoring ecosystem health through the use of animal sentinels (Rabinowitz and Conti, 2013). Marine mammals are a good sentinel species in marine and aquatic research due to their physiological relationship to humans, their position as apex predators, and their visibility as a keystone species in the ecosystem (Bossart, 2006; Wong, 2002).

Fecal sampling can be employed as a non-invasive alternative to capture/release in study of threatened species or in areas where tracking an animal for other biological indicators is difficult. Feces contain a wealth of information on the animal, including genetic, hormonal, and toxin loading, and the nutrient-rich intestinal environment is particularly conducive to microbiological research (Kohn and Wayne, 1997; Miranda and Zemelman, 2001; Taberlet et al., 1999).

Research using the intestinal bacterial flora of marine vertebrates as indicator species for ARB pollution has shown varying degrees of resistance. ARB presence has been studied in predatory fish, marine birds, pinnipeds, and various whale and dolphin species by culturing bacteria from rectal or fecal swabs (Blackburn, 2010; Grieg, 2007; Johnson et al., 2008; Lockwood et al., 2006; Miller et al., 2008; Miranda and Zemelman, 2002; Rose et al., 2008; Schaefer et al., 2011; Schroeder et al., 2009; Stoddard et al., 2002). The levels of resistant samples and the complexity of resistance patterns has been positively correlated by sampling site to suspected contamination points, ex. higher number of wastewater treatment plant (WWTP) outflows (Grieg et al., 2007), number of septic tanks (Schaefer et al., 2011), human population density (Blackburn et al, 2010; Grieg et al., 2007), and freshwater outflows (Johnson et al., 2008). Rainfall

and weather events have also been related to increase antibiotic resistance findings in short-term temporal analyses (Grieg et al., 2007; Schaefer et al., 2011).

The Salish Sea is an ecologically diverse and economically important estuary in the Pacific Northwest region of the United States of America and southwestern British Columbia, Canada (Fraser et al., 2006; Gaydos et al., 2009). This glacial carved fjord inland marine ecosystem stretches along the inland waters from Olympia, WA in the United States to the Campbell River in Canada, and is the home to over 7 million people and many endemic and rare marine species (Jackson and Kimerling, 2003). Like many of the world's coastal zones, population growth and increased toxin and fuel loading has amplified pressure on the marine habitat and wildlife in the Salish Sea (Gaydos et al. 2009; Puget Sound Partnership, 2011). Population growth has increased loading to septic and waste treatment systems, increasing likelihood that harmful substances, including pathogenic bacteria, toxins, and other chemical compounds will reach the water column without being properly broken down (Take Back Your Meds, 2011). Groundwater, storm water, and combined sewer overflows discharge untreated and industrial wastewater and compounds leached from leaky septic drainage systems when capacity is exceeded or water levels rise above the drainage fields also contribute to surface water pollution (Puget Sound Partnership, 2011; Dougherty et al., 2010). Research on marine bacterial resistance in Washington shows that ARB can be found in marine sediment near aquaculture sites, the sea-surface, and public beaches (Herwig et al., 1996; Roberts et al., 2008; Soge et al.,

2009). When analyzing sand samples from Washington beaches, five distinct strains of multi-drug resistant methicillin-resistant *Staphylococcus aureus* (MRSA) and 33 methicillin-resistant coagulase-negative *Staphylococcus* ($MR_{CON}SA$) spp were identified (Soge et al., 2009) Roberts et al. (2008) identified 18 resistant strains of vancomycin-resistant *Enterococcus* in Washington public beaches. The presence of these resistant and pathogenic strains makes a case for the possibility of ARB genes in offshore marine waters, and understanding the transmission of these genes is critical to assessing the risk of the marine environment serving as a reservoir of resistance genes transmittable to pathogens.

In the Salish Sea, abundant data is available the endemic ecotype of the *Orcinus orca*, or Southern Resident Killer Whale (SRKW). Concerns over decline in the SRKW population resulted in a large collection of data on the life history, family lineage, geographic range, and individual identity of most whales. The population consisted of 86 individuals at the 2012 spring census and declined to 80 by the fall of 2013, during the time of this study (Center for Whale Research, 2013). Declines in this population are primarily thought to be due to increased toxin accumulation from human pollutants, stress from lack of nutrition with declining salmon population, and underwater noise pollution, and other proposed threats include pathogens (NOAA, 2011).The importance of this species as a cultural icon, recreational draw, and keystone species make them ideal for the study of antimicrobial resistance in marine mammals. Prior studies using marine vertebrates as sentinel species have shown positive correlation between human influence and ARB colonization to some degree (Blackburn, 2010; Grieg, 2007;

Johnson et al., 2008; Lockwood et al., 2006; Miller et al.,2008; Miranda and Zemelman, 2002; Rose et al., 2008; Schaefer et al., 2011; Schroeder et al., 2009; Stoddard et al., 2002), but no research has been able to draw robust conclusions on anthropogenic effects on ARB acquisition due to the lack of a natural history of the animals surveyed and a vague sense of their geographic range, confounding variables not applicable to the study of ARB in this species.

In this study, those confounding variables were limited by studying bacterial resistance caused by pollution of antimicrobials and wastewater by analyzing prevalence and patterns of ARB colonization in the feces of the well-studied *Orcinus orca.* By using a sentinel species for colonization with ARB and pairing prevalence and patterns with demographic, geographic, and anthropogenic risk factors, the relative influence of human impacts as well as traits intrinsic to ARB susceptible wildlife can be assessed. Results indicate how other species could be affected by exposure to waters affected by pharmaceutical pollution and determine if ARB are a problem of relevance to public health in the Salish Sea marine environment.

CHAPTER ONE

LITERATURE REVIEW

An evaluation of current literature is needed to frame the research question in a manner that validates the problem and contributes to an explanation of the chosen study design. For this, sufficient background on antimicrobial drugs, complications of their entry into the environment, and how the phenomenon could potentially impact the Salish Sea and wildlife therein is critical, as well as the findings from similar research in order to adopt methods and establish anticipated results for this study.

Pharmaceutical Pollution in the Salish Sea

Study Site Characteristics and Vulnerabilities

The Salish Sea is an ecologically diverse and economically important estuary in the Pacific Northwest region of the United States of America and southwestern British Columbia, Canada (Fraser et al., 2006; Gaydos et al., 2009). This glacial carved fjord inland marine ecosystem stretches along the inland waters from Olympia, WA in the United States to the Campbell River in Canada, and is the home to over 7 million people and many endemic and rare marine species (Jackson and Kimerling, 2003). Like many of the world's coastal zones, the Salish Sea is declining environmentally as human population pressure and pollutants begin to accumulate (Gaydos et al. 2009; Puget Sound Partnership, 2011). Human population growth in the Pacific Northwest is putting pressure on aquatic habitat and marine mammals and industrial effluent has resulted in increased toxin and fuel concentration. About 100 municipal and industrial

wastewater treatment plants discharge over 430 million gallons of treated wastewater to imperiled waters of Puget Sound and the surrounding area each day (Puget Sound Partnership, 2011). Groundwater, storm water, and combined sewer overflows can discharge untreated wastewater, and harmful compounds leach from leaky septic drainage systems when capacity is exceeded or water levels rise above the drainage fields (Dougherty et al., 2010; Puget Sound Partnership, 2011). Added input to septic and sewage systems from population growth increases the chances that harmful substances, including pathogenic bacteria, toxins, and other chemical compounds will reach the water column without being properly broken down (Take Back Your Meds, 2011).

Pharmaceutical Pollution

Personal care product and pharmaceutical pollutants (PCPPs) are classified as emerging contaminants of concern in water quality, and have become an increasingly significant focus area in water quality research. Investigation of pharmaceutical pollution is important because most drug wastes are biologically active, readily mobile and not easily biodegraded (Kümmerer, 2008). A biologically active compound can have direct physiological effects on living cells, according to the theory that small molecules can change cellular phenotypes in cells of non-target organisms, thereby altering cellular activity and function and imitating the effects of genetic mutations (Klekota et al., 2005). The high mobility of drugs and drug metabolites is due to a high water solubility, and results in high dispersal rates of drugs once released to surface waters (Kümmerer, 2008). These properties are exacerbated by the persistence certain drugs and their metabolites

in many environments, the unregulated use of many drugs in animal agriculture, inefficient drug absorption by target species, the incomplete removal of excreted or dumped compounds by water treatment facilities, and the myriad pathways of indirect exposure of antimicrobials to the environment (Kümmerer, 2004; Kümmerer, 2009). Pharmaceutical exposure to the environment can come from WWTPs, untreated sewage, hospitals, runoff from croplands, animal feces, and aquaculture activities (Bushman et al., 2002; Dougherty et al., 2010; Johnson et al., 2004; Puget Sound Partnership, 2011; Take Back Your Meds, 2011).

The effectiveness of water treatment in removal of these contaminants depends on the volume and type of chemicals received in waste as well as the design on the treatment center (Lubliner, 2010). There are 96 publicly owned waste water treatment plants (WWTPs) in the Puget Sound Basin, processing over 124 million gallons of sewage from over 3.5 million people each day (WA Department of Ecology, 2010). A study conducted by the Washington State Department of Ecology in 2008 analyzed 172 PCPPs in influent, effluent, and holding tank grab samples from five WWTPs in the Puget Sound Basin (Lubliner, 2010). The study found that 56% of the analytes were detected in at least one sample, and each sample had measurable concentrations of some combination of PCPPs (Lubliner, 2010). About 21% of the compounds detected in the influent were reduced below detection levels by secondary treatment alone, and 32% of the remaining compounds were below detection levels after treatment with an advanced tertiary technology (Lubliner, 2010). Three pharmaceuticals, carbamazepine, fluoxetine, and thiabendazole, were identified as being untreated

by tertiary treatment and recommended as environmental tracers for PCPP pollution (Lubliner, 2010). Most of the WWTPs in Puget Sound use secondary treatment, which focus on "removing pathogens, biochemical oxygen demand, toxic chemicals, and suspended solids, with the primary objective of protecting human health" (Puget Sound Partnership, 2011). In other words, targeted removal of pharmaceuticals is not typically conducted by the WWTPs emptying into the Puget Sound Basin.

Examining the presence and concentration of PCPPs in urbanized areas and comparing findings to a more remote "pristine" area helps to develop a sense of the extent and severity of these pollutants in the Salish Sea. Researchers compared concentration and incidence of detection of 37 dissolved anthropogenic compounds such as in the surface waters of Barkley Sound and south Puget Sound, where population density is approximately six times higher in the latter site (Keil et al., 2011). In the control area of Barkley Sound, 28 of the 37 chemicals were detected at least once, but in the more populous Puget Sound region all 37 chemicals were detected. Only two plasticizers were detected in Barkley Sound more frequently than in Puget Sound, and average concentrations of most the three most abundant compounds in both fjords was approximately 20 times higher in Puget Sound. The survey found the pharmaceuticals ibuprofen and 17a-ethynylestradiol, a hormone commonly found in birth-control pills in both the natural and altered study sites. These pharmaceuticals were hypothesized to originate from human sources, with higher concentrations of the chemicals correlating to higher human population density (Keil et al., 2011).

A similar study conducted in Liberty Bay, WA found herbicides, pharmaceutical and other personal care products in both ground water and surface water in an area lacking a WWTP, supporting the hypothesis that non-point source pollution of human waste contributes to this phenomenon (Dougherty et al., 2010). Liberty Bay is a small embayment of the Puget Sound section of the Salish Sea, with varying degrees of urbanization and use of 70% septic disposal. The authors found that seven of the 12 compounds detected were detected in other studies examining septic systems as potential sources for PPCPs, suggesting that non-point pollution of surface waters in pristine areas could also contribute to drug pollution in the Salish Sea. The concentrations of these drugs in both studies were in sub-toxic levels, but the occurrence of such drugs in any quantity in relatively pristine areas lacking a clear route for point-source contamination speaks to the magnitude of the problem (Dougherty et al., 2010; Keil et al., 2011).

Antimicrobial Drug Pollution

Within the category of PCPPs, antimicrobial drugs are of special concern in the environment. Antimicrobials are substances produced by microorganisms to hinder growth of other microorganisms and thereby favor the propagation of their own species (Hogg, 2005). Antimicrobials used in medical treatment are either the metabolites of these organisms, known as allelochemicals, natural chemical compounds analogous to these substances, or synthetic compounds mimicking the inhibition of specific bacterial growth to target undesirable bacteria (Grunden, 2013).

Antimicrobials are the third-largest group of medicines prescribed for humans and the largest group used in veterinary medicine (Thiele-Bruhn, 2003). Worldwide, annual consumption of antimicrobials is estimated to be between 100,000 and 200,000 tons per year (Wise, 2002). In the United States alone, 1860 tons of antimicrobials are used in human medicine per year, resulting in 1.9 ug/L appearing in sewage and 0.73 ug/L appearing in surface waters (Kümmerer, 2008). In the US, outpatient antimicrobial usage is relatively steady in the ranking of top drugs (Bearden, 2013). The most commonly consumed antimicrobials are the β-lactam family group of drugs which include penicillin, ampicillin, amoxicillin and others, trailed by tetracycline, macrolide, fluoroquinolone and sulfonamide family groups (see Table 1) (Bartholow, 2012; Kümmerer, 2008).

Although no data on prescription rate and use of antimicrobials is kept for a state or regional basis, the Center for Disease Control is expanding its scope to begin doing so (Bearden, personal communication). National data on most common outpatient antibiotic prescriptions is available yearly, and hospitals do track prescription rates, but outpatient antimicrobial use dwarfs that of the hospital, making hospital estimates for a region an underrepresentation of true use (Bearden, 2013).

Antimicrobial concentrations in the environment are found in some orders of magnitude lower than in therapeutic use (Kümmerer, 2004). The environmental persistence of an antimicrobial varies based on its chemical structure and the environmental compartment where it is accumulating. B-lactams are less stable in the environment because of their easily hydrolyzed ring structure, but drugs like

the sulfonamides, fluoroquinolones and macrolides are especially severe environmental contaminants because of their higher persistence and greater stability (Cha, 2006; Segura et al., 2009). The primary method of pollution elimination in most environmental environments is bacterial decomposition, which is less effective against synthetic or semi-synthetic antimicrobial drugs or in bacterial populations that have become less diverse due to unnatural selection from the presence of resistant bacteria or pharmaceuticals (Barbosa and Levy, 2000; Kümmerer, 2004).

Continued exposure to most of these chemicals even at trace levels has unknown consequences, and the combined action of this exposure in addition to other routes is possibly cumulative (Smith, 2012). Continuous low doses to an environmental system are thought to be more detrimental than high concentrations, because the presence of antibiotics over a longer period of time stimulates the transfer of resistance genes between bacterium and the propagation of already resistant bacteria is favored by the decline in numbers and decreased competition from susceptible species (Barbosa and Levy, 2000; Kümmerer, 2004). This concept is covered more thoroughly in the next section.

Water, sediment and fish tissue in Puget Sound have been previously shown to have measureable concentrations of antimicrobials (Corcoran and Tyler, 2010; Johnson, Carey and Golding, 2004; Nilsen et al., 2007). As was the case in the Keil et al. (2011), there seems to be spatial relationships between urbanization and higher chemical detection rates of antimicrobials in the environment (Dougherty et al., 2010). In Liberty Bay, WA, data show that the community, which

predominately uses septic tanks rather than centralized WWTPs, is receiving detectable levels of trimethoprim in surface and ground waters, with the occurrence of detections increasing as population density increased (Dougherty et al., 2010). The authors speculate that detections are likely to increase as populations, prescriptions, and household product uses increases (Dougherty et al., 2010).

Revisiting the Washington State Department of Ecology study, of the 172 compounds tested in WWTP influent and effluent, 24 made the department's short list for the highest priority PCPPs to test in environmental studies based on use, toxicity, consumption, properties, and persistence (Lubliner, 2010). Six of these 24 high-risk compounds have antimicrobial uses and included erythromycin, sulfamethoxazole, tetracycline, triclosan, triclocarban, and trimethoprim (Lubliner, 2010). Study results indicate that secondary and tertiary treatments remove a majority of antimicrobial concentrations in waste water, but detectable levels of these chemicals are still present in effluent released to surface waters (see Table 2). Other antimicrobials not designated as high risk were also detected in the effluent, including azithromycin, ciprofloxacin, clarithromycin, clinafloxacin, cloxacillin, enerofloxacin, lyncomycin, lomefloxacin, norfloxacin, ofloxacin, ormetoprim, oxacillin, oxolinic acid, penicillin G, penicillin V, roxithromycin, sarafloxacin, virginiamycin, anhydrochlortetracycline, chlortetracycline, demeclocycline, doxycycline, and other tetracycline derivatives in effluent (Lubliner, 2010). Interestingly, veterinary drugs were intentionally left off of this list with no reasoning given.

Introduction of antimicrobial resistance to the environment is thought to only be partially caused by pharmaceutical pollution, with other resistance being attributed to natural resistance or introduction of resistant bacteria through wastewater or fecal pollution. A review of resistance is necessary to better understand how bacteria transfer this trait in the natural world.

A Review of Antimicrobial Resistance

Antimicrobial Drugs

As noted above, antimicrobials are biochemical compounds derived from or mimicking complexes designed to promote the growth of a particular microorganism at the expense of another. The natural phenomenon of allelopathy has been harnessed by scientists to produce a variety of drugs that suppress or eliminate the growth of undesirable bacteria in human and veterinary medicine. The five basic mechanisms of antimicrobial action are (Rollins and Joseph, 2000):

1. Inhibition of cell wall synthesis, caused by a compound inhibiting peptidoglycan synthesis, precursors, or linkages, which terminates cell wall growth and lyses the cell.
2. Alteration of cell membranes, Antimicrobials that employ this method essentially injure the plasma cell wall membrane, which disrupts the cell cross-membrane potential and causes leaks and imbalances.
3. Inhibition of translation. This process disturbs protein synthesis by binding proteins or peptides to ribosomal subunits, which

disrupts peptide elongation and results in the cell being unable to correctly copy and pass on genes. This mechanism is primarily bacteriostatic, except for the aminoglyceride class of antimicrobials.

4. Inhibition of nucleic acid synthesis. Drugs that inhibit DNA gyrases or DNA-dependent RNA gyrases, which prevent DNA coiling and replication.
5. Antimetabolite activity. These drugs prohibit the synthesis of folic acid, thereby preventing the synthesis and repair of DNA within the cell.

The success or failure of an antimicrobial against a bacterium is dependent on the natural defenses of the target cell, cellular metabolism, and cellular growth processes. Therefore, not all antimicrobial mechanisms are effective against any particular microbe, and different antimicrobial families using different mechanisms can be used to target specific bacterial types (see Table 3 for a basic summarization). This becomes important in the study of antimicrobial resistance in the environment, as resistance rates can be overrepresented if the predominate bacteria in the ecosystem are intrinsically resistant to antimicrobials used (Kümmerer, 2004; Lorian, 1996).

Antimicrobial Resistance Acquisition and Mechanisms

Resistance is acquired by natural mutations or the transfer of genetic material, known as vertical and horizontal transfer, respectively. Vertical transfer of resistance genes is the result of spontaneous mutations for resistance within the

bacterial genetic code, which occur at an estimated frequency of 1 out of every 108-109 alleles (Todar, 2011). During DNA replication, resistance genes are passed on to all descendants and the population gains resistance over time (Barbosa and Levy, 2000; Khachatourians, 1998).

Horizontal transfer mechanisms are of primary importance to this study. Horizontal transfer is the acquisition of heritable traits by the modification or transfer of genetic material from one individual bacterium to another (Todar, 2011). The three methods of genetic material transfer are conjugation, transduction, and transformation. Conjugation is the transfer of plasmids containing DNA packets from one bacterium to another via direct cell-to-cell contact (Todar, 2011). The transfer of resistance via plasmids is thought to be the main mechanism of horizontal gene transfer, and bacterial species need not be similar for conjugation to occur (Alekshun and Levy, 2007). Transduction is the transmission of resistant DNA by bacteriophages, or bacterial viruses that infect similar bacterial species (Todar, 2011). Transformation occurs when free DNA from the environment is absorbed by a microbe. Resistance genes present in the environment from the lysis or death of a bacterium can be assimilated into the DNA of a completely different bacterial species, often through transposons or gene cartridges (Alekshun and Levy, 2007).

The genetic codes for resistance manifest as a number of different mechanisms that prepare a bacterium to survive antimicrobial treatment. Three major mechanisms of bacterial adaptation have been identified to combat the five mechanisms of antimicrobial action. These mechanisms are the enzymatic

breakdown or inactivation of antimicrobials, modification of receptor sites and development of efflux pumps or revision of metabolic pathways (Hogg, 2005).

Spread of Resistance

Resistance is led into the environment by the introduction of resistant bacteria or by unnatural selective pressure resulting from antimicrobial drug pollution. Antimicrobial resistance to some drugs is natural in certain bacteria, but clinical antimicrobial medication and human waste disposal methods favored the selection and propagation of bacteria with genes encoding additional resistance (Baquero et al., 2008; Kümmerer, 2004). Over time, genetic drift of microbial populations subject to this selective pressure will increase the number of resistant bacteria in the environment because only bacteria with these traits will survive to multiply (Barbosa and Levy, 2000). The artificial selective pressure to maintain resistance genes in a bacterium is documented to be increased with contact of human wastes in environments, including water (Greig et al., 2007; Kümmerer, 2004; Kümmerer, 2008; Miller et al., 2008; Miranda and Zemelman, 2002). This is because low and continued trace levels of antimicrobials, such as those emitted from waste water treatment, are even more effective in conveying resistance, which makes the emission of small doses even more concerning (Lorian, 1996). This is known as subtherapeutic dosing, and this process kills out the weaker strains of bacterium, naturally selecting for stronger and more virulent strains in a very short amount of time (Lorian, 1996; Shnayerson and Plotkin, 2002).

Reversal of Resistance

There is evidence that by decreasing the input of antimicrobials to a system, the bacteria will gradually shed the genetic material that makes resistance advantageous, and order can be restored over time (Kümmerer, 2004). The majority of studies on reduction of resistance have been conducted in clinical environments, with some successful cases in decreasing the number of resistant pathogenic bacterial strains after alterations in hospital policy (Klare et al., 1999; Smith, 1999; van den Bogaard et al., 2000). Additionally, Corpet et al. (1998) showed that after feeding volunteers a near sterile diet, the numbers of resistant bacilli in their fecal flora decreased almost 1000-fold. These findings offer some hope that means that by reducing antimicrobial use, it is possible to reverse some of the effects of continued resistance.

The banning of agricultural antimicrobials in the European Union has provided an interesting case study for tracking productivity in agricultural animals and human pathogenic resistance after the removal of selective pressure. Sweden banned of all food animal growth-promoting antibiotics by in 1986, and the European Union banned avoparcin in 1997, followed by bacitracin, spiramycin, tylosin and virginiamycin in 1999 (Casewell et al., 2003). Recent studies performed in several European countries following the ban in the use of avoparcin report an encouraging and sometimes dramatic decrease in the frequency of certain pathogens resistant to the banned antimicrobials in animals and food products as well as the microbial flora of humans (Klare et al., 1999, van den Bogaard et al., 2000).

However, contrary evidence exists to discredit the theory that bacteria will naturally shed genetic material coding for resistance as pressure is reduced. Studies have shown that resistance can be found in bacteria in people and animals without recent history of antimicrobial drug use (Calva et al., 1996; Gilliver et al., 1999). And though there may be reductions in the frequency of resistance detection, research suggests that the resistance does not return to a pre-exposure level and that decreases in resistance are much slower to evolve than increases (Austin et al., 1999; Barbosa and Levy, 1992). The delay in return to normal susceptible bacterial flora provides opportunity for resistance to return if the antimicrobial is reintroduced, as well as the development of cross-resistance to other antimicrobial agents (Barbosa and Levy, 1992).

Antimicrobial Resistance in the Environment

Antimicrobial misuse is common and considered a serious problem (CDC, 2011; FDA, 2012; Harris, 2009; Shnayerson and Plotkin, 2002; Union of Concerned Scientists, 2012). The misuse by humans in physicians (Harrison and Svec, 1998) and patients (CDC, 2011) in human clinical medicine has created virulent strains that are not able to be combated with typical, or even multiple, antimicrobials. Commonplace products used in cleaning and health products also contribute to increased antimicrobial exposure to the environment, in addition to unregulated antimicrobial use in animal agriculture and aquaculture.

Resistance in Humans and Health Implications

Antimicrobial resistance bacteria are most often considered a problem from the standpoint of clinical medicine. The formation of resistant bacterial strains pose a serious threat to human health (da Costa et al., 2006; Kunin, 1993, Shnayerson and Plotkin, 2002). Virulent pathogens are transmitted through community infections (particularly in hospitals), infected resistant bacterial-contaminated foods, and through contact with water, soil or wildlife that is contaminated. Any number of these situations can lead to infection of the consumer, which is more difficult and costly to treat because of the ineffectiveness of readily available antimicrobials.

A recent study focused on costs in medical treatment and mortality from antimicrobial resistant bacterial infections in a Chicago, Illinois hospital. The researchers concluded that 13.5% of 1391 patients surveyed were infected with an antimicrobial resistant microbe (Roberts et al., 2009). Medical costs incurred by each patient due to their resistance ranged from $18,588 to $29,069, and the hospital stay for these patients was 6.4 – 12.7 days longer (Roberts et al., 2009). Most alarmingly, mortality rates in this hospital alone due to antimicrobial resistant bacteria were 6.5% which is twice the rate for patients without antimicrobial resistant infections (Roberts et al., 2009). The anticipated societal costs, or costs for the families of the ARB infected patients was estimated to fall between $10.7 and $15 million (Roberts et al., 2009). Roberts extrapolated this cost to hospitals nationwide, and conservative estimates of antimicrobial resistant infection were at 900,000 in the year 2000, equating to $16.6 to $26 billion

dollars spent on the treatment of these preventable infections (Roberts et al., 2009).

This data is from the year 2000, and the rise of antimicrobial resistant infections implicates that this problem has only grown. A more recent report from the World Health Organization found that death toll from the pathogen methicillin-resistant *Staphylococcus aureus* (MRSA) is approximately 18,000 people per year in the United States alone (Braine, 2011). Other emerging resistant infections include vancomycin-resistant *Enterococcus,* vancomycin-intermediate/resistant *Staphylococcus aureus,* carbapenem-resistant *Enterobacteriacaea,* carbapenum-resistant *Klebsiella pneumonia,* fluoroquinolone-resistant *Neisseria meningitides,* isoniazid, rifampicin and floroquinolone resistant *Mycobacterium tuberculosis,* multi-drug resistant *Acinetobacter baumannii, Bacillus anthracis, Neisseria gonorrhoeae,* Group B *Streptococcus, Shigella spp., Streptococcus pneumonia, Salmonella* spp. (CDC, 2013).

The discovery of the New Delhi Metallo-β-Lactamase group of enzymes is especially disturbing, and has attracted significant scientific and media attention. This finding deserves consideration, as the gene encoding for resistance to the beta-lactam family of drugs is very mobile, complex, and adaptable to many different pathogens (Dortet, 2012). The promiscuity of the gene known as bla_{NDM-1} has eliminated the use of carabapenems, which in the 1980s and 1990s were considered a last resort against extended-spectrum β-lactamase gram-negative bacteria (Bonomo, 2011).

Concern over growing antimicrobial-resistant pathogen strains, the loss of emergency antimicrobials for resistant infections, and the lack of development of new antimicrobial drugs has resulted in a panic over global 'superbugs'. Policies opposing the USDAs lenient rules regarding agricultural antimicrobials, the imprudent prescription of antimicrobials for unrelated illnesses in clinical medicine, and encouraging research and development of new antimicrobials that would be more strictly regulated have been proposed to help ameliorate the growing health crises (Interagency Task Force on Antimicrobial Resistance, 2011).

Resistance in Animal Agriculture

The inappropriate use of antimicrobials in animal agriculture is a major contributor to the emergence of ARB. The annual quantity of antimicrobials in agriculture is 100 to 1000 times the use in human populations. In agriculture, antimicrobials are added in trace amounts to food or water to prevent illness in animals kept in close quarters or as a growth promoter (Khachatourians, 1998; Levy, 1998; Peak et al., 2007). Ninety percent of the drugs administered are given at continual subtherapuetic levels rather than to treat illness, which promotes the selection of ARB in the gut of the animals and promotes the spread of resistance (Chadwick et al., 1997; Khachatourians, 1998). In the United States, current regulations have restricted the use of certain antimicrobials that are used in human medicine to quell the rate that the medicine becomes ineffective for human use, but the majority of drugs used to treat animals remain unregulated and do not require a prescription from a veterinarian to dispense (FDA, 2012).

Many agricultural animals have been shown to harbor antibiotic resistant bacteria, including cattle, poultry, and swine (Chee-Sanford et al., 2001; Peak, 2007; van den Bogaard, 2002). Moreover, the soil and waste water in pastures where treated animals are raised has been shown to have increased resistance levels, and runoff of animal waste from farms is a suspected contributor of ARB to surface waters (Dougerhty et al., 2010; Santamaria et al., 2011; Yang et al., 2010). Additionally, it has been shown that meat produced from animals treated with antibiotics can transfer genetic resistance (Teubner, 1999).

Resistance in Aquaculture

Prophylactic antimicrobials are used in aquaculture to compensate for overcrowding of fish farming sites, increased density of fish numbers in aquaculture, unsanitary conditions, and the failure to isolate sick fish (Cabello et al., 2006; Naylor et al., 2000). It has been shown that the heavy use of antimicrobials in aquaculture causes residual traces of chemicals to remain in the local sediment (Kruse and Sorum, 1994; Miranda and Zemelman, 2002; Sorum, 2006) and can increase the proportion of resistant bacteria in nearby sediment (Herwig et al., 1997; Huys et al., 2000; Miranda and Zemelman, 2002; Schmidt et al., 2000; Sorum, 2006). The most concerning impact of antimicrobial use in aquaculture is that unconsumed food reaches the sediment and drugs can be leached directly from the food pellets in addition to passing through fish feces, and they can be washed by currents to distant sites (Cabello, 2006). Additionally, research is showing decreased diversity of bacteria in sites with continuous use of

antimicrobials (Cabello, 2006; Davies et al., 2009; Herwig et al., 1997; Smith, 2008; Sorum, 2006).

Resistance in the Environment and Ecosystem Implications

Antimicrobial resistance is increasingly framed as an ecological problem. Understanding and documenting the transmission of nonpathogenic ARB is important because these bacteria can serve as reservoirs of resistance genes to pass on to pathogens (Okeke and Edelman, 2001). Additionally, bacteria are an ecologically critical group of organisms. They are essential in the biochemical cycling of nitrogen, phosphorus, sulphur and oxygen, and are key decomposers, with the resulting nutrients released often fueling primary production in the ocean and stimulating the global carbon cycle (Kümmerer, 2008). Documentation of resistant strains and presence of antimicrobials in the environment is an important step in understanding the impact human wastes are having on bacterial mutation rates, and consequently the health of the marine environment and public health in the region.

ARB in the Marine Environment

This problem is significant in marine and aquatic environments in particular because the persistence of chemicals in the sediment or in small concentrations in the water column has the potential to alter the normal bacterial flora and plankton in the affected areas, shifting the diversity of the microorganisms in a way that potentially disrupts other processes necessary for ecosystem health. For instance, decreased diversity has the potential to alter

trophic and metabolic functions and promote anoxic environments, contributing to algal blooms and fish kills (Cabello, 2003; Valiela, 1995). These and other environmental disturbances are not as well defined by media sources when discussing antimicrobial pollutants, but are as, if not more, critical to the health of humans and the ecosystem as a whole.

Research indicates that antimicrobial resistant bacteria can be found not only in marine sediment near aquaculture sites (Herwig et al., 1996), but also in public marine beaches on the west coast. Soge et al. (2009) collected water and intertidal sand samples from 10 public beaches in Washington State and optimized growth for *Staphylococcus* spp. The resultant 51 isolates from 9 of the 10 beaches were exposed to chloramphenicol, trimethoprim/sulfamethoxozole, erythromycin, and tetracycline by disk diffusion analysis. PCR assays were then used to identify *erm*A, *erm*B, *erm*C, *msr*A, *tet*M and *tet*K, specific genes encoding for antimicrobial resistance in *Staphylococcu*s exposed to the aforementioned antimicrobials (Soge et al., 2009). Outcomes identified five distinct strains of multi-drug resistant MRSA, which were more phylogenetically similar to hospital-acquired MRSA infections than community acquired infections, as compared to 4 methicillin susceptible strains of *Staphylococcu*s (Soge et al., 2009). At the time of press, this was the first report of MRSA and $MR_{CON}SA$ isolated from marine water and intertidal beach sand, suggesting that the marine environment may serve as a reservoir for antimicrobial resistant genes (Soge et al, 2009). Nine of the MRSA strains had characteristics commonly associated with hospital MRSA, though the beach sites were not near hospitals

and the cool water temperatures make infected human swimmers unlikely as the source of the contamination, and the cause of contamination is unknown but not ruled out as coming from a single source (Soge et al, 2009).

This study was expounded upon in 2012, with water and sand samples collected from two marine water beaches [A and B] and one freshwater beach [C] in the Seattle WA area (Levin-Edens et al., 2012). A total of 31 MRSA isolates representing 21 different strains were identified, 71% from the fresh water drainages and creeks surrounding marine Beaches A and B and/or fresh water Beach C (Levin-Edens et al., 2012). MRSA isolates in this study were 67.7% SCC*mec* IV, which often originates in untreated wastewater, while in the Soge et al. study 83.3% of the isolates were SCC*mec* type I indicating hospital MRSA, which implies a change in the relative contribution of methicillin resistance genes, or possibly an artifact from changes in sample size and the addition of a freshwater beach (Levin-Edens et al., 2012). The two studies agree that the diversity of MRSA isolates support the hypothesis that MRSA is progressively distributed in the environment via multiple sources including human WWTP effluent, hospitals, human contact, and wildlife (Levin-Edens et al., 2012).

This conclusion is supported by research from Roberts et al. (2009), who used a similar sampling protocol to identify vancomycin-resistant *Enterococcus* (VRE) on beaches in Washington and California. This study detected 18 isolates with *van*A and/or *van*B genes, in addition to *tet*M genes capable of plasmid-mediated transfer (Roberts et al., 2009). The VRE strains also expressed resistance to clindamycin, minocycline, tetracycline and teicoplanin (Roberts et

al., 2009). VRE were isolated from the samples collected in Washington in 2002, 2003 and 2008, but not from 2001, suggesting a temporal accumulation of resistance genes in the environmental pool (Roberts et al., 2009). Samples from a WWTP emptying into Puget Sound in 2001 were not positive for *van*A or *van*B, but without retesting effluent in the years of positive identification of VRE there is no relationship attributable to human influence (Roberts et al., 2009). A later study conducted by Roberts et al., in 2013 evaluated 296 recreational beach samples for MRSA, of which 31 (10.5%) were positive for MRSA with 22 isolates (71%) coming from fresh water streams running into the marine and freshwater beaches. Again, the MRSA strains had characteristics commonly associated with hospital MRSA but with no nearby hospitals, suggesting that upstream freshwater influx into marine environments significantly contributes to the pool of resistance in marine near-shore waters (Roberts et al., 2013).

A separate study examined differences in all antibiotic resistance gene signals across the surface water samples of the Salish Sea as related to a WWTP water sample. Seven samples were taken from six locations in the surface waters of Puget Sound and a single effluent sample was collected from a WWTP emptying into the basin of Puget Sound (Port et al., 2012). Eighteen resistance genes were identified from the samples, with tetracycline resistance being most common, and the abundance of genes increase in resistance from open waters to the WWTP, suggesting a spatial relationship between resistance gene abundance and human impacts (Port et al., 2012).The WWTP effluent resistance gene profile showed similarities to resistance gene taxonomy from human infections,

suggesting human impact, and the spatial differences across the open water samples did not alter their taxonomic similarity or profiles of mobile genetic elements (Port et al., 2012). The authors suggest that resistance gene signals were underestimated, which they attribute to the methodology of pyrosequencing (Port et al., 2012).

The findings from these studies show that antimicrobial resistance in the marine environment exists on the West Coast and waters of the Salish Sea, including multi-drug resistant pathogens of clinical importance. This is concerning because evolved bacterial resistance to an antimicrobial usually manifests as a single resistance gene, and the abundance of multi-drug resistant bacteria identified along public beaches implies that horizontal transfer of mobile resistance genes is occurring in Washington's marine waters (Miller et al., 2008).

ARB Colonization in Animals

Terrestrial Animals

Concern over antimicrobial resistant bacteria has led to studies on the presence of resistant strains present in human and livestock, but resistance of strains in wildlife are slower to be studied (Blackburn et al., 2010). Antimicrobial resistance has been previously documented in various animals primarily from fecal or intestinal sampling, due to the richness of animal enteric systems being a prime site for bacterial colonization and the simultaneous presence of many pathogenic or zoonotic bacteria being commonly found in the gut.

Ahmed et al. (2007) studied the occurrence of gram negative AR genes in bacteria from zoo animal fecal and anal swabs and found that captive animals are a potential reservoir for clinically important resistance genes. This study highlighted the incidence and wide range of species affected, testing a variety of birds, turtles, tortoises, monkeys, snakes, salamanders, foxes, giraffes, badgers, tigers, and other reptilian and mammalian species (Ahmed et al., 2007). These results show that feces from animal species can be used to isolate antimicrobial resistant bacteria to monitor resistance in a contained environment. However, this research was not intended to apply resistance as an indicator of ecosystem health, expand upon possible reasons for varied findings in or between species, or relate the findings to any environmental phenomenon.

To relate resistance to environmental factors, Edge and Hill (2005) studied the relative contribution of fecal pollution sources to resistance in surface waters. They studied resistant *E. coli* strains in surface waters and used a discriminant function to distinguish the bacteria from the two sources. They concluded that feces from seabirds had lower levels of resistance, but contributed more to fecal pollution in the surface waters, highlighting the importance of considering wild fecal sources in environmental analysis of resistance. Conversely, Parveen et al. reported a lower incidence in fecal samples from terrestrial wild animals than found in the surrounding waters. In agreement with this finding, Rose et al. reported a higher resistance occurrence in seabirds than in marine mammals and fish from nearby waters, speculating that the greater contact of the seabirds with coastal communities and their diet of refuse may contribute to this phenomenon.

However, the sampling method depended on bycaught animals to represent a healthy standard population, which by their own admission needs more investigation. This study was limited in the number of samples, and swabbed different areas for different species depending on their provenance (live, bycaught or stranded), further confounding results and accuracy in measuring the occurrence of ARB in coastal waters.

Marine Animals

To further explore the distribution of ARB in marine waters, study has expanded to include different types of bacteria in off- and near-shore marine species, in an attempt to elucidate reasons behind varied patterns of resistance with other phenomenon.

Studies on penguins and polar bears have been used to spatially relate resistance to human population, since they are primarily located in remote areas. Sea turtles, fish, sharks, marine birds, pinnipeds and cetaceans have also been used as environmental indicators for antimicrobial and fecal pollution, and resistant bacteria have been detected in all of these animals (Al-Bahry et al., 2010;Blackburn et al., 2010; Glad et al., 2010; Greig et al.,2007; Lockwood et al., 2006; Miller et al., 2008; Miranda and Zemelman, 2001;Rose et al., 2008; Schroeder et al., 2009; Stoddard et al., 2008).

Antimicrobial resistance in bacteria from seawater and penguin fecal samples was studied in Antarctica, where international treaties have limited antimicrobial contamination. Thus, this study serves as a measure of human

impacts on the environment and a potential snapshot of the natural resistance in the pre-antimicrobial world (Miller et al., 2008). The authors found that drug resistance was higher in introduced than endemic microbes, and that the ratio of introduced to endemic bacterial spp. and resistance bacteria increased with proximity to Palmer Station (Miller et al., 2008). The research concludes that even in relatively pristine areas, the frequency of drug and multi-drug resistance is low among native bacteria, and can be increased by even the most regulated of human habitation (Miller et al., 2008).

Another study of resistance in an area of relatively little impact was conducted on the microbiome of Polar bear (*Ursus maritimus*) fecal (Glad et al., 2010). The researchers amplified the beta-lactam resistant bla_{TEM} gene, and discovered that only 4 out of 144 isolates were positive for bla_{TEM} genes from the wild polar bears (Glad et al., 2010). The bla_{TEM} gene is increasingly found in clinical and commensal bacteria, suggesting that proximity to development may contribute to resistance in fecal samples, along with phylogeny and diet (Glad et al., 2010). However, culturing fecal material from the polar bears on ampicillin-infused agar plates resulted in no growth, which signifies that all bacteria were susceptible to ampicillin. The use of molecular techniques in addition to bench culturing was necessary to elucidate the exact proportion of bacteria capable of resistance, which is important to consider in the development of further research. Additionally, it was discovered that that more fecal samples than rectal swabs showed evidence of bla_{TEM} genes, suggesting that fecal samples are superior for the study of resistance (Glad et al., 2010).

ARB were studied as bio-indicators of contaminated effluent in green turtles in Oman using oviductal fluid as the biological test agent. To test for exposure of turtles to pollution, fluid was acquired from forty nesting turtles, resulting in 132 species of bacteria from 7 genera (Al-Bahry et al., 2010). Of these bacteria, the Kirby-Baur disk diffusion method was used to test resistance to 15 antimicrobials, and 60.6% showed multi-drug resistance, mostly expressed for ampicillin, trailed by streptomycin and sulphamethoxazole (Al-Bahry et al., 2010). The authors concluded that testing exposure to polluted effluents using wildlife bacteria as bio-indicator is a valuable way to assess endangered species and ecosystem health (Al-Bahry et al., 2010).

It was also observed that green turtles are exposed to many different types of effluent and pollutants in their wide geographical and migratory regions, and since it is not practical to investigate all along the migratory route, monitoring of ARB is useful to find the extent of pollution (Al-Bahry et al., 2010). This assumption is important, because the authors accept that resistance travels with the turtles during migration rather than being acquired at the nesting sites, though the time period of travel and spatial range of each individual turtle is unknown. This assumption is unproven and unbacked in the text.

Another study using marine species as an indicator for effluent pollution used fish caught in Concepcion Bay, Chile (Miranda and Zemelman, 2001). This study was designed to determine the frequency of resistant bacteria in fish, to evaluate potential differences in the frequencies based on pelagic or demersal habitat, and to determine resistance patterns of some selected specific bacteria of

human health interest. Miranda and Zemelman (2001) showed that bacteria isolated from demersal fish showed higher multi-resistance levels than fish from pelagic habitats, demonstrating resistance to up to 10 antimicrobials while pelagic fish samples yielded bacterial strains resistant to seven antimicrobials or less, with high frequencies of antimicrobial resistance for ampicillin, streptomycin, and tetracycline. (Miranda and Zemelman, 2001). This difference was not significantly significant, but suggests that feeding and ecological habits, as well as exposure to the high content of resistant bacteria in polluted sediment (Herwig et al., 1997) may lead to higher contact of antimicrobial agents.

Another study concentrating on antimicrobial resistance in marine vertebrates by Blackburn et al. (2010) focused on predatory fish and sharks off the coast of Florida. The study goals were to determine if prevalence of ARB decreases as distance from shore increases, if animals from the same species in different locations exhibit different resistance patterns, and if resistance patterns differ within different species in the same area. Anal swabs of fish were cultured and resulting isolated subjected to the Kirby-Baur test for drug resistance to antimicrobials (Blackburn, 2010). Results showed that resistance was ubiquitous in the marine environment and multidrug resistance was common in areas with larger human populations (Blackburn et al., 2010). Older fish had higher incidences of resistance, and bacteria were most often resistant to penicillin, ticarcillin, cefitofur, doxycycline, and chloramphenicol (Blackburn et al., 2010).

The most common resistances were not the same in fish from Chile to fish in Florida; in fact, chloramphenicol resistance was uncommon in Chile and the

most common in Florida, suggesting that local intrinsic resistance or antimicrobial use may play a role in the development of resistance.

Both the Miranda and Zemelman paper and the Blackburn study found resistance in marine fish through culturing in agar and testing isolates for resistance using the standard clinical Kirby-Baur disk diffusion test. The exclusion of anaerobic bacteria and lack of investigation into the genes encoding for resistance is likely misrepresenting the proportion of resistant bacteria in marine fish. Anaerobic bacteria account for up to 99% of bacteria in the intestinal tract of most animals (Moore, 1969), thus improvements in both studies would be to attempt to culture both aerobic and anaerobic bacteria or utilize biochemical methods like Glad et al. (2010) to identify resistance genes so as to not underrepresent resistant bacterial numbers. However, investigating the expression of resistance through the Kirby-Baur method is also important, as genes for resistance may not always be expressed, although they can still be passed on to other bacterium.

Another shortcoming in both of these studies in respect to drawing conclusions about human impacts is the lack of a natural history of the animals and a vague sense of their geographic range. Small sample sizes and lack of repeated sampling of an individual fish or the coupling of resistance findings with a life history that would support age approximations, sex determination and actual geographic range of the fish would support the analysis of fish risk-factors for colonization with ARB. These confounding variables makes geographic analysis

less robust and weakens the ability of the author to draw conclusions on the human contribution to the resistance rates.

Marine Mammals

Additional research has been conducted on the antimicrobial resistance of bacteria gathered from marine mammals. Revisiting Rose et al. (2008), swabs from the anus, feces, and tissue of marine fish, birds, pinnipeds and cetaceans were gathered along the coast of the Northeastern United States. The goal of the study was to determine differences in ARB bacteria type and occurrence among different marine vertebrates and to determine if the provenance of the animal affects the ABR pattern. From 64 marine mammals, 174 isolates were collected, 50% of which showed resistance to at least one antimicrobial. While this is lower than the 68% of resistant isolates collected from seabirds, and marine mammal sampling may be biased due to sampling bycaught and stranded animals, the study indicates that there is a high incidence of ARB in the marine environment. Carbenicillin, augmentin, ampicillin, and cepholothin were ineffective against more that 25% of the isolates, denoting a large environmental pool of ARB in the marine environment. This study did make use of anaerobic culturing, providing a more accurate estimation of the culturable ARB in the samples.

Several studies have concentrated on ARB in pinnipeds. Stoddard et al. (2008) used elephant seals on the coast of California to determine potential environmental and demographic factors associated with ABR in three specific pathogenic bacteria. Sex, weight, county, month, coastal human population,

exposure to sewage or freshwater outflow, and precipitation in the previous 24hr, 7 d, 30 d, 90 d, and 180 d were variables considered in risk analysis using rectal swabs from live and stranded seals as the source of bacteria. Broth microdilution methods to test for resistance against many antimicrobials were employed, resulting in high resistance to ampicillin and tetracycline (Stoddard et al., 2008). Elephant seals that were stranded, closer to a freshwater outflow, or in areas of high human population density demonstrated higher rates of resistance and were resistant to more drugs.

In a study from Lockwood et al. (2006), bacterial cultures collected over a period of 12 years from stranded harbor seals in Puget Sound were evaluated to define common pathogens and their ARB patterns. Cultures from wounds, umbilici, ears, conjunctiva, nares, oral lesions, and feces bore 134 pathogenic isolates, which were most resistant to amikacin (99%) and gentamicin (97%), and least affected by ampicillin (26%). Again, the possible bias related to the provenance of the seals and the use of culturing rather than biochemical analysis may muddle the results, but the findings of resistant pathogens in marine mammals in Puget Sound is encouraging for the prospect of using marine mammals as an indicator species for resistance in the study site basin.

Study of cetacean antimicrobial resistance patterns is more relevant to this study. Greig et al. (2007) studied *E. coli* isolates from bottlenose dolphins in Florida and South Carolina to determine if resistance between sampling sites is homogenous and applied a population genetic analysis to estimate within-animal isolate diversity, and identify reason for different resistance patterns in different

study sites. Findings indicated that in ARB, prevalence and complexity of ARB patterns increased in rectal samples taken near more developed areas compared to rural areas, implying WWTP outflows influence ARB presence in cetaceans (Greig et al., 2007). Resistance was detected in 19 of 25 antibiotics, with resistance to penicillin being most common followed by cephalothin, ampicillin, and amoxicillin (Greig et al., 2007). These antimicrobials represent the beta-lactam and cephalosporin drug classes, some of the oldest and most commonly used drugs in human medicine.

An important assumption made by these authors is that the dolphins that were captured had spent considerable time in the locale in which they were sampled. The range of the bottlenose dolphin is considerable, and there is no reason to believe that the sampled dolphins were native to the Florida coast or South Carolina bay. As seen in all of the papers regarding resistance and marine vertebrates, the range and natural history of the animals were assumed, which weakens links between geographic location and human influence to ARB carriage in these animals.

The lack of information on geographic range of the individuals prior to the sampling event calls into question the relationship between time and the retention of resistant bacteria. It is currently unknown how long ARB may colonize a cetacean and if the effects of human influence are long-term or immediately apparent once an animal enters ARB or antimicrobial-poisoned waters. Arguments for the quick loss of resistance genes in a bacterium once the artificial pressure is removed are viable, because a bacterium wants to expend all possible

energy and genetic material on the ability to reproduce, and can shed these genes as they become obsolete (Kümmerer, 2004). Contrarily, ARB colonization and resistance patterns may be long lasting due to pathogenic nature and research indicating the specificity of resistance patterns. Firstly, if an animal is a carrier of ARB genes and those genes have taken hold in a pathogen, the virulence of the pathogen is increased and potentially make the animal sick. Once the animal is infected, bacterial infections can spread throughout the pod, and whether or not this results in sickness or mortality the genes for resistance may continue to be passed back and forth between family groups (Gaydos et al., 2004). Second, researchers have been able to trace the sources of fecal contamination in subtropical and other water bodies to specific sources by analyzing the resistance patterns of enteric bacteria collected from the water (Harwood et al., 2000). This was possible due to the astounding specificity of the genetic patterns for resistance genes in contamination from a specific site. If these resistance genes were able to be analyzed and traced from water in an oligotrophic system, a more nutrient-rich environment, such as the enteric system of a cetacean, would retain more bacterial populations and thus may contain more bacterial density and/or diversity.

The critique of studies above suggests that improvements to the research of antimicrobial resistant drugs and bacteria using marine sentinels would begin by choosing an animal with a known geographic range, a well-studied natural history, known dietary preferences, and that can be resampled over time. An ideal marine sentinel for in the Salish Sea would be the southern resident killer whale

(SRKW), an ecotype of *Orcinus orca* with annual census reports dating back to the 1970s. This ecotype has a long lifespan similar to humans, eat almost strictly salmon, with high preference for Chinook, and spend most of the summer months in the Salish Sea basin from the Strait of Georgia south to Bainbridge Island and from the San Juan Islands west to the mouth of the Strait of Juan de Fuca (Ford et al., 2000). Thus, to study the phenomenon of spreading resistance due to human pollution in the south Salish Sea, the similarities between humans and the SRKW and the degree of information available on their life history makes them an ideal indicator species.

One experiment on ARB in SRKW has been conducted, concentrating on the blow (exhalation upon rising to the surface to breathe) respiratory bacteria. Schroeder et al. (2009) collected orca blow samples in Puget Sound by closely following individual whales and holding exposed petri dishes with various agars above surfacing orcas, catching the exhalation as they rose (Schroeder et al., 2009). Instances of ARB were discovered in the respiratory tract, comprised of different bacteria of clinical and veterinary importance, including *Salmonella, Vibrio, Pseudomonas,* and *Bacillus* spp (Schroeder et al., 2009). The most common drugs to which resistance was expressed were lincomycin, sulfa-trimethoprim, and ampicillin-sulbactum (Schroeder, 2009).

Animal Sentinels in Environmental Surveillance

Marine Mammals as Sentinel Species

A sentinel species is evaluated to identify negative trends in an ecosystem's health and to better manage the possible impacts of these effects on human and animal health (Bossart, 2006). Marine mammals are probably one of the best sentinel organisms in aquatic and coastal environments because of their closer physiological relationship to humans, long life spans, their position as apex predators in their food chain, have extensive fat stores that can serve as depots for anthropogenic toxins and their visibility as a sentinel species for aquatic health (Bossart, 2006).

Use of Scat as Biological Indicator

The use of scat samples as a method of biological sampling of wild animal populations has become popular and even standard in the field of conservation biology. While researching existing research on ARB in marine vertebrates, fecal or anal swabs were used by the majority of researchers (Blackburn et al., 2010; Glad et al., 2010; Grieg et al., 2007; Miller et al., 2009; Schaefer et al., 2011; Stoddard et al., 2008), with gill (Miranda and Zemelman, 2001; Rose et al., 2009), breath (Schroeder et al., 2009), or stomach contents (Miranda and Zemelman, 2001) used less often. Thus, fecal sampling is the most logical choice for this study to make it comparable to current literature.

There are several benefits to using scat for study of a wild species. First, fecal sampling can be employed as a non-invasive alternative to capture/release in study of species that are threatened or endangered. Under Endangered Species Act law, permitting for study of wild animals is required. These permits are specific

contracts with particular numbers (called “takes”) that limit the disturbance a field crew may make to an animal population. Compliance with this permit is critical for the completion of the study, and the right to research endangered species may be revoked at any time. The non-invasive nature of scat sampling removes many of these problems, and although Endangered Species Permits may still be required, permit violation and more importantly animal harm may be reduced by using non-invasive techniques.

Second, feces contains a wealth of information on the animal, including genetic, hormone, and toxin levels (Kohn and Wayne, 1997; Taberlet et al., 1999). Using advanced molecular biology techniques, population size, genetic variation, kinship, paternity, sex determination, pathogen sequences from bacteria, viruses, protists and other macroparisites, and food sources can be determined (Kohn and Wayne, 1997). From hormone levels, physiological stress, reproductive status, pregnancies, and aborted pregnancies can be determined for an animal (Wasser et al., 2004). Toxin levels are also determinable through organic chemistry methods and solid-liquid extraction of chemicals. The suite of biological information that can be determined from a scat sample makes its collection useful for an array of research goals, and thus this method is mutually beneficial to scientist of differing disciplines.

Third, and particular to this study, the nutrient-rich intestinal environment is particularly conducive to microbiological research (Kohn and Wayne, 1997; Miranda and Zemelman, 2001; Taberlet et al., 1999). The nutrient-rich stomach of marine fish has been shown to harbor more bacteria than surface waters, making

use of fecal samples more practical for identification of bacteria that may be in low density in surface waters (Miranda and Zemelman, 2001).

The findings of Jakobsson et al., (2010) that increased levels of resistance in human gut bacteria can persist for up to two years after treatment support the use of scat as an indicator for contact with antimicrobials or ARB. Information about the rate of shedding of resistant bacteria in the feces is not readily available, but findings that long-term effects persist after antimicrobial treatment cessation makes the analysis of ARB in scat worthwhile, and perhaps findings from this study can even help answer the question of ARB residence time in mammalian gut flora.

Characteristics of Orcinus orca SRKW Ecotype

The SRKW population that makes its home in the Salish Sea and the PNW coast are some of the best-studied population of whales in the world, and consequently their health and habitat is fairly well documented, which makes them an ideal subject.

The SRKW is a subspecies of *Orcinus orca* that are genetically, morphologically, and culturally distinct from other killer whale ecotypes of the world, though a review of the endangered species listing did not consider them a discrete population segment (Gaydos et al., 2004; Krahn et al., 2002). This subset of a larger population of killer whales that inhabit all of the world's oceans has yet to receive a taxonomic distinction, which has led to the opposition of its listing as an endangered species by some groups. However, this oversight is

apparent in the community of marine mammal scientists and currently some researchers focus on cataloguing subspecies of the killer whale (Gaydos et al., 2004). The SRKW population was devastated in the 1960's, when capture of animals for aquarium exhibits resulted in the death of 13 whales and the live-capture of 45 juveniles (Center for Whales research, 2013). The population declined to approximately 70 animals in the mid-1970's, spurning Endangered Species Act listing of the SRKW. In line with recovery efforts, the population rose as high as 100 individuals in 1995 after a trend of increasing population throughout the 1980's and 1990's, but numbers are again falling (Center for Whale Research, 2013). The SRKW population consisted of 86 individuals at the 2012 spring census, decreasing to 80 individuals by the fall 2013 census (Center for Whale Research, 2013). The population is divided into three family groups, called pods, dubbed J, K and L (Ford et al., 2000). This particular ecotype does not breed or associate with the transient or offshore killer whales off the Washington coast. The SRKW populations is also distinct in that the diet is composed almost entirely of Chinook salmon, while off-shore and transient killer whales are known to attack and consume harbor porpoise, seals, sea lions, and occasionally other whales (Ford et al., 2000). This population are known as 'residents' because they return each summer and fall to the Salish Sea from wintering habitats that are varied and less well defined (NOAA, 2011). The pods spend 18-65% of their days in the San Juan Island area of the Salish Sea from April to October, ranging to the Strait of Juan de Fuca, the Fraser River, further south into Puget Sound, or in unknown waters for the remaining time (Hanson,

2010). Winter sightings of the SRKW since 2005 suggest a range from Monterrey, California, USA, to Langara Island, British Colombia, CA (Center for Whale Research, 2013). Toxin analysis of blubber biopsy samples have shown differences in the proportion of toxins in the three pods, indicating that they likely occupy different winter ranges (Krahn et al., 2007). California sightings of L and K pods correspond to higher DDT/PCB ratios, suggesting that these groups travel further south (Krahn et al., 2007). J pod has a higher PBDE/PCB ratio, which suggests they winter where they consume prey closer to an urban source (Krahn et al., 2007).

Family groups of whales are known as pods. Pods are all descended from a central female ancestor, as the SRKWs are a matriarchal society and calves stay with their mothers for life. A SRKW pod travels, feeds, and hunts together, and their communication "language" is distinctive from other pods (Center for Whale Research, 2012; Gaydos et al., 2004). Within pods, family groups are even more tightly bound, and female descendants of the matriarch and their young will virtually always be in close contact. Commonly, SRKWs are presumed dead if they do not return to Puget Sound in the summer with their family group, since deceased whales are rarely recovered.

One of the characteristics of the SRKW is the highly developed cultural bonds (Gaydos et al., 2004). Scientists monitoring the behavioral ecology of the whales have noted the complex community structure and familial attachments. When all three pods converge, there is an elaborate greeting ceremony of sorts, in which they form two lines facing one another and swim until they are nearly face

to face. This is followed by contact and touching, which seems to be similar to play. The great matriarch and eldest of the whales, J-2, or "Granny", is over 100 years old (Center for Whale Research, 2013).

Another interesting cultural distinction of this particular species of killer whale is the return of the pods to Puget Sound from less well-defined wintering habitats each summer. Historically, large salmon runs made the trip through the Strait of Juan de Fuca energetically beneficial for the whales, when they could feast on fat Chinook returning to the Fraser River and Hood Canal. The steep decline in salmon populations in these runs have made the continued presence of the whales in Puget Sound over summers seemingly unlikely, especially when salmon stocks elsewhere are in boom. The return of the SRKW is thought to be a cultural tradition more than a benefit to the whales, because the pods go different places in the winter and only meet back up in Puget Sound during summers. It is thought that most mating and calving is done in the relatively calm waters of the Sound.

Unfortunately, this amazing tradition may be hurting the population. The species is at a critically low number, and nutritional stress is proven to decrease pregnancy rates (Ayres et al., 2011). Studies comparing birth rates to fish counts have found more pregnancies occur when hormonal stress levels that indicate nutritional deficiencies are absent, leading to the conclusion that more fish would equal more whales (Ayres et al., 2011; Gaydos et al., 2004). Declines in this population are primarily thought to be due to increased toxin accumulation from human pollutants, stress from lack of nutrition with declining salmon population,

and underwater noise pollution (Taylor et al., 2013).The importance of this species as a cultural icon, recreational draw, and ecosystem component makes them ideal for the study of antimicrobial resistance in marine mammals.

Implications for Findings of Resistance

The significance of discovering antimicrobial resistance in the microbiome of an indicator species are four-fold. Firstly, an increased number of resistant bacteria suggest that human wastes are not being sufficiently treated in a way that does not change the biological composition of the waters. A level of resistance higher in developed or polluted areas than undeveloped or "pristine "areas would show that human impacts are causing changes to the environment.

Secondly, the expression of resistance to anthropogenic antimicrobials indicates genetic material in the ecosystem is actively encoding for resistance. This becomes a natural reservoir of resistance genes that are capable of transferring resistance to pathogenic or environmentally significant bacteria.

Similarly, if resistance is present in an indicator species, this organism has become a vector for pathogenic resistance. Since the easy and quick genetic exchanges between bacteria are so common, contact with the organism could mean transfer of exotic resistant genes or pathogenic bacterial strains that are harmful to human beings. Of the 1,461 recognized human diseases, roughly 60% are pathogens able to move across species from animal to human lines and in the past 30 years, approximately 75% of new emerging human infectious diseases are zoonotic (King et al., 2008; Taylor et al., 2001; Torrey and Yolken, 2005).

The fourth implication of resistance in an indicator organism is the possibility of bioaccumulation of antimicrobial compounds in the food web. If antimicrobials are lipid-soluble, they may accumulate in small amounts in aquatic primary producers, which are eaten by herbivorous, omnivorous, and then predatory fish, such as the Chinook salmon. A study was conducted on the bioaccumulation of tetracycline and oxolonic acid in blue mussels to assess their potential for biomonitoring of antimicrobials in the marine environment (Le Bris and Pouliquen, 2004). The discovery that these antimicrobials accumulated in the soft tissues of the mussels indicates that biomagnification of antimicrobial compounds in the marine ecosystem is a realistic possibility (Le Bris and Pouliquen, 2004). Another study assessed the occurrence, distribution and bioaccumulation of 22 antimicrobials in surface water, sediment and fish samples and found that both ciprofloxacin and erythromycin exhibited potential bioaccumulation in carp, with bioaccumulation factors of 3262 L/ kg and 4492 L/ kg (Gao et al., 2012). The study also found that sediments retained the majority of antimicrobials, indicating that they could be a large reservoir of antimicrobial compounds in marine environments (Gao et al., 2012).

Essentially, findings of resistance in the environment demonstrates the presence of natural reservoirs of resistance and the phenotypic expression of resistance in the environment, indicating modification of bacterial properties by antimicrobial inputs to surface waters.

We can tell if antimicrobials are in the water column by using chemical analysis, but to show a relationship between chemical occurrence and active

phenotypic expression of antimicrobial resistance, both chemical and biological analysis from an indicator organism are necessary. The use of an indicator organism is critical for creating this connection.

Table 1. Antimicrobials ranking in the top 200 drugs by number of prescriptions in the United States for 2011. Repeat compounds produced by different manufacturers not included. Some prescription totals unavailable (NA) (Bartholow, 2012).

Antimicrobial Rank	Drug Name	Overall Rank Drug	Total Prescriptions
1	Azithromycin	9	26,427,000
2	Amoxicillin	20	19,764,000
3	Sulfamethoxazole/ Trimethoprim	28	NA
4	Amoxicillin Trihydrate/Clavulanate Potassium	114	NA
5	Penicillin VK	166	NA
6	Cephalexin	173	NA
7	Clindamycin HCl	175	NA

Table 2. Concentration range of six antimicrobials in WWTP influent and effluent in the Puget Sound Basin, WA, 2008 (Lubliner, 2010). All US WWTPs are required to have secondary treatment of wastewater, which includes degradation of the biological content of sewage, often through aerobic biological processes, as opposed to primary treatment, which is merely screening of waste water. Tertiary treatment includes some mechanism of water quality improvement before release to surface waters, including removal of nutrients, additional filtration, or other disinfection or odor-control methods.

Compound	Influent Concentration (ng/L)	Secondary Effluent Concentration (ng/L)	Tertiary Effluent of Reclaimed Water Concentration (ng/L)
Erythromycin	255-556	154-327	nd-343
Sulfamethoxazole	2,770-4,010	2-1,830	2-104
Tetracycline	13-186	10-40	Nd
Triclosan	1,480-2,770	nd-805	nd-77
Triclocaraban	289-541	31-78	3-103

Trimethoprim	611-1,400	308-791	nd-294

Table 3. Antimicrobial drug families, key drugs, mechanisms and targets for action. This list, while not all-encompassing, is demonstrative of the majority of drugs covered in this research (adapted from EB Medicine, 2005).

Drug Family	Sub-families	Key drugs	Mechanism	Target Bacteria
Penicillin	Aminopenicilllins, antipsuedomonal penicillins, anti-psuedomonoal penicillins with beta-lactamase inhibitor, penicillinase-resistant penicillins	Penicillin G, methicillin, ampicillin, amoxicillin, ticarcirillin, pipracillin, ticarcillin/clauvanate	Bactericidal Inhibits cell wall synthesis	Gram + Some Gram – Some Anaerobes
Cephalosporins	1st generation, 2nd generation, 3rd generation, 4th generation	Cephalexin, cefoxitin, ceftriaxone, cefepime	Bactericidal Interferes with cell wall synthesis	Gram + Some Gram – Some Anaerobes
Carbapenems		Imipenum, meropenum	Bactericidal Inhibit cell wall synthesis	Gram + Gram – Anaerobes
Floroquinolones	Extended spectrum floroquinolones	Ciprofloxacin, norfloxacin, levofloxacin, moxifloxacin	Bactericial Inhibit DNA gyrase	Some Gram + Gram – Atypicals
Macrolides		Erythromyin, azithromycin, clarithromycin	Bacteriostatic Inhibit protein synthesis	Gram + Some Gram – Atypicals
Aminoglycerides		Gentamycin, tobramycin, amikacin	Bactericidal Inhibit protein synthesis	Gram -

Tetracycline		Tetracycline, oxytetracycline	Bacteriostatic Inhibit protein synthesis	Some Gram + Some Gram – Atypicals Some Anaerobes
Clindamycin			Bacteriostatic Inhibits protein synthesis	Gram + Anaerobes
Vancomycin			Bactericidal Inhibits cell wall synthesis and inhibits RNA synthesis	Gram + Some Anaerobes
Trimethoprim/ sulfmethoxazole			Bacteriostatic Inhibits folate synthesis	Some Gram + Some Gram – Some protozoans
Metronidazole			Bactericidal Interferes with electron transport	Anaerobes Some protozoans and parasites
Chloramphenicol			Bacteriostatic Inhibits protein synthsis	Gram + Gram – Anaerobes

CHAPTER TWO

MANUSCRIPT

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Influences on antimicrobial resistant bacteria colonization in *Orcinus orca* scat: Using marine sentinel indicators for anthropogenic pollution in the Salish Sea

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Abstract

Antimicrobial drugs revolutionized health care, but drugs enter surface waters directly through drain disposal or indirectly through unmetabolized compounds in human and animal wastes. This input of antimicrobials and resistant bacteria to the environment may increase resistance, alter bacterial populations, or create reservoirs of resistance genes transmittable to pathogens. This study assessed risk factors for colonization with antimicrobial resistant bacteria (ARB) in the scat of an *Orcinus orca* ecotype endemic of the Salish Sea, to determine if geographic, temporal, or animal traits related to prevalence and/or patterns of resistance. Samples were collected June-October 2012 and July-October 2013, using a scat detection dog to locate whale feces as they floated in the water. Eleven samples were plated on agar infused with ampicillin, chloramphenicol, erythromycin, and tetracycline for colony count and multidrug resistance assessment. Polymerase chain reaction (PCR) amplification of the resistance-conferring genes erythromycin ribosomal-methylase B (*erm*B), methicillin-resistance gene A (*mec*A), and tetracycline-resistance genes B and M (*tet*B and *tet*M), was performed on 32 samples from at least 27 animals. The study area was divided into segments based on watershed traits, and distance from shore, number of septic tanks, wastewater treatment plants, land area and human population density were analyzed for each sample based on segment. Animal age, sex, and pod were

analyzed as organism risk factors. Number of colonies, presence/absence of ARB gene, and multidrug resistance (MDR) rates were independent variables. A total of 1730 resistant colonies were cultured from all animals, with erythromycin ranking first in prevalence and total resistant colonies, followed by ampicillin, tetracycline, and chloramphenicol. The effect of sampling location on number of colonies was significant ($F_{2, 8}$= 6.78, p = 0.019), and a sample obtained from the Southern Gulf Island site had more ARB colonies and was significantly higher in bacteria resistant to ampicillin ($F_{2, 8}$ =19.75, p=8.05×10^{-4}), erythromycin ($F_{2, 8}$ =5.36, p=0.03), and enteric bacteria resistant to ampicillin ($F_{2, 8}$ =37.07, p=8.99×10^{-5}). This site has more WWTPs, including a plant that recently used only primary treatment, but no environmental factors were statistically related to colonies or MDR by regression analysis. Results showed 4 samples positive for the *tet*M gene, and although all 4 were from females of the same pod, results were not significant. The positive samples were also from the San Juan study area, in contrast to culturing results. This study is the first to report positive identification of ARB in the feces of *Orcinus orca*, and though no specific environmental relationships were identified, the prevalence of ARB warrants further research.

1. Introduction

Since the introduction of antimicrobials in the 1940's, bacteria have shown an increased response in resistance. Antimicrobials are the third-largest group of medicines prescribed for humans, and the largest category of medicines used in veterinary practices (Thiele-Bruhn, 2003). A 2002 estimate of global antimicrobial consumption reports between 1 and 2 $\times 10^8$ kg annually (Wise, 2002), and the FDA reported annual consumption in the United States as approximately 1.6 x 10^7 kg, 80% of which is consumed by livestock for non-therapeutic treatment (FDA, 2009). These antimicrobial drugs enter the environment directly through flushing unused drugs down the drain, and indirectly through unmetabolized compounds excreted by human and animal waste through wastewater effluent and leaking septic tanks,, and runoff and drainage from agricultural lands and aquaculture sites (Cabello, 2006; Kümmerer, 2004; Okeke and Edleman, 2001; Zhang et al., 2009).

Increased input of antimicrobial drugs creates opportunities for environmental and pathogenic bacteria to develop selective resistance to pharmaceuticals due to the ease with which bacteria exchange genetic material and the speed at which they reproduce. More virulent and resistant bacteria are artificially selected by this increased exposure, and also threaten water quality through altered environmental bacteria populations. Given that increased levels of resistance in human gut bacteria can persist for up to two years after treatment has stopped (Jakobsson et al., 2010), the massive amount of antimicrobials that we are adding to our environment now may have long term consequences.

Antimicrobial resistance is increasingly framed as an ecological problem in addition to a human health problem. The use of synthetic antimicrobials is potentially devastating to natural populations of bacteria that provide important ecosystem services because xenobiotic compounds are recalcitrant, especially when they are broad spectrum and effective against more bacterial species, and thus they tend to be more persistent in soils and waters (Kümmerer, 2004). Understanding and documenting the transmission of non-pathogenic antimicrobial resistant bacteria (ARB) is important because environmental bacteria can serve as reservoirs of resistance, passing genes to pathogens through conjugation, transduction, or transformation (Barbosa and Levy, 2000; Khachatourians, 1998).

Increased human population and decreased wild habitat has intertwined the health of humans, wildlife, and the environment, breaking down divisions between these disciplines in the assessment of ecosystem health (King et al., 2008). This new paradigm, exemplified by the growing One Health movement, has created a more collaborative and holistic approach to monitoring ecosystem health through the use of animal sentinels (Rabinowitz and Conti, 2013). Marine mammals are a good sentinel species in marine and aquatic research due to their physiological relationship to humans, their position as apex predators, and their visibility as a keystone species in the ecosystem (Bossart, 2006; Wong, 2002). Fecal sampling can be employed as a non-invasive alternative to capture/release in study of species that are threatened or endangered. In addition, feces contains a wealth of information on the animal, including genetic, hormonal, and toxin loading, and the nutrient-rich intestinal environment is particularly conducive to

microbiological research (Kohn and Wayne, 1997; Miranda and Zemelman, 2001; Taberlet et al., 1999).

Research using the intestinal bacterial flora of marine vertebrates as indicator species for ARB pollution has shown varying degrees of resistance. ARB presence has been studied in predatory fish, marine birds, pinnipeds, and various whale and dolphin species by culturing bacteria from rectal or fecal swabs (Blackburn, 2010; Grieg, 2007; Johnson et al., 2008; Lockwood et al., 2006; Miller et al., 2008; Miranda and Zemelman, 2002; Rose et al., 2008; Schaefer et al., 2011; Schroeder et al., 2009; Stoddard et al., 2002). The levels of resistant samples and the complexity of resistance patterns has been positively correlated by sampling site to suspected contamination points, ex. higher number of wastewater treatment plant (WWTP) outflows (Grieg et al., 2007), number of septic tanks (Schaefer et al., 2011), human population density (Blackburn et al, 2010; Grieg et al., 2007), and freshwater outflows (Johnson et al., 2008). Rainfall and weather events have also been related to increase antibiotic resistance findings in short-term temporal analyses (Grieg et al., 2007; Schaefer et al., 2011).

The Salish Sea is an ecologically diverse and economically important estuary in the Pacific Northwest region of the United States of America and southwestern British Columbia, Canada (Fraser et al., 2006; Gaydos et al., 2009). This glacial carved fjord inland marine ecosystem stretches along the inland waters from Olympia, WA in the United States to the Campbell River in Canada, and is the home to over 7 million people and many endemic and rare marine

species (Jackson and Kimerling, 2003). Like many of the world's coastal zones, population growth and increased toxin and fuel loading has amplified pressure on the marine habitat and wildlife in the Salish Sea (Gaydos et al. 2009; Puget Sound Partnership, 2011). Population growth has increased loading to septic and waste treatment systems, increasing likelihood that harmful substances, including pathogenic bacteria, toxins, and other chemical compounds will reach the water column without being properly broken down (Take Back Your Meds, 2011). There are 96 publicly owned WWTPs in Washington State emptying into the Salish Sea and processing over 124 million gallons of sewage from over 3.5 million people each day (WA Department of Ecology, 2010). Groundwater, storm water, and combined sewer overflows discharge untreated and industrial wastewater and compounds leached from leaky septic drainage systems when capacity is exceeded or water levels rise above the drainage fields also contribute to surface water pollution (Puget Sound Partnership, 2011; Dougherty et al., 2010). In Liberty Bay, WA, data show that the community, which predominately uses septic tanks rather than centralized WWTPs, is receiving detectable levels of trimethoprim in surface and ground waters, with the occurrence of detections increasing as population density increased (Dougherty et al., 2010).

Research on marine bacterial resistance in Washington shows that ARB can be found in marine sediment near aquaculture sites, the sea-surface, and public beaches (Herwig et al., 1996; Roberts et al., 2008; Soge et al., 2009). When analyzing sand samples from Washington beaches, five distinct strains of multi-drug resistant methicillin-resistant *Staphylococcus aureus* (MRSA) and 33

methicillin-resistant coagulase-negative *Staphylococcus* ($MR_{CON}SA$) spp were identified (Soge et al., 2009) Roberts et al. (2008) identified 18 resistant strains of vancomycin-resistant *Enterococcus* in Washington public beaches. The presence of these resistant and pathogenic strains makes a case for the possibility of ARB genes in offshore marine waters, and understanding the transmission of these genes is critical to assessing the risk of the marine environment serving as a reservoir of resistance genes transmittable to pathogens.

In the Salish Sea, abundant data is available the endemic ecotype of the *Orcinus orca*, or Southern Resident Killer Whale (SRKW). Concerns over decline in the SRKW population resulted in a large collection of data on the life history, family lineage, geographic range, and individual identity of most whales. The SRKW population was devastated in the 1960's, when capture of animals for aquarium exhibits resulted in the death of 13 whales and the live-capture of 45 juveniles (Center for Whales research, 2013). The population declined to approximately 70 animals in the mid-1970's, spurning Endangered Species Act listing of the SRKW. In line with recovery efforts, the population rose as high as 100 individuals in 1995 after a trend of increasing population throughout the 1980's and 1990's, but numbers are again falling (Center for Whale Research, 2013). The population consisted of 86 individuals at the 2012 spring census and declined to 80 by the fall of 2013, during the time of this study (Center for Whale Research, 2013). Declines in this population are primarily thought to be due to increased toxin accumulation from human pollutants, stress from lack of nutrition with declining salmon population, and underwater noise pollution, and other proposed

threats include pathogens (NOAA, 2011). The species is at a critically low number, and compound factors of nutritional stress and toxin loading can increase likelihood for infections in these animals (Ayres et al., 2011). The importance of this species as a cultural icon, recreational draw, and keystone species make them ideal for the study of antimicrobial resistance in marine mammals. Prior studies using marine vertebrates as sentinel species have shown positive correlation between human influence and ARB colonization to some degree (Blackburn, 2010; Grieg, 2007; Johnson et al., 2008; Lockwood et al., 2006; Miller et al.,2008; Miranda and Zemelman, 2002; Rose et al., 2008; Schaefer et al., 2011; Schroeder et al., 2009; Stoddard et al., 2002), but no research has been able to draw robust conclusions on anthropogenic effects on ARB acquisition due to the lack of a natural history of the animals surveyed and a vague sense of their geographic range, confounding variables not applicable to the study of ARB in this species. Here the issue of bacterial resistance caused by pollution of antimicrobials and wastewater was examined by studying prevalence and patterns of ARB colonization in the feces of *Orcinus orca.* By using a sentinel species for colonization with ARB and pairing prevalence and patterns with demographic, geographic, and anthropogenic risk factors, the relative influence of human impacts as well as traits intrinsic to ARB susceptible wildlife can be assessed. Results indicate how other species could be affected by exposure to waters affected by pharmaceutical pollution and determine if ARB are a problem of relevance to public health in the Salish Sea marine environment.

2. Methods

2.1 Ethics Statement

Fecal samples from orcas were collected in United States waters under National Marine Fisheries Service permits 532-1822-00, 532-1822 and 10045 and in Canadian waters under Marine Mammal License numbers 2008–16 and 2009–08 as well as Species at Risk Act permits numbered 91 and 102. Sample collection methods were approved by the University of Washington's Institutional Animal Care and Use Committee (IACUC) although no permit was required, because research was non-invasive.

2.2 Study Site

Sampling was conducted in the Salish Sea from May-October 2012 and June-October 2013. The research team was based off the western coast of San Juan Island, and sampled when SRKWs were confirmed within approximately 1 hour travel time from harbor. This limited the study site to approximately the Strait of Georgia to Vancouver, BC in the north, the Strait of Juan de Fuca to Sooke, BC in the west, Smith Island, USA to the south, and Rosario Strait through the western shore of Washington's interior coast to the east. This study range breaks down into six major segments based on watershed and environmental characteristics as determined by the Puget Sound Watershed Characterization Project in the US and the Marine Environment Monitoring and Assessment program in CA (Figure 1) (Capitol Region District, 2013; Stanley, 2010). The Canadian regions are Juan de Fuca, Saanich Peninsula, and Southern Gulf Islands and in the USA the study site breaks down into the San Juan Islands (including all islands in the San Juan archipelago), Nooksack, and the Coastal Skagit Basin.

This classification was appropriate because of the prior use of these divisions in water quality research, the grouping of major watersheds which help make non-point pollution assessment more accurate, and the extent of the study site which is covered by these segments.

The land area, population density, number of WWTPs, and best current estimate of the number of septic tanks in each region are the environmental variables of relevance for this study and values for the six segments in the study site are noted in Table 3 (Capital Regional District, 2013; ESRI, 2010; ESRI, 2013; Whatcom County Health Department, 2013; Skagit County Health Department, 2013; Wiseman et al., 2000). The number of WWTPs and number of septic tanks are important variables for relating the direct input of antimicrobials or ARB to surface waters through sewage treatment or leaking septic systems. The variables of land area and population density are to approximate indirect anthropogenic pollution that could result in elevated ARB, including run-off from large land masses, urban storm-water, and if the number of WWTPs or septic systems is significant, if this is related to human population density or other factors.

2.3 SRKW Profile

The SRKW population is a distinct ecotype of *Orcinus orca*, a sub-order of *Cetacea Odontoceti* in the family *Delphenidae*, closely related to other toothed whales such as pilot whales, sperm whales, and oceanic dolphins (Taylor et al., 2013). The SRKW is genetically, morphologically, and culturally distinct from

other killer whale ecotypes of the world, though endangered species listings do not yet consider the differing forms discrete population segments (Gaydos et al., 2004; Krahn et al., 2002). There are three distinct killer whale groups in the north eastern Pacific Ocean, commonly known as 'resident', 'transient' and 'offshore' Killer Whales, who maintain social isolation from each other despite overlapping ranges (Ford, 2002). The SRKW population diet is composed almost entirely of Chinook salmon, while off-shore and transient killer whales are known to attack and consume harbor porpoise, seals, sea lions, and occasionally other whales (Ford, 2002).

The population is divided into three pods, dubbed J, K and L, which are descended from a central female ancestor, as the SRKWs are a matriarchal society and calves stay with their mothers for life (Ford et al., 2000). A SRKW pod travels, feeds, and hunts together, and their communication "language" is distinctive from other pods (Center for Whale Research, 2012; Gaydos et al., 2004). This population are known as 'residents' because they return each summer and fall to the Salish Sea from wintering habitats that are varied and less well defined (NOAA, 2011). The pods spend 18-65% of their days in the San Juan Island area of the Salish Sea from April to October, ranging to the Strait of Juan de Fuca, the Fraser River, further south into Puget Sound, or in unknown waters for the remaining time (Hanson, 2010). Winter sightings of the SRKW since 2005 suggest a range from Monterrey, California, USA, to Langara Island, British Colombia, CA (Center for Whale Research, 2013). Toxin analysis of blubber biopsy samples have shown differences in the proportion of toxins in the three

pods, indicating that they likely occupy different winter ranges (Krahn et al., 2007). California sightings of L and K pods correspond to higher DDT/PCB ratios, suggesting that these groups travel further south (Krahn et al., 2007). J pod has a higher PBDE/PCB ratio, which suggests they winter where they consume prey closer to an urban source (Krahn et al., 2007).

2.4 Sample Collection

Samples were collected using a scat detection dog in the same procedure using modifications outlined by Ayres et al., 2012, which was revised from Wasser et al., 2004, and Rolland et al., 2006. Briefly, a detection dog rode on the bow of a 6m fiberglass hull vessel with a professional dog handler. The boat was positioned downwind from the whales, and the driver and handler assessed the wind direction, strength, and water conditions to set the boat perpendicular to the "scent cone" to optimize the ability of the dog to smell the feces. The dog was selected for his obsessive tendencies to play with a ball, and his identification of a sample was rewarded by play with the ball and the handler. This encouraged the dog to associate sample detection with the play reward, and resulted in a change in behavior when the target scent was perceived. This anticipation of reward caused a change in behavior upon scent observation, which was noted by the professional handler, who communicated with the driver as the scent changed from high to low concentration. The handler and the driver worked together to direct the course of the boat as the dog stood erect, turned, slobbered, and whimpered as the concentration of the strength of the scent changed.

Simultaneously, crew members scanned the surface for whale fecal samples, which were identified by algae-like appearance and distinct fishy smell.

Once identified, samples were scooped using 1 L polypropylene beakers and brought back onboard to immediately discard the water while retaining the fecal pellet in a 50 ml polypropylene screw-top tube. This process was repeated until all floating sample was collected up to 30 mL. The tube was capped and centrifuged on the boat at 1,000 rpm for five minutes and excess water poured off.

For culturing, 4-5 sterile cotton swabs were inserted into the fecal pellet and stirred slightly to gain a heterogeneous sample of approximately 0.5 mL. The swabs were stored in 10mL of buffered sterile peptone water and placed on wet ice until they could be transferred to the lab, with times ranging from 1.5 to 6.5 hours and averaging 4 hours. Two control samples were taken by recreating the sampling process with water scooped from the sea surface, passed through the processing equipment, and swabbed in the same manner as the fecal pellets.

For PCR analysis of resistance genes, the same procedure was used with a single swab, which was then carefully inserted into a 2 ml micro tube and the stick sterilely broken off to close the lid. Samples were stored in 20% glycerol in MilliQ water or dry for method comparison. Once back on land, samples were immediately stored at -20º C until DNA extraction.

2.5 Plate Culturing Methods

All culturing was done at Friday Harbor Labs in Friday Harbor, San Juan Island, WA, USA. Samples in peptone broth were vortexed for 10 seconds. A

1mL disposable pipette was used to add 0.1 mL of fecal/peptone mixture to plates. When dilution was necessary, 0.1mL of fecal/peptone was added to 9.9mL of sterile saline. The whole or diluted mixture was spread on tryticase soy agar (TSA) + 2% NaCl plates infused with ampicillin (Amp), chloramphenicol (Cm), erythromycin (Erm) and tetracycline (Tc), and MacConkey without Crystal Violet plates with Amp, Cm, and Tc at the values shown in Table 1, using the plate spreading method and standard bench techniques (Hurst, 2002). Plates were incubated at 36.5°C for 24-48 hours under aerobic conditions. After the samples had been incubated, the number of colonies on each plate was recorded and color was noted for colonies on MacConkey agar. Plates were parafilmed and stored for future research.

2.6 PCR Assay Methods

Validation for optimum rRNA extraction from fecal swabs was conducted by comparing 16S rRNA content from two extraction kits and using two different Taq polymerase master mixes for resistance gene amplification (see supplemental material for additional methods validation procedures). The DNAeasy Blood and Tissue Kit and Qiagen HotStarTaq reagent were used with primers amplifying the resistance genes *erm*B, *met*A, *tet*B, and *tet*M (Wasser et al., 2011). These genes were selected because the common use of erythromycin, methicillin, and tetracycline in animal and human medicine, the promiscuity of these particular genes in genetic exchange, and previous discovery of these resistance genes in mammalian gut flora made them likely candidates for colonization of the SRKW. Gel electrophoresis on 1% agarose gel with 5% TBE buffer at 100 volts for 45

minutes, and visualized with 2µL of stop mix and UV light excitation was used for identification of the resistance genes.

Genotyping for whale identification, including pod and sex, was performed by NOAA's Northwest Fisheries Science Center at 2725 Montlake Boulevard, Seattle, WA. Presumptive sex was identified for samples lacking positive genotyping identification by comparing progesterone and testosterone levels gained through radioimmune-assay analysis in the Department of Conservation Biology Laboratory, University of Washington, Seattle, WA.

2.7 Data Management and Statistical Analysis

Culturing bacteria

The number of antimicrobial resistant colonies were totaled for each antimicrobial and sample as an indicator of ARB density in fecal samples. Approximate quantitative assessment of resistant colony forming units (CFU) per milliliter of sample was calculated by dividing the number of colonies by the volume plated and dividing that number by the total dilution factor. This number was multiplied by total colonies on each plate to approximate the number of ARB in each mL of whale feces. Multidrug resistance (MDR) was calculated by summing the total number of drugs to which each sample expressed resistance. Ordination was performed to analyze patterns between resistances within samples, using freely available R software (The R Foundation for Statistical Computing, 2013). Principle component analysis (PCA) was selected because the primary purpose was to identify and compute composite resistance numbers for any trends in antimicrobial resistance patterns between samples.

Each sample was assigned a land location based on the GPS coordinates taken at time of sampling and shortest distance from shore as calculated using ArcMap 10.1 (ESRI, Redlands, Calif., USA).

Environmental risk analysis of culturing results

The environmental risk factors included in statistical evaluation were distance from shore, population density (people/km^2), watershed area size (km^2), number of septic tanks, and number of WWTPs in the geographic segment nearest to the GPS location of each sample that was cultured for bacterial growth.

For each geographic segment, human population density was estimated from and land watershed area was obtained using ArcGIS Online population density maps (ESRI, 2010; ESRI, 2013). Number of septic tanks in each area were obtained from San Juan, Skagit, and Whatcom County USA Health Department Records, San Juan County Conservation District, and from the Capitol Regional District in British Columbia (Capitol Regional District, 2013; personal communications, 2013; Wiseman et al., 2000). Distance from shore and number of resistant colonies was compared by correlation.

Total ARB for each sample were compared by ANOVA separated by geographic segment to see if sampling area affected the number of colonies. ANOVA was also performed on ARB colonies totaled for each antimicrobial and each antimicrobial plate, separating the MacConkey bacteria from the TSA growth to see if patterns in resistance were reflected in the type and amount of resistant bacteria.

Total colonies were averaged by each area and linear regression analysis against the environmental variables was conducted to assess if there is a relationship between number of colonies and environmental parameters. The MDR and environmental variables were analyzed for similarity trends using a contingency table.

Temporal change in ARB prevalence based on total and number of consecutive days the whales had been in the basin was assessed. The Julian date of each sample was correlated to the total ARB and ARB for each antimicrobial to look for trends in ARB prevalence as a factor of time. The total days the whales were present in the study area before sampling occurred was calculated based on field effort log entries. For days when new whales entered the study area and joined previously sampled whales, the count was reset because sampling protocol focused on alternating pods and family groups as much as possible. The number of consecutive days was analyzed by correlating against total ARB and ARB by antimicrobial to seek trends in number of resistant bacteria as days in the basin increased, and an influence function plot was constructed to reduce the effect of influential data points. ANOVA was conducted to compare means of total ARB and ARB by antimicrobial by number of days in study site.

PCR genetic assay

For each sample undergoing genetic analysis, the sample distribution of sex and pod was compared to the entire SRKW population by X^2 goodness-of-fit test to test for sample distribution bias. A representative age distribution of

samples was tested with Student's t-test. When resistance genes were identified in an assay, all samples from that assay were assessed for risk factors of sex and pod by X^2 testing, and using ANOVA for distance comparison.

3. Results

3.1 Sample Collection Results

Genetic analysis samples were collected during July-October 2012, and culturing samples were collected during August-October 2013. A total of 11 fecal samples plus two control water samples were collected for culturing, sampling from a single family group for 2 days and from the entire SRKW populations for seven days. Samples were assumed to be independent due to the sampling focus on collecting feces from all whale groups. Two water samples were also collected as controls.

For PCR assay analysis, 32 fecal samples were collected for genetic analysis in 2012. DNA genotyping by NOAA allowed for identification of individual whales for 23 (71.9%) samples (Hemplemann, J, unpublished data). From the 23 samples, 19 individuals were identified, so samples are not considered independent. Age range was 13-79 years old, with a mean age of 28.5 and standard deviation of 16.5. Sex information on individuals lacking identify confirmation was supplemented by hormonal analysis, bringing total sex identified animals to 29 (90.6%) of samples (Wasser, unpublished data). The PCR assay samples were representative of the known individuals in the SRKW population by age distribution ($t=1.34$, $p=0.18$, d.f. $=105$), and sex ($X^2= 1.93$, $p=$

0.38) (Figure 4), but the variance of individuals were not evenly distributed by pod, with more samples attributed to the J family pod (X^2= 34.85, p= 1.31 × 10^{-7}) (Figure 5).

3.2 Plate Culturing Results

Each of the 11 fecal samples resulted in bacterial growth on at least one antimicrobial plate, while the two control water samples showed no growth on any plate (Table 2). For this reason, bacteria are considered to be from the fecal source rather than surface water or laboratory contamination, and the environmental data for the control samples was not analyzed. Samples were assumed to be independent in statistical analysis because of sampling protocol dictating to move between whale family groups for maximum distribution of samples and from track logs recording the specific whale groups followed at time of sampling.

The total number of ARB colonies by antimicrobial is represented in Figure 6, with TSA and MacConkey plates differentiated by color. Amp25 plates colony growth ranged from 0-87 colonies ($\bar{x}$ =14.91, stdev=26.22), Cm25 ranged 0-9 ($\bar{x}$ =1.36, stdev=2.66), Erm10 ranged 0-520 ($\bar{x}$ =138, stdev=167.57) and Tc25 0-16 colonies ($\bar{x}$ =3, stdev= 5.46).

CFU per milliliter of fecal quantification by sample ranged from 2.10×10^2 CFU/mL fecal for sample 3 to 1.27×10^6 CFU/mL for sample 4, with $\bar{x}$ =1.37×10^5 and stdev=3.78×10^5. The mean CFU/mL grouped by antimicrobial was 9.08×10^4 (stdev=1.53×10^5). Total CFU grouped by antimicrobials were: Amp25 =3.44×10^4,

Cm25 =3.15×10^3, Erm10 =3.19×10^5, and Tc =6.93×10^3. The CFU/mL notation was not used in subsequent analysis because the homogenous nature of bacteria within the fecal was not verified by replication of culturing; however, the CFU/mL measurement is the best current estimation based on peer-reviewed data on cetacean fecal.

ANOVA testing revealed that there was a significant effect on the number of colonies by antimicrobial drug type with $p<0.05$ ($F_{6, 84}= 2.21$, $p = 1.38\times10^{-5}$). Tukey's Post-Hoc results show Erm10 plates ($\bar{x}$ =116.77, stdev=161.51) was significantly higher in colony numbers than all other plates. ANOVA analysis of effect on total colony growth by sample was not statistically significant at $p<0.05$ ($F_{10, 66}= 0.82$, $p = 0.61$).

Single drug resistance (SDR) was expressed by three (27.2%) samples and MDR on the remaining eight (72.8%), with $\bar{x}$ = 2.45 and stdev=1.17 (Figure 7). This is interesting because the maximum MDR is to all four antimicrobials.

Ordination analysis of similarities in number of ARB resistant bacteria for each sample are represented by the spatial distance between sample numbers on the PCA plots, and the weight and direction that each of the antimicrobial variables was given is shown in the loading plots. For both PCA analyses, samples 12 and 13 (Control 1 and Control 2) are overlapping.

PCA was run with the variable of colony count by antimicrobial and plate type to examine similarities in samples of relative types of resistant bacteria in addition to antimicrobial resistance (Figure 8). The loading plot shows that there

is similar ARB growth patterns in MacConkey and TSA plates for Amp and Tc, but that the two plate types are not similar in ARB growth pattern for Cm (Figure 8). Samples are not spatially related by numerical order (i.e. date). Samples 4 and 5 are the most unrelated to any other samples. Sample 4 is influenced by the high number of Erm resistant bacteria along with Amp resistant growth on both plate types, while the distance of Sample 5 from other samples is best explained by the resistance to Erm, Tc for both plates, and MacConkey Cm. This combination of resistant growth is unique to this sample alone.

PCA for joined antimicrobial was also conducted to examine differences in patterns among the resistance by drug disregarding agar type (Figure 9). Again, Sample 4 is widely separated from other samples, but Samples 5 and 8 are also outliers and closely related since the separation between Cm resistances by plate is not recognized.

Aside from demonstrating relationships in resistance patterns between samples, the comparison of loading charts shows the relationships between ARB prevalence for antimicrobial. In Figure 8 it is demonstrated that Cm resistance is often opposing to Tc and MacTc resistance and Erm, Amp and MacAmp resistance are closely related. Figure 9 shows a weakened relationship between Erm and Amp, and a more closely aligned behavior in resistance patterns of Tc and Cm in the samples due to combining the two plate types.

3.3 PCR Assay Results

Four of 43 total samples (including duplicates) tested positive for the *tet*M gene encoding resistance to tetracycline. No other resistance genes were detected. All four *tet*M positives were from female J pod animals and in the San Juan Island geographic segment. Analysis of the relationship between sex and presence/absence of the *tet*M gene returned no significance (X^2= 3.35, d.f. = 2, p= 0.18). Analysis of positive genetic identification of *tet*M by pod showed no significant relationship (X^2=6.68, d.f. = 3, p= 0.08) for $p < 0.05$, but findings would be significant at a $p<0.15$ acceptance of error. An age and ARB colonization two-tailed student's t-test for unequal variances also determined no significant differences (t=3.18, 0.47).

The ANOVA conducted to test variance between positive and negative groups and distance from shore resulted in no significant difference between groups ($F_{1, 39}$=0.73, p=0.38). Chi-square testing of the geographic segment relationship to positive resistance gene identification resulted in no significant relationship for $p<0.05$ (X^2=1.05, d.f. = 2, p= 5.99).

3.4 Data and Spatial Analysis

Geographic assessment based on sample locations resulted in the use of three of the six geographic segments in the study area: San Juan in the USA, and Southern Gulf Islands and Juan de Fuca in CA. The tested environmental risk factors related to each of these segments and sample numbers that correspond to each is shown in Table 3. Of the 11 samples, eight were categorized as San Juan, two as Juan de Fuca, and one as Southern Gulf Islands. The low representation of

geographic distribution makes spatial and environmental analysis less robust due to low number of data points and lack of variability, but analyses still seek trends in data.

Single-factor ANOVA was used to compare the effect of sampling location on total number of colonies in Southern Gulf, San Juan, and Juan de Fuca locations because the 2013 culture samples fell only within these three sectors (Figure 10). There was significant effect on the mean number of colonies at the $p<0.05$ level for the three locations ($F_{2, 8}= 6.78$, $p = 0.019$). Tukey's Post Hoc test revealed that the mean number of colonies for the San Juan ($\bar{x}$ =115.25, stdev=128.44) and Juan de Fuca ($\bar{x}$ = 100.5, stdev=126.57) areas were not significantly different, but both differed widely from the Southern Gulf sample ($\bar{x}$ =607, stdev=0).

Colony growth for each site was compared by one-way ANOVA to assess if geographic segment had influence on an antimicrobial's resistance prevalence. This was done in two ways: by growth on each plate, and by growth numbers on all plates for each antimicrobial. For growth by plate, TSA Erm10 ($F_{2, 8}$ =5.36, p=0.03) and MacConkey Amp25 ($F_{2, 8}$ =37.07, $p=8.99\times10^{-5}$) were found to be significant, both indicating that the sample from the Southern Gulf Islands location produced more bacteria resistant to these antimicrobials. Accounting for combined antimicrobial prevalence, Amp25 ($F_{2, 8}$ =19.75, $p=8.05\times10^{-4}$) and Erm10 ($F_{2, 8}$ =5.36, p=0.03) were found to be significant, again indicating more colony growth in the Southern Gulf Islands.

Total number of colonies by sample was compared to the environmental variables of WWTP, septic tank, land area, and population density using linear regression analysis. There were no statistically significant environmental factors for $\alpha=0.05$. The values for the insignificant relationships are as follows: number of WWTPs ($y=176.68x-138$, $r^2=0.87$, $F_{1,2}=7.09$, $p=0.23$), number septic tanks ($y = -0.0521x + 772.74$, $r^2 = 0.106$, $F_{1,2}=0.12$, $p=0.78$), land area in km^2 ($y = -0.282x + 480.98$, $r^2 = 0.451$, $F_{1,2}=7.09$, $p=0.23$), and population density in inhabitants/ km^2 ($y = 3.9112x + 195.35$, $r^2 = 0.05$, $F_{1,2}= 0.05$, $p=0.86$).

Correlation of total number of resistant colonies as a function of distance from shore showed no significant relationship ($y = -0.0083x + 178.1$, $r^2 = 0.0175$, $F_{1, 9}=0.16$, $p=0.69$) (Figure 11).

An analysis of the relationship between MDR rank (ranging from 1-4 drugs) and environmental risk factors was performed by creating contingency tables and using the Williams (1976) adjusted X^2-statistic for small sample size. Analysis revealed no statistical significance between MDR and any environmental variable for $p < 0.05$ (adjusted $X^2= 4.04$, d.f. = 6, $p= 0.30$).

Revisiting the PCA analyses charts (Figure 8 and Figure 9), there is no close relationship between Samples 10 and 11, the Juan de Fuca samples, over their relationship to samples from the San Juan geographic segment. The single Southern Gulf Islands sample, Sample 4, is an outlier in both PCA analyses, as explained above. From the data, no relationship between ARB patterns and geographic segment can be made.

Correlation of the relationship between time and total ARB colony number growth assessed by Julian date showed no significant relationship ($y = -0.2561x + 217.48$, $r^2 = 0.0004$) (Figure 12). Analysis of time and ARB number by antimicrobial was also not significant (Figure 13).

The relationship between the number of days the whales had been in the basin before the sample was collected yielded no statistically significant results by ANOVA by total or single drug resistance, or by regression analysis (Figure 14) ($y = -19.628x + 216.16$, $r^2 = 0.0935$). However, due to the small sample size and low variance in number of days before sampling, the decreasing trend in ARB shedding as the whales remain in the basin warrants further investigation, as it contradicts the hypothesis that the Salish Sea is a source of drug pollution and resistant bacteria.

4. Discussion

Antimicrobial resistance is ubiquitous in the environment and resistant bacteria have been isolated from beaches, surface water, and SRKW blow in the Salish Sea in prior research (Roberts et al, 2009; Schroeder et al, 2009; Soge et al, 2009). This research presents the first isolation of ARB from the feces of the SRKW, with 11 (100%) of samples showing resistance to ampicillin, chloramphenicol, erythromycin, or tetracycline and 8 (72.8%) samples growing colonies of bacteria resistant to multiple drugs. The findings of no growth in control samples support the assumption that all bacteria colonies originate from

the collected fecal rather than water surface, processing equipment, or laboratory contamination.

Compared to other studies, resistance in SRKW fecal matter is at first glance significantly higher. Stoddard et al studied ARB prevalence in *Escherichia coli* from fecal bacteria of stranded and healthy elephant seals in California, and found that only 6.7% of the free-ranging seals showed resistance, and a mere 1.2% showing resistance to more than one antimicrobial in an assessment of 12 different drugs (Stoddard et al., 2008). However, their study concentrated on a specific bacterial species while this study was indiscriminate to bacterial identification, so comparisons are hard to draw. Blackburn et al. (2010) study of predatory fish returned a total of 130 bacterial isolates from 63 total animals sampled, approximately 2.1 isolates per sample. Specific isolate identification was not performed in either experiment, and this study shows and average of 157.3 colonies per sample, indicating more resistance. It must be noted that our sample size of 11 is considerably smaller, which could alter the eventual outcomes. However, the SRKW has been referred to as the most polluted cetacean in the world (Ross et al., 2000), so the increased resistance in this study should be taken seriously regardless of small sample size.

This study found that resistance to erythromycin was most common for samples on non-differential media, followed by ampicillin and tetracycline, then chloramphenicol. The differential media showed highest resistance to ampicillin, chloramphenicol and tetracycline, in that order. Erythromycin was not used on MacConkey plates. The high instance of ampicillin resistance is common in

papers researching marine fecal bacteria (Miranda and Zemelman, 2001; Rose et al., 2008; Stoddard et al., 2008). However, other researchers have found higher instances of tetracycline resistance, and there is no analogous explanation for the high erythromycin resistance observed in this experiment (Blackburn et al., 2010; Miranda and Zemelman, 2001; Stoddard et al., 2008). This study tested significantly less antimicrobials for resistance, and this incongruity may be an artifact of the laboratory analysis.

Growth of 1635 (94.5%) of the total colonies occurred on the TSA 2% NaCl plates rather than MacConkey 2% NaCl plates. The TSA agar is a general purpose growth medium used for cultivation of a wide variety of bacteria, while MacConkey agar is selective for gram-negative bacteria and typically used in investigation of mammalian gut enteric flora (BD Diagnostics, 2009). While the SRKW is a marine mammal, the genetic analysis of the SRKW microbiome shows a rather low number of *Bacteroidetes* and other gut flora common in microbiota of terrestrial mammals, (Bik, Elisabeth, personal communication) so less growth on media designed for human flora is not entirely unexpected particularly when plated with salt solution.

A vast majority (87.7%) of colonies proliferated on TSA 2% NaCl Erm plates, suggesting that bacteria within the fecal microbiome of the SRKW may have some intrinsic resistance to erythromycin. Current research on the SRKW microbiome shows a bacterial flora dominated by *Clostridium sordelli, Clostridium perfringens, Cetobacterium ceti, Fosobacterium mortiferum, Photobacterium damselae, Escherichia coli, Edwardsiella tarda,* and

Actinobacillus delphinicola, but without deeper investigation into the specific resistance genes that these bacterial species commonly acquire, it cannot be ruled out that this resistance has developed as a result of outside factors such as pharmaceutical pollution or contamination with ARB (Bik, Elisabeth, personal communication). In a study by the Washington State Department of Ecology, erythromycin concentration in WWTP effluent was the second highest antimicrobial in tertiary treated effluent, at concentrations of up to 343 ng/L, and secondary treatment effluent showed concentrations of 154-327 ng/L (Lubliner, 2010). Tetracycline was also discovered in concentrations considerably lower than erythromycin in both influent (13-186 ng/L) and secondary treatment effluent (10-40 ng/L), and was non-detectible in tertiary effluent (Lubliner, 2010). These patterns fit the incidence of ARB resistance found in SRKW scat, and the contribution of large concentrations of unmetabolized erythromycin compounds in WWTP is a possible explanation for the high rate of resistance seen in this study.

The results from this study related to the 2009 study of orca blow resistance show interesting comparisons. In 11 samples from male killer whale breath, erythromycin resistance is low, expressed in only two samples (Schroeder et al., 2009). However, the expanded macrolide-lyncomycin-streptogramin (MLS) family of antimicrobials includes lyncomycin, and high resistance to this drug is high in orca blow, with eight samples expressing resistance (Schroeder et al., 2009). This suggests that there could be intrinsic resistance to antimicrobials with the bacteriostatic mechanisms of protein-inhibiting synthesis on the 50s ribosomal

sub-unit, or that there is a gene that proliferates MLS resistance in the waters of the Salish Sea. Tetracycline resistance was comparatively low, only demonstrated in two of the 11 samples (18.2%), and resistance to beta-lactams was shown in 7 samples (63.6%) (Schroeder et al., 2009). The ratio of tetracycline to beta-lactam resistance is similar in the study of feces, with 36.4% of samples showing tetracycline resistance and 72.7% of samples resistant to ampicillin. The breath study did not include chloramphenicol, but did include florfenicol, which is in the same amphenicol family of antimicrobials. Resistance to these two drugs was not similar within these two studies, with 27.2% of whale breath samples showing resistance compared to 45.4% of scat samples (Schroeder et al., 2009). The similarities in relationships for beta-lactams, tetracycline, and MLS drugs could point to common drug resistance colonization in the waters or wildlife of the Salish Sea and is an interesting trend worth further analysis.

The comparison of loading charts from the PCA ordinations demonstrate resistance and Erm, Amp and MacAmp resistance are closely related. Increased Amp resistance for differential and nondifferential media growth could be a result of large numbers of Amp resistance genes or resistant bacteria. Amp falls within the beta-lactam family of antimicrobials, which includes penicillin. These are the oldest antimicrobial families, and as a result many bacterial species have developed resistance, in addition to some naturally resistant species. The alignment of macrolide bacterial growth with beta-lactam growth is not explained. It has been shown that bacteria resistant to a single antimicrobial are more likely to show resistance to other drugs after exposure (Livermore, 2003), and it is also

possible that there are genes resistance to Erm and Amp impacting the fecal bacteria of the whales. Alternatively, beta-lactam and erythromycin are popular human drugs, and the stability of erythromycin in wastewater treatment has already been demonstrated, so this phenomenon could be a result of more pollution of these two drugs in tandem in the Salish Sea. More research is needed to confirm these explanations.

Again, the PCA loading charts indicating that Tc and MacTc have similar patterns in resistance is expected and explained by tetracycline resistance genes. However, the opposition of tetracycline resistance to chloramphenicol resistance is unexpected. The above explanation that a bacterium developing resistance to one drug is more likely to be multi-drug resistant would seemingly directly oppose this finding. However, the overall low expression of chloramphenicol-resistant bacteria may better explain this opposing ordination than any intrinsic drug or bacterial properties. Again, this requires more study.

Although the relationship between time in basin and total ARB growth was not significant, the low number of data points makes the likelihood of a statistical relationship low. The downward trend is of interest because it directly opposes the hypothesis that pollution in the Salish Sea increases ARB colonization, and there are several possible explanations for increased ARB shedding when animals reenter the basin. First, the long travel from the open ocean to the basin could be taxing to the animal, resulting in an immune compromised condition once they reach the basin, which is reflected by their fecal. Secondly, this could be a factor of the water quality the whales encounter

on their travel from the ocean through the Strait of Juan de Fuca. Canada's WWTP regulations do not require secondary treatment, and two large WWTPs that only screen sewage release to surface waters are located on Vancouver Island, where the Strait of Juan de Fuca meets the Salish Sea and the whales must pass to enter the basin. This theory supports the hypothesis that loading of pollution over time might be affecting ARB more than immediate geographic sampling area, important to note for other studies which relate indicator organism sampling site to geographic location. A third explanation is that the fecal composition is richer in ARB when the whales return from the ocean because of food sources. Field observations show that scat samples are larger and appear fattier in the first day after the whales return to the basin, decreasing in size and fat content as the animals remain in the basin. This has been unofficially attributed to differing food sources, and the pollution of oceanic salmon or increased consumption of farmed salmon treated with antimicrobials could explain the increased ARB when the whales re-enter the basin. The larger size of the samples could also contribute to more ARB colonies, because the intestinal system of the whale is voiding more completely more bacteria could be shed from intestinal walls. Again, the relationship between return to basin and total ARB colonization was not statistically significant, but the trend lacks data points and warrants further study. Additionally, this theory opposes prior use of immediate sampling location to assess water quality and geographic relationship to ARB prevalence and suggests that the factors of time and recent geographic travel should be considered in other studies of animal indicator organisms.

Genetic analysis revealed a smaller rate of resistance, yielding only *tet*M positive samples in 4 (9.3%) of samples. Four of the cultured samples expressed resistance to tetracycline (36.36%), indicating that the true resistance to tetracycline could be higher. The variation in genetic resistance and cultured resistance is curious, but could be a result of the time delay in DNA extraction for the samples collected for genetic analysis. Multiple instances of freezing and thawing occurred over the minimum 4 month period between sample collection and DNA extraction, which denatures DNA and decreases the ability for conventional PCR assays to detect genes (Qiagen, 2013). This theory is supported by spectrophotometry of the nucleic extracts, which revealed that most samples concentrations of DNA with the volume of template used for PCR reactions resulted in less than the recommended 50-500ng DNA per reaction recommended in general PCR guides (see Figure 13)(Palumbi et al., 2002). In contrast, the samples collected for culturing were processed within hours of collection, leading to the conclusion that quick transportation of samples results in better assessment of ARB in SRKW fecal samples. Tetracycline and oxytetracycline are often used in aquaculture, and the findings of *tet*M could be indicative of infiltration of these antimicrobials to the food web.

Although there were no statistically relevant whale traits relating *tet*M colonization to age, sex, or pod, all positives came from three female J pod whales off the west side of San Juan island. The three females, J8, J17, and J31, belong to different sub-family groups within J pod, and other J females sampled in this region during this time did not result in positive resistance gene

identification. Two sampling incidents of J31 over a month apart resulting in two positive identifications of the *tet*M gene confirm the hypothesis that using a well-studied and identified marine mammal population is beneficial for research seeking to study the residence time of ARB, and may indicate that effects are more specific to individual health than outside factors. This is corroborated by the study of marine animals on the east coast, which indicated that animal provenance (healthy, stranded, or bycaught) was a better indicator of ARB colonization than animal type or traits (Rose et al., 2009). However, J31 has not been known to have health problems, while J8, who was also sampled, was missing and presumed dead as of October 2013 (Center for Whale Research, 2013). Despite limited explanations due to low positive results, the usefulness of a repeatable and known population of animals in a study utilizing sentinel species is supported.

Examination of the microbiome for 3 of the SRKW fecal samples was performed to examine the functional metagenomic resistance genes with more sensitivity and precision. Qualitative polymerase chain reaction (qPCR) was performed on a J pod female, a K pod female, and an unknown sample. Genes for resistance to tetracycline, methicillin, extended-spectrum beta-lactam (ESBL), vancomycin, and sulfonimides antimicrobial resistance were observed (Table 4) (Roberts MC, personal communication). The detection of the *mec*A gene through metagenomic analysis and not the PCR assay of this study indicates that low PCR assay precision and time elapsed between sampling and gene amplification may have flawed the genetic analysis in this study. The number and relative rarity of some of the resistance genes, particularly the genes encoding for resistance to

ESBL genes, indicate more investigation into the array and scope of resistance genes in SRKW fecal is of scientific interest, particularly if paired with measurements of functional ARB genes in water or wastewater of the Salish Sea.

The Salish Sea is an interesting study site due to the discrepancies in wastewater treatment and pharmaceutical disposal practices on either side of the international border. Eight WWTPs empty into the Salish Sea in British Columbia, CA, two of which use only primary treatment of wastewater before discharging into surface waters (Capitol Region District, 2013). The remaining Canadian WWTPs use secondary treatment, and all dispose of effluent directly into the Salish Sea (Capitol Region District, 2013). In contrast, nine WWTP facilities serve the US side of the Salish Sea, eight of which release effluent to the Salish Sea. All US WWTPs have at least secondary treatment as required by the 1979 US Clean Water Act (Washington State Department of Ecology, 2007) However, the Capital Regional District of British Columbia's pharmaceutical take-back program has been widely successful, with 95% voluntary participation of pharmacies, collecting 60.32 kg of unused drugs in 2010 (Post-Consumer Residual Stewardship Program, 2010). Drug take-back programs in Washington State exist in 17 of 39 counties, and have been less successful, averaging just 18.96 kg/year in six years of surveillance (Take Back Your Meds, 2010). Therefore, more unmetabolized drugs might be entering surface waters in WWTP effluent from the US because of lack of medicine-disposal alternatives, despite stronger water treatment standards.

Due to the lack of spatial variability in the cultured samples and the small number of positives in the genetic samples, it is difficult to draw conclusions based on human impact to the total resistance in SRKW fecal. To improve upon this research, more detailed information on the environmental risk factors within the study area, particularly the western coast of San Juan Island, would be beneficial. The current breakdown of sample sites to six main land locations is too broad a scale for the high concentration of samples collected in that specific area. Additionally, other environmental risk factors that are thought to contribute to increased ARB such as rainfall, agricultural lands, hospitals, freshwater outflows, combined sewer overflows, and large on-site sewage systems were not included in this study, partially due to difficulty obtaining records on the scale needed for analysis and in part due to the difficulty of working with differing policies, government divisions, and record keeping standards across international borders. A future paper will include a more detailed spatial evaluation of these environmental variables.

Further research is needed with complimentary genetic identification of the SRKW to better identify trends in whale risk factors for colonization with ARB. Other improvements would be a more thorough evaluation of ARB genes for samples from both years, a larger suite of antimicrobial drugs tested, and increased specificity on environmental risk factor data.

5. Conclusion

The findings of resistance, both cultured and genetic, in SRKW fecal is of importance to environmental and public health fields, conservation biologists, and wildlife veterinarians. The prevalence of resistance in cultured samples is higher than in previous studies of sand, water, and orca blow in the Salish Sea. Whether this is a result of small sample numbers and a small suite of antimicrobials tested or is evident of increasing resistance in the waters and wildlife is not clear, but further research with finer-scale geographic analysis could improve our understanding of the development of ARB in the Salish Sea, along with the public, environmental, and veterinary health implications that could result from increases in resistance.

Tables

Table 1. Names, doses, and abbreviations of antimicrobial drugs added to agar plates in this study.

Drug Name	Dose Used (μg/mL)	Abbreviation
Ampicillin	25	Amp
Chloramphenicol	25	Cm
Erythromycin	10	Erm
Tetracycline	25	Tc

Table 2. Total growth from fecal sample cultures, summed in last row and column by number of resistant colonies by antimicrobial and by sample. C1 and C2 correspond to the two control samples. Refer to Table 1 for antimicrobial abbreviations.

Sample	TSA 2% NaCl				MacConkey 2 %NaCl			Total # Resistant Colonies
	Amp25	Cm25	Erm10	Tc25	Amp25	Cm25	Tc25	
1	22	0	19	1	0	0	1	43
2	0	0	32	0	0	0	0	32
3	0	0	0	0	1	0	0	1
4	28	0	520	0	59	0	0	607
5	0	0	279	15	0	1	1	296
6	0	0	2	0	0	0	0	2
7	0	0	28	11	1	2	0	42
8	0	9	267	0	4	0	0	280
9	14	0	193	0	19	0	0	226
10	12	1	177	0	0	0	0	190
11	0	0	1	4	4	2	0	11
C1	0	0	0	0	0	0	0	0
C2	0	0	0	0	0	0	0	0
Total Resistant Colonies	76	10	1518	31	88	5	2	1730

Table 3. Geographic segment division and environmental risk factors for analysis.

Segment Name	Country	Land Area (km^2)	Population Density (people/km^2)	Number WWTP	Number Permitted Septic Tanks	Sample Numbers Assigned
Coastal Skagit Basin	USA	8029	14.60	2	25000*	NA
Juan de Fuca	CA	1512	2.96	2	11606	10,11
Nooksack	USA	3651.9	55.72	1	30000**	NA
Saanich Peninsula	CA	103	365.72	1	6345	NA
San Juan	USA	471.38	33.94	1	8168	1,2,3,5,6,7,8,9
Southern Gulf Islands	CA	216	23.61	4	8946	4

*Best current estimate based on information from Skagit County Health Department. **Best current estimate from Whatcom County Health Department.

Table 4. Functional metagenomic results for resistance genes in 3 SRKW fecal samples from 2012 field season.

Whale ID	Sex	Age 2012	Resistance Genes
J31	F	17	sul2, sul3, blaCMY, ereA, oxa2, AAC6
K16	F	27	tetA, tetU, mecA, AAC6, catB8, aac31A, blaCMY, vatE, vatA, blaMOX-CMY9
Unknown	NA	NA	ctxM1, ctxM2, mox, oxa9, ermB per2, cmy, mecA, AAC6

Supplemental Material

Figure 1. Map of study site divided into six primary watershed units. Boat docking location noted with red star.

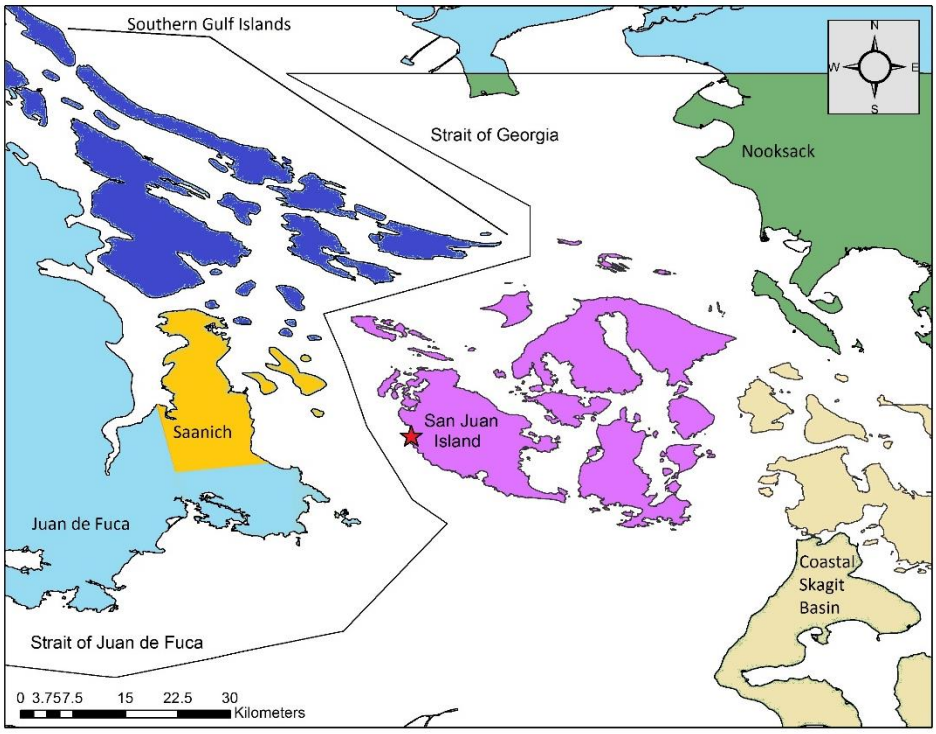


Figure 2. Map of culturing sample locations, 2013.

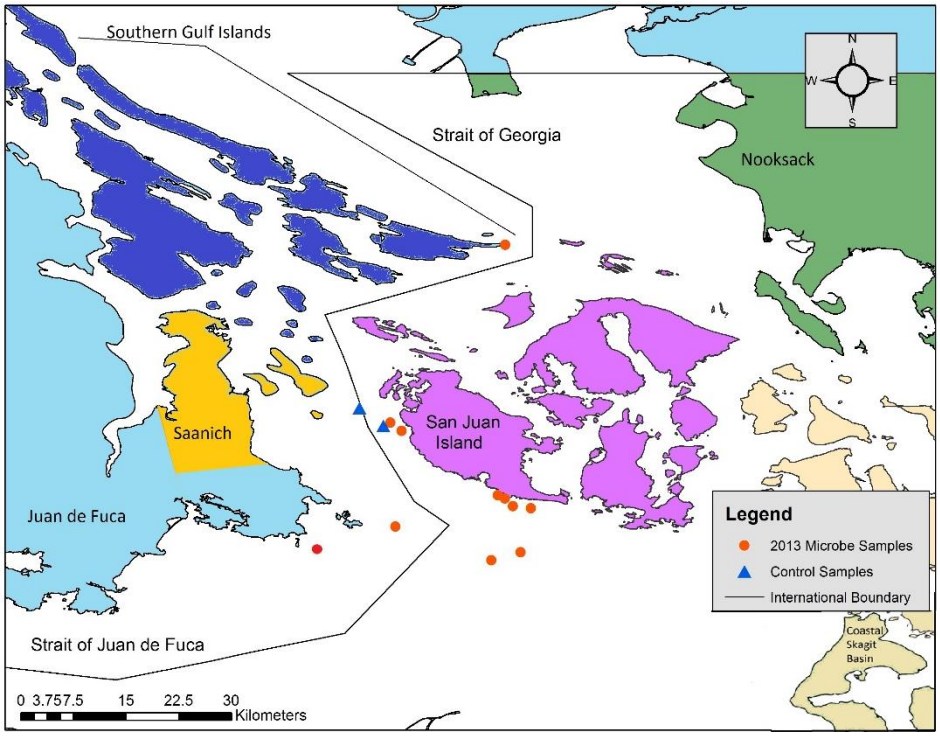


Figure 3. Map of genetic analysis sampling locations, 2012. Samples positive for *tet*M gene are denoted with red pentagons.

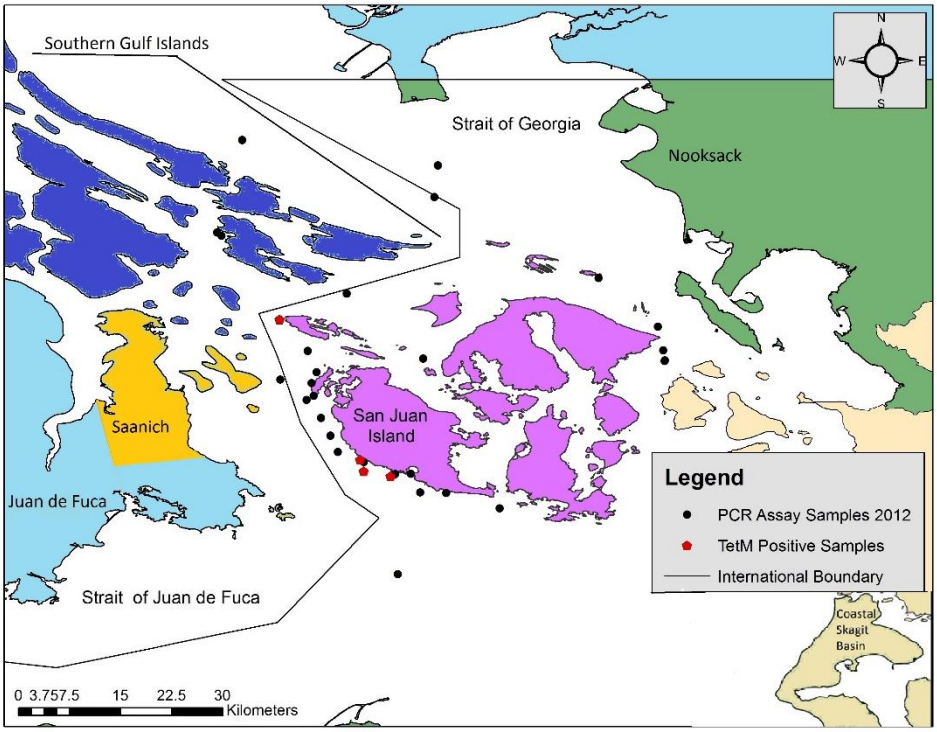


Figure 4. Sex distribution from DNA and hormone data for 2012 samples (n=32)

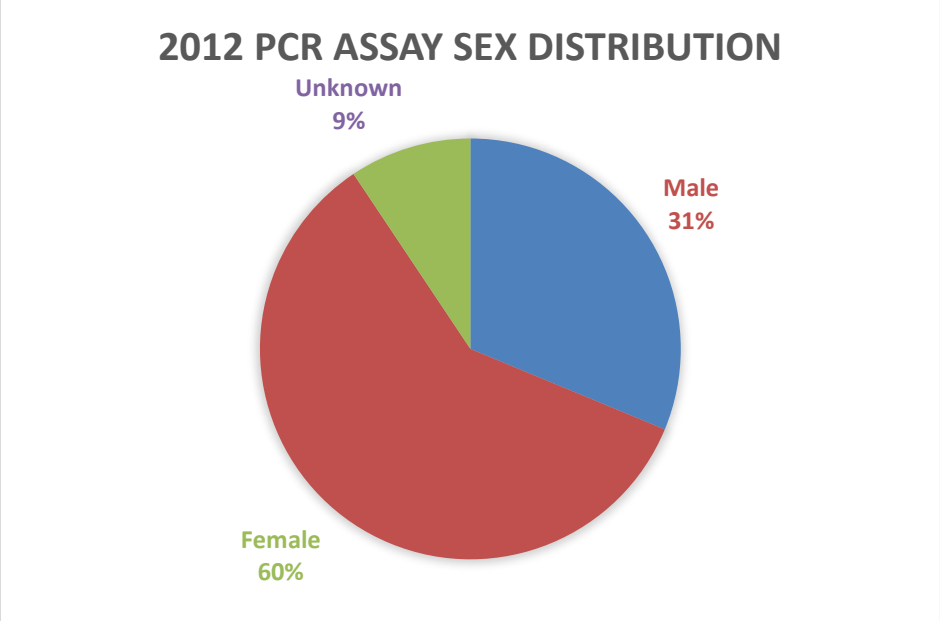


Figure 5. Pod representation from DNA genotyping identification of 2012 PCR assay samples (n=32).

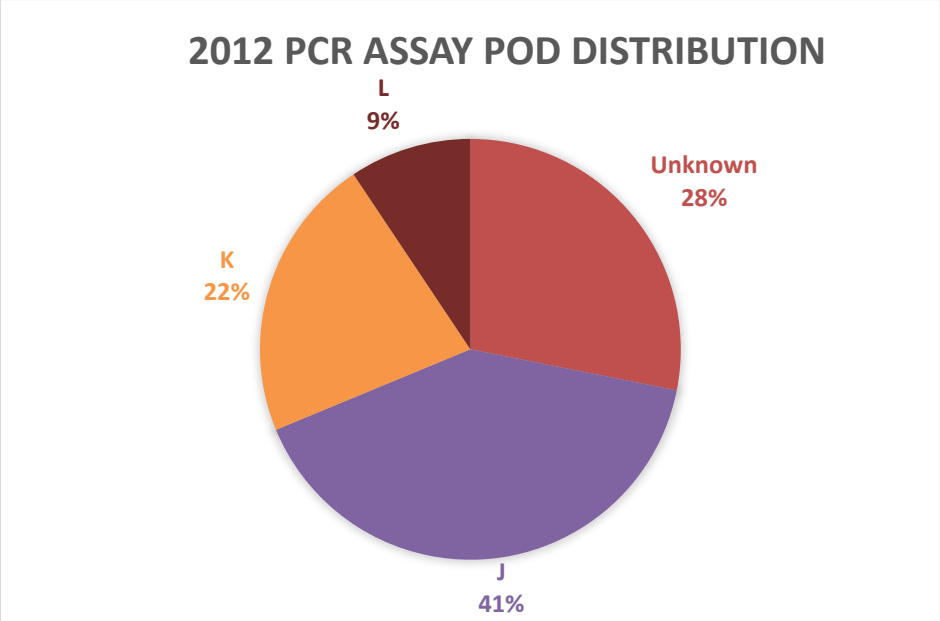


Figure 6. Total number of colonies grown on antimicrobial-infused plates by drug type represented on logarithmic scale. Growth on TSA and MacConkey plates differentiated by color, actual colony counts on bar.

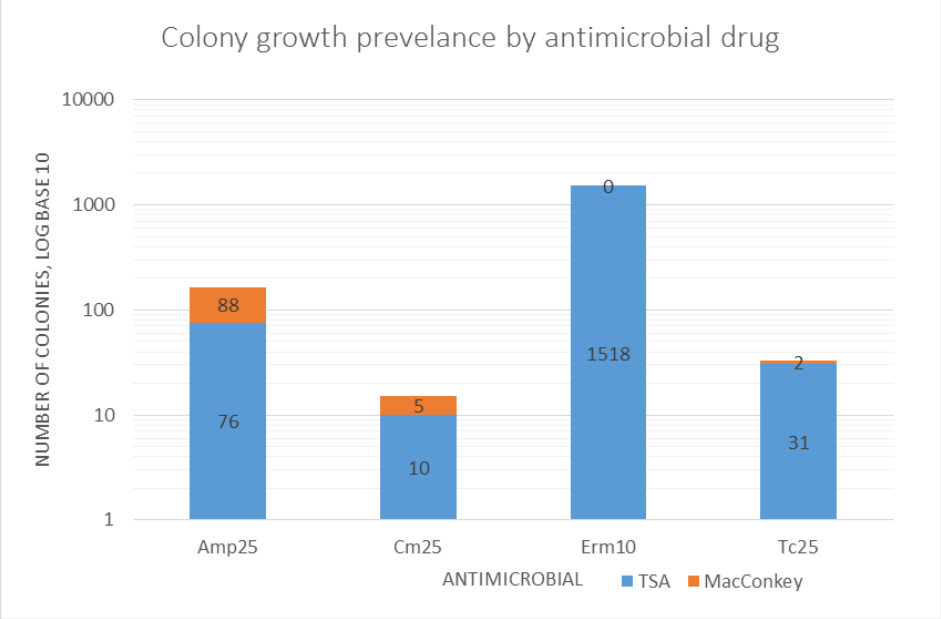


Figure 7. Incidence of MDR in cultured samples 1-11. Four antimicrobials were used in agar plates, thus four is the maximum MDR number.

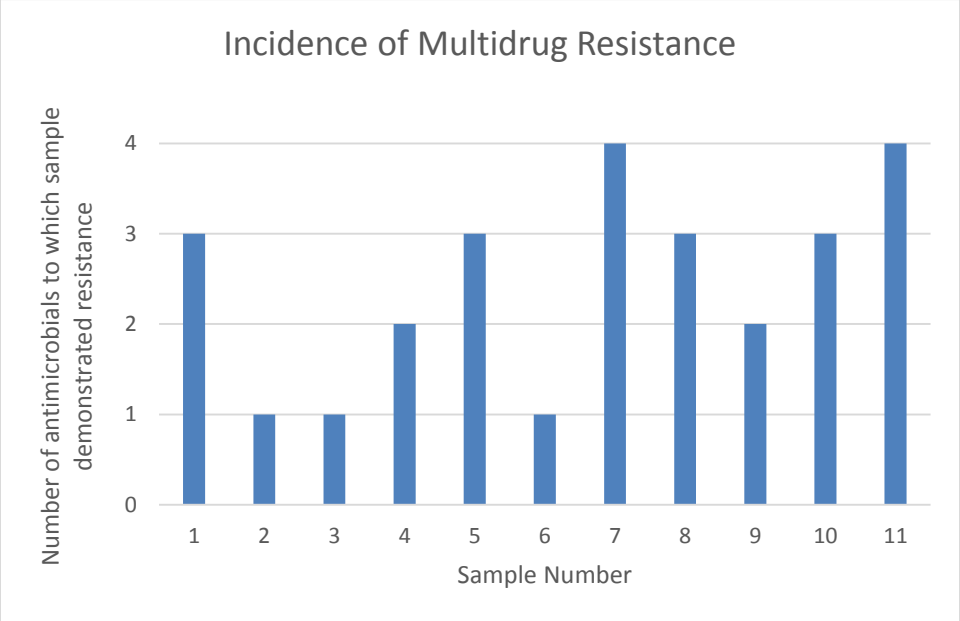
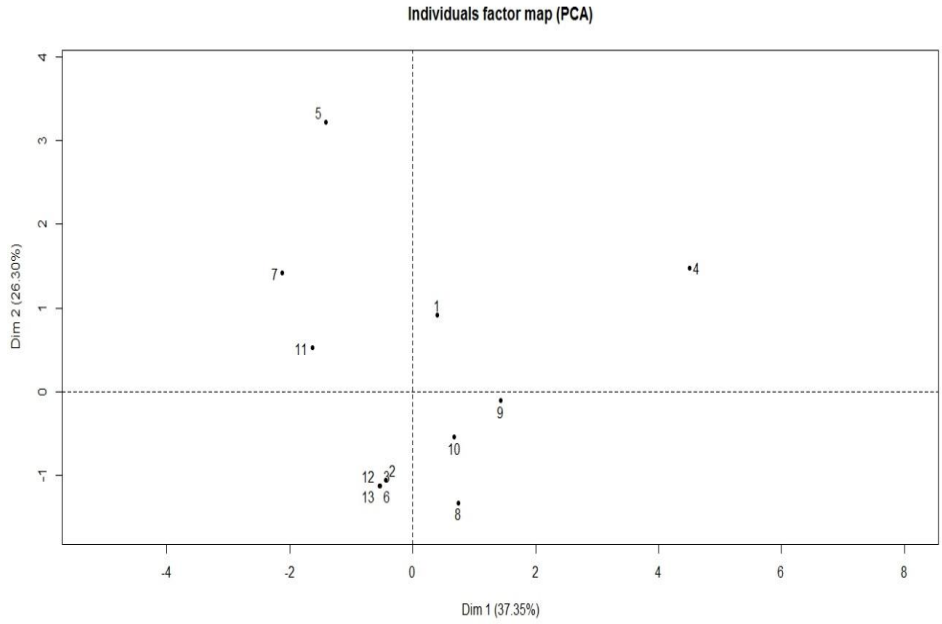


Figure 8. Principle component analysis for samples separated by antimicrobial and bacterial growth type (MacConkey or TSA) representing patterns in abundance of ARB colonies cultured in samples 1-11 and control samples 1-2 (12 and 13). The relationship between samples is determined by the similarity of ARB colony growth number for each antimicrobial plate, and the direction determining spatial distance is demonstrated in the loading plot below.



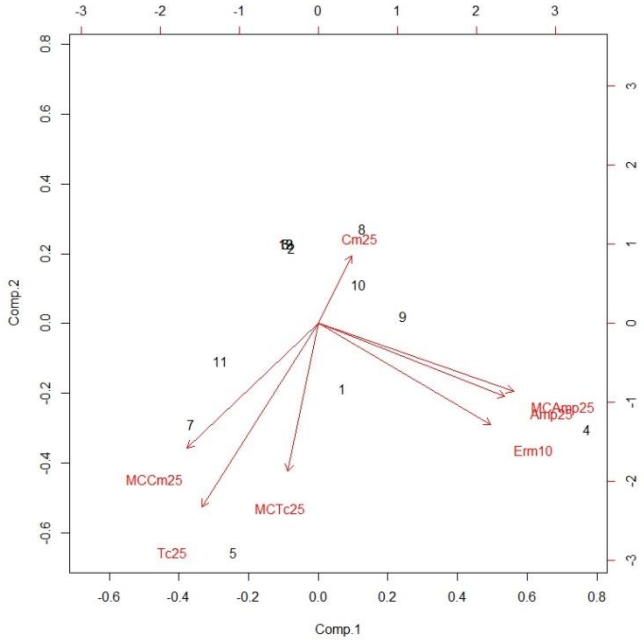
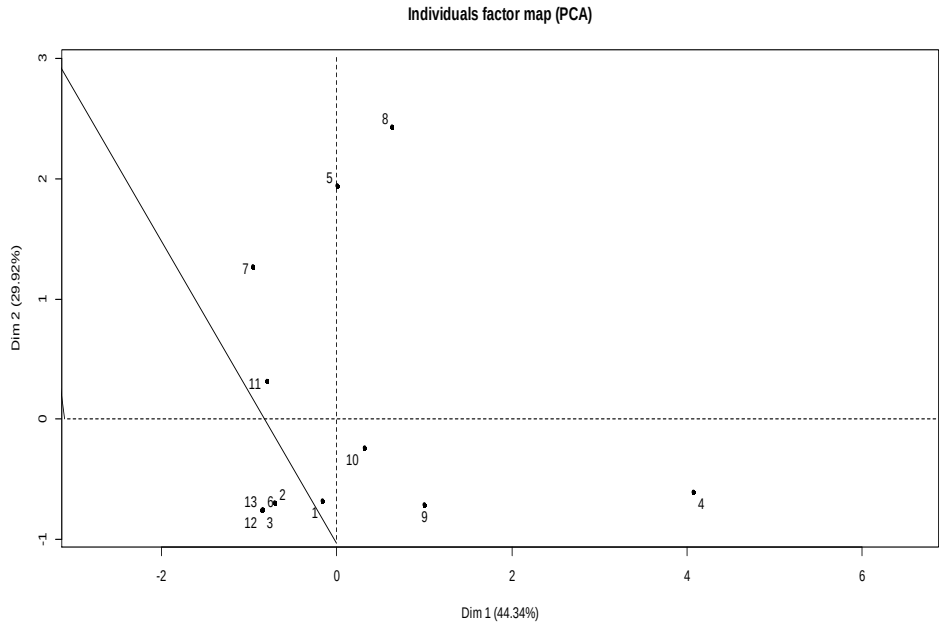


Figure 9. Principle component analysis for samples separated by antimicrobial representing patterns in abundance of ARB colonies cultured in samples 1-11 and control samples 1-2 (12 and 13). Loading factor map below shows the dimensions between variables which determined the distance between samples in the individual factors map.



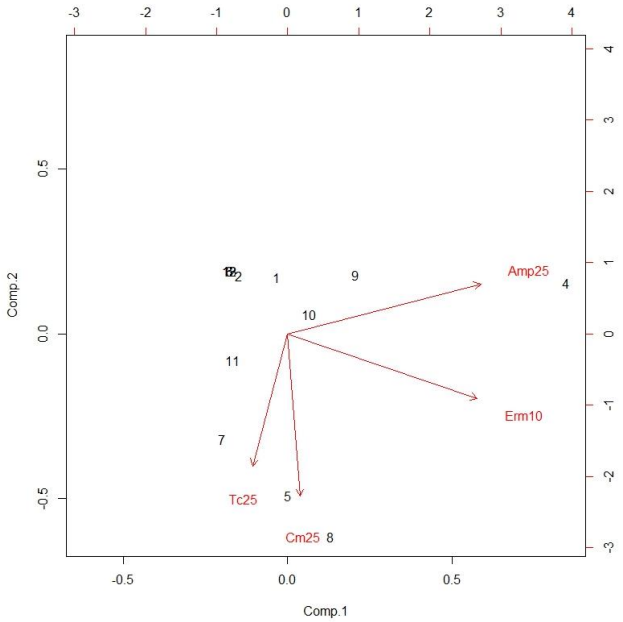


Figure 10. Averaged numbers of ARB colonies by study site segment.

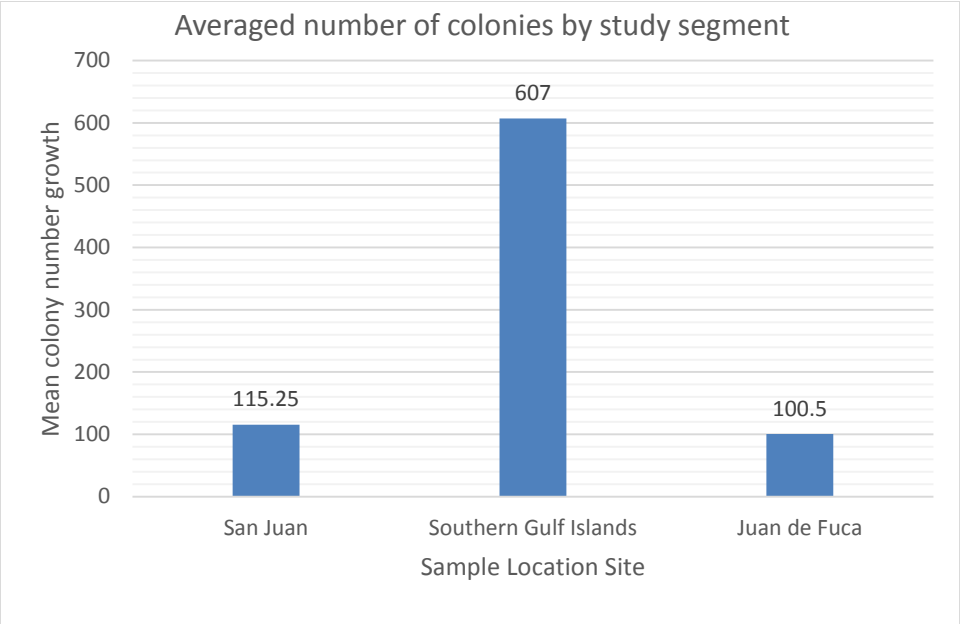


Figure 11. Total number resistant isolates in each samples as function of distance from shore.

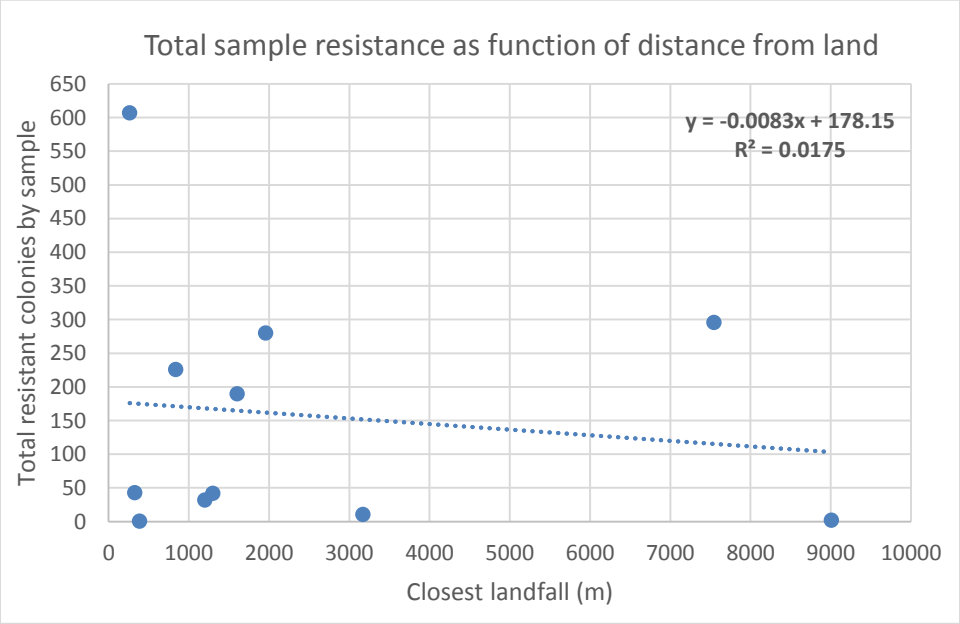


Figure 12. Total ARB cultured as a factor of time using Julian date. No significant relationship was found (y = -0.2561x + 217.48, R^2 = 0.0004)

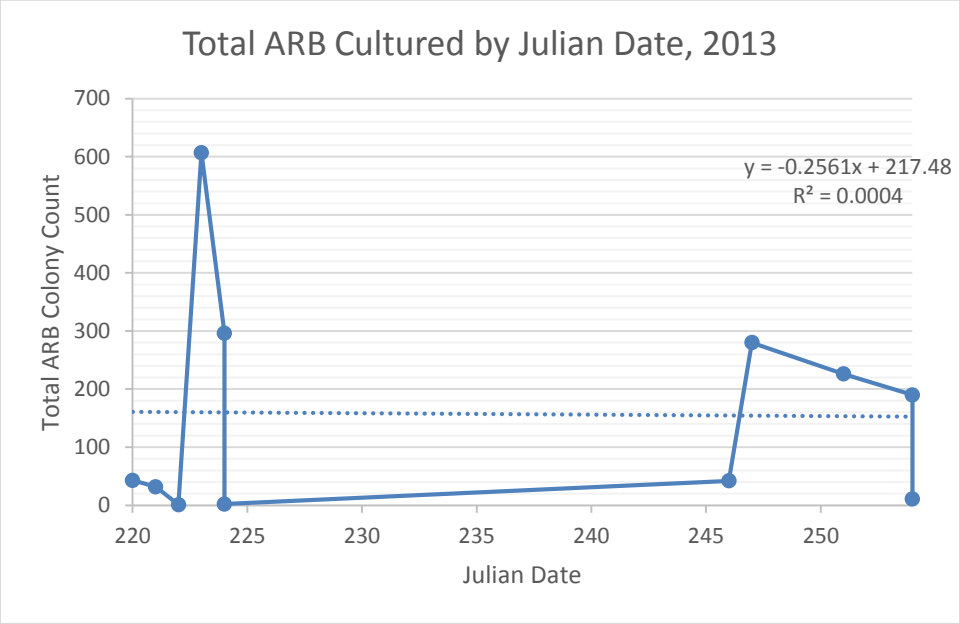


Figure 13. ARB colony prevalence by drug resistance type related by Julian date, 2013. No significant relationships found and thus not reported.

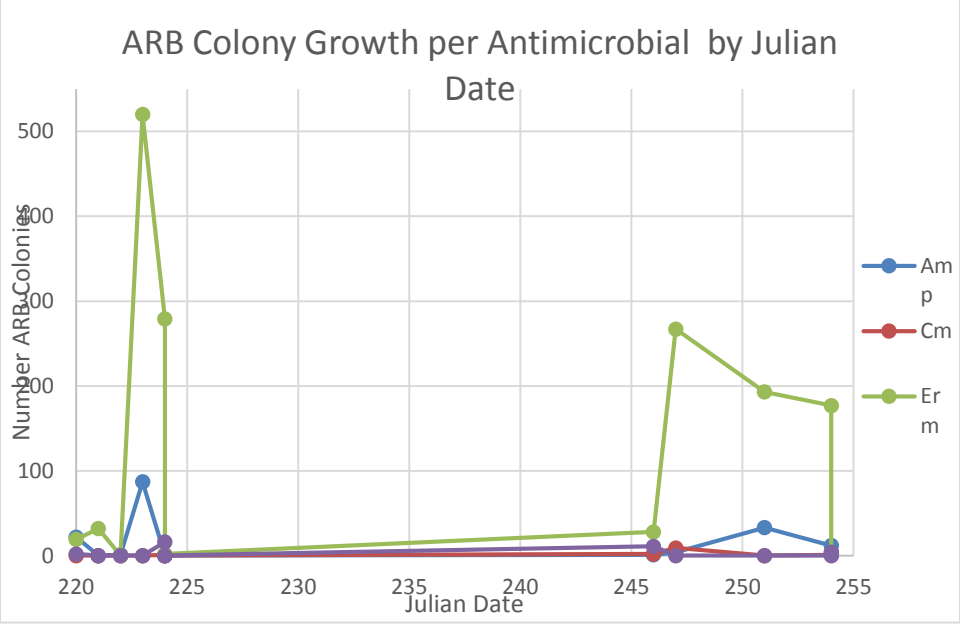


Figure 14. Scatterplot representing number of days the whales had been in basin before sampling by total ARB count. Although the r^2 value is low and suggests no relationship, the small sample size (n=11) and uneven distribution between the number of days before sampling may have had an effect on the decreasing trend of ARB shedding in fecal as time in the Salish Sea progressed. Further examination of this aspect of resistance should be studied.

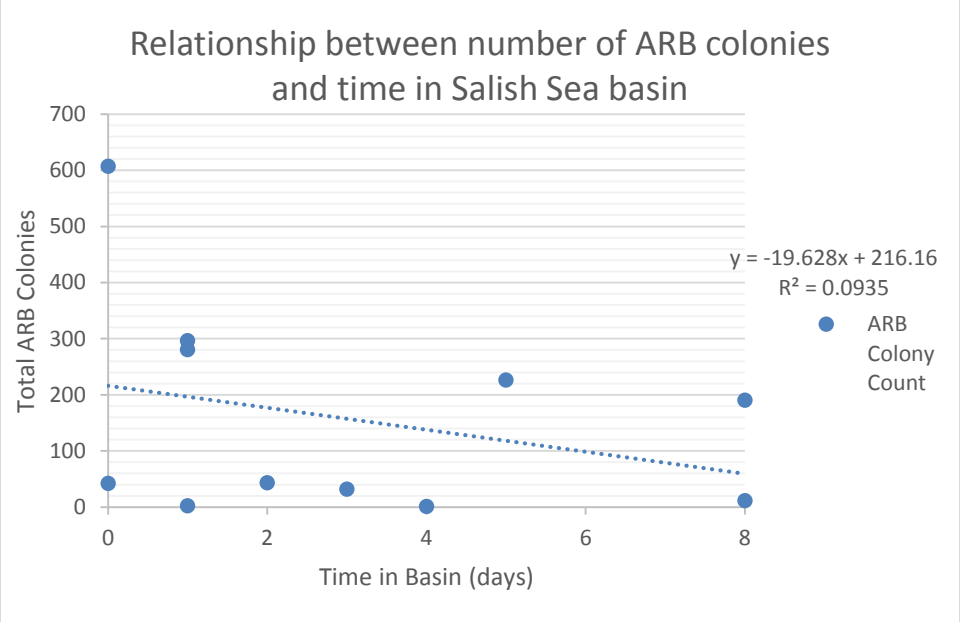
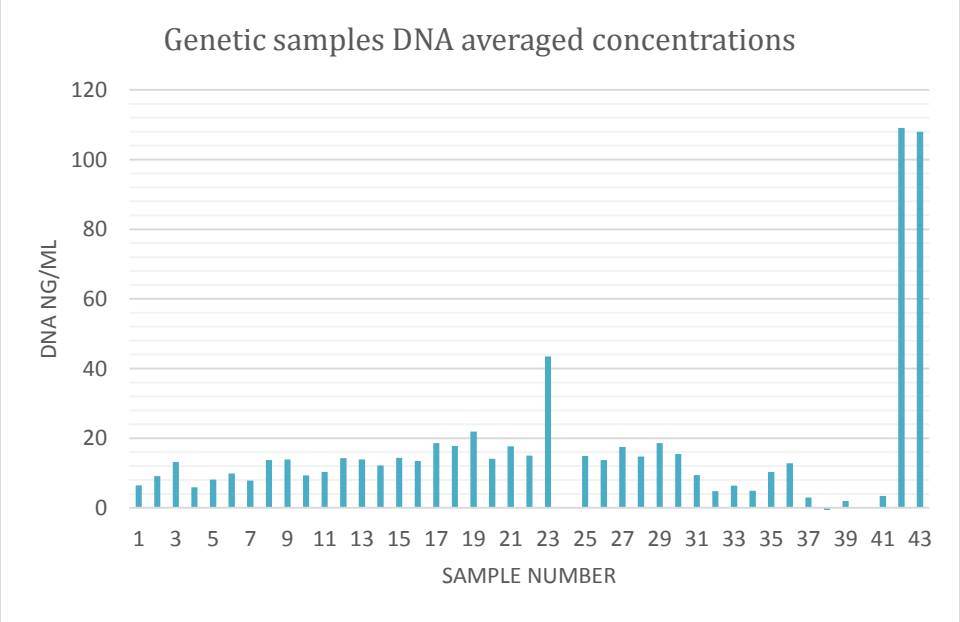


Figure 15. DNA concentration in ng/L of extract used as PCR template.



PCR Methods Validation Protocol

To identify and validate the most efficient method of biochemically identifying ARBs in preserved scat and swabs, methods of preservation, storage, DNA extraction and PCR amplification methods had to be optimized. Duplicates were taken in the field to compare recoveries in swabs preserved in 20% glycerol in MilliQ water against swabs stored dry. Microtubes containing swabs were placed immediately on wet or dry ice, which varies the temperature at which they were stored. Additional swabs of whole fecal samples taken in the laboratory after 2-3 months of storage were taken as replicates to assess DNA degradation over time.

A subsample of fecal swabs were extracted using Qiagen's QiaAmp DNA Stool Mini Kit and DNeasy Blood and Tissue Kit (catalog # 69506). Each swab was extracted according to the directions of the manufacturer with tissue kit modifications as noted in Wasser et al., 2011, and stool kit modifications found in Wasser et al., 2004, which are optimized to run in 96-well plate formats rather than individually. Blank negative controls were included in every extraction to rule out contamination.

The extractions were compared on gel electrophoresis for whole DNA extraction content by running 1 and 5 µL of extraction product and 2 µL of stop dye for 45 minutes on 0.7% agarose gel with 5% TBE buffer at 100 volts for 45 minutes, and visualized by UV light. Bacterial content within extraction was analyzed by quantifying bacterial genes after amplifying 3uL of each extraction. The bacterial DNA was amplified using PCR with universal bacterial primers (63f

and 1387r) to obtain the 16S rRNA content, indicating the presence of prokaryotes; PCR program set to denature for 15 min at 95°C, followed by 35 annealing cycles of 30 sec at 94°C, 90 sec at 55°C, and 1 min at 72°C, followed by a 30 min extension at 60°C, and a 4°C hold.

Comparisons between the quality of PCR amplification was conducted using two different Taq polymerase master mixes. Red Taq and Qiagen HotStarTaq DNA Polymerase master mixes were tested in the amplification of waste water treatment plant effluent, crow and cow feces along with a *tet*M positive control to evaluate which master mix was better for amplifying the *tet*M gene which encodes for tetracycline resistance. For the Red Taq, 12.5 µL of taq was added to 10.5 µL of autoclaved PCR water and 0.5 µL of M4 and M6 primers along with 1 µL of template for each reaction. For the optimized Qiagen HotStarTaq, 5 µL of polymerase was added to 1 µL of PCR water along with 0.5 µL of each primer, and 3 µL of the DNA template (Qiagen, 2013). Both were run on a PCR program set to denature for 15 min at 95°C, followed by 35 annealing cycles of 30 sec at 94°C, 90 sec at 55°C, and 1 min at 72°C, followed by a 30 min extension at 60°C, and a 4°C hold according to the Qiagen manual. Again, PCR product in 1 and 5 µL concentrations was run on 1% agarose gel with 5% TBE buffer at 100 volts for 45 minutes, and visualized with 2 µL of stop mix and UV light excitation.

CHAPTER THREE

DISCUSSION AND INTERDISCIPLANRY STATEMENT

These results are the result of a multidisciplinary study approach and will be of interest to professionals from differing academic and professional spectrums. Conservation biology, veterinary science, microbiology, water resources, and environmental and public health field research contributed to the literature research and theory that made this project possible.

Field research on this project was made possible by the University of Washington's Center for Conservation Biology (CCB) and the non-profit organization Conservation Canines (CK9). Their monitoring of the SRKW's health has occurred seasonally from 2007-2009 and 2010-2013 and uses hormones extracted from the fecal samples of the killer whales to derive stress levels for malnourishment, ambient noise, and pregnancy and testosterone rates to understand relative impact of salmon population, commercial and recreational boaters, and endocrine-disrupting compounds on the decline of SRKW population. This research on ARB in the fecal of the killer whale is not geared primarily to monitoring health of the population, but the data gathered can help support recovery efforts nonetheless.

Veterinary science is intertwined with conservation efforts because the evaluation of the health of the individual whales is both indicative and dependent on the health of the entire population. The samples preserved for microbial analysis are of interest to researchers from the National Oceanic and Atmospheric

Association (NOAA), the SeaDoc society, and researchers from the University of British Columbia and University of California Davis that are highly engaged in recovery efforts for the SRKW. Currently, a project is in planning that researches the virus and whale pathogens in the fecal sample to assess infections that may be endangering the population using the 2013 samples collected for this study.

An additional factor for veterinary science that has been incorporated in this study is the use of scat detection dogs to local samples. Just as dogs can be trained to sniff out drugs, bombs, and missing persons, they can learn to cue in to the scat of a particular animal. CK9 was the first program to train dogs on the scent of animal targets and use scat, scale and hair shedding, and other materials from target animals. The data is used to monitor population numbers, extent of habitat, sex distribution, family relationships, pregnancy and abortion rates, reactions to environmental stress, and health of the species in question. The target animals, which have included elephants, tigers, arboreal iguanas, moose, deer, caribou, wolf, black bear, wolverine, Pacific fisher, marten, Northern Spotted Owl, sharp-tail snake, cougar, and other animals, ultimately benefit from the scientific research being conducted, as do the Conservation Canines that work for the program. CK9 only accept dogs from shelters or that are owner-surrendered. This is because the traits that make a great scat-sniffing dog, like boundless energy, constant desire to play, and obsession with their ball or other toys, often make these dogs incompatible pets. Additionally, many of the dogs have special needs, aggressive tendencies, or health problems from trauma inflicted upon them before they were adopted by the program. The dogs are trained, cared for by

professional animal trainers, and travel the world assisting in conservation biology research. Dogs sometimes bond with a particular handler, providing them with human companionship as well as a chance to expend their energy and play as much as possible. And as a CK9 dog becomes too old or infirm to work in the field, they are retired to good homes with the approval of the CK9 director Heath Smith. This program is truly remarkable for the services it provides to scientific research as well as the benefits to shelter dogs who would likely be euthanized without their adoption by CK9. I have been truly honored to work with this program, feel that it is a valuable and innovative use of resources, and sincerely hope that the non-profit will survive the recession to work on future conservation biology studies.

A drastically different set of disciplines that contributed to the design of this study and will benefit from the findings are microbiology and biochemistry. Bench microbiological techniques were required to complete this research, as was a great deal of background research in biochemical and microbiological theory. Research in these disciplines are sometimes considered a narrow field utilizing primarily controlled laboratory experiments to gather information on specific bacterial strains, genes, or processes of interest, typically geared toward human health research. However, the growing field of environmental microbiology is contributing to more *in-situ* (in place) studies that examine how bacterial organisms and species interact with one another to create a process. The development of more sensitive and deep PCR techniques has made genome sequencing more affordable and practical. This technology has given rise to the

research of the "microbiome", the assemblage of bacterial species present in a given area. Studies on the microbiome of the sea surface, soils, and environments such as caves, sediment, and intestinal flora of many species have been conducted. These studies have revealed how interconnected the bacterial array in an area are and that the assemblage, not the species, creates the ultimate function and health of the environment. For example, a recent study on the relationship between intestinal microbiota and obesity showed a direct link between bacterial assemblage and weight gain. Separate groups of germfree mice were fed uncultured microbiota from each member of four pair of human twins, one of which of each pair was lean and one obese (Ridaura et al., 2013). The study concluded that laboratory mice receiving fecal transplants from the bacterial component of the obese co-twins' microbiota gained significantly more weight eating the same diet as the mice receiving the lean twin bacterial communities (Ridaura et al., 2013). Increased understanding in the dynamics of bacterial ecology paired with advances in technology make microbiological and biochemical experiments increasingly applicable to real-world problems and more-often relating to environmental processes. Research has used the antimicrobial resistance patterns in fecal coliform bacteria to identify sources of fecal contamination to water sources, for instance (Bernhard and Field, 2000; Burnes, 2003). The research conducted on ARB occurrence and rates in SRKW fecal was designed as a pilot project for the use of marine mammals in the Salish Sea to serve as indicators of fecal and antimicrobial pollution, with the sample location and wintering habits of the whale pods helping to identify "hotspots" for

acquisition of resistance genes, and in that aspect this study is very much aligned to the field of applied environmental microbiology. By understanding the spread and transmission of bacteria and resistance genes, microbiologists can work with water resource professionals to trace contamination and learn more about the extent of antimicrobial pollution and begin to create solutions for remediation.

The field of water resources connects to this project because of the introduction of PCPPs and ARB into surface waters through WWTPs, leaky septic tanks, agricultural land, and CSO. Reduction of anthropogenic compounds and bacteria introduced to surface waters is directly tied to proper management and regulation of these sources of pollution. Minimization of pharmaceuticals and ARB in wastewater and runoff preserves water quality and decreases potential problems that decrease sustainability of water resources, reducing need for large scale engineering projects. Monitoring of ARB in water animals is one method that engineers, hydrogeologists, and water scientists could observe the spread of PCPP pollution through surface waters and thereby learn which systems are being properly managed and how to improve resources.

Finally, the fields of environmental and public health are critical to the development and application of this research on ARB in the Salish Sea. These fields are grouped together because there is truly no environmental health problem that will not soon become a human health problem. The framework for this integrated concept that the health of humans is connected to the health of animals and the ecosystem has been coined the "One Health" movement (CDC, 2013b). The One Health movement relies on interdisciplinary, collaborative

approaches to address potential risks at the forefront of human, animal, and environmental health (One Health Global, 2012). The One Health method monitors and controls public health threats by working with physicians, ecologists, and veterinarians to create an overall landscape-scale understanding of how diseases and environmental contaminants move through the ecosystem, and uses this information to reduce potential hazards in the interactions of the three spheres. Animals are used as indicator organisms for zoonotic pathogens, such as West Nile virus in birds, and serve as an early warning sign of human disease threats (CDC, 2013b). This thesis research was based on the idea that the marine mammal serves as an indicator organism for ARB pollution in surface waters. It was the hope of this researcher that this study could connect marine mammal veterinary health measurements to the environmental health risk of ARB in surface waters, which has important consequences for human health if these ARB enter our water supply or transmit genes to human or zoonotic pathogens. The holistic approach to environmental and public health that has been adopted by the One Health movement was a major motivation for the design of this project, and the interdisciplinary and holistic measurement of contamination and disease in the environment based on the interconnectedness of these three domains is important for future studies.

Lastly, environmental policy becomes important to this study because of the myriad ways antimicrobial drugs and PCPPs are infiltrating the environment, and the number of opportunities that arise to prevent the pollution.

First, policies regulating discharge of WWTPs and private industry under the Clean Water Act, known as National Pollutant Discharge Elimination System (NPDES) permits, does not cover emerging pollutants like PCPPs. The Clean Water Act required the monitoring of other water quality parameters, such as fecal coliform, nitrogen, and total suspended solids, and made at least secondary treatment practices mandatory, which in the Washington Department of Ecology's report was shown to decrease the concentrations of PCPP compounds in WWTPs (Lubliner, 2010). Tertiary treatment methods decreased the concentrations even further, making the requirement for tertiary treatment one possibility for reducing the amount of pharmaceuticals discharged into surface waters. This would have to be accompanied by monitoring for PCPPs in stormwater and non-point runoff as well to be effective.

Another way to minimize prescription drugs entering the environment is decreasing the amount of drugs that enter the wastewater system. This could be accomplished by assuring that only compounds that have been partially degraded by the human waste system are flushed. The promotion of drug take-back programs would help people dispose of unused or expired medicines safely and without environmental harm. However, there is no consistent drug take-back program in the USA, and the Take Back Your Meds program in Washington operates only intermittently. In Canada, pharmacies provide free drug disposal to customers, and the services are relatively well publicized. Implementing and advertising permanent drug take-back programs could prevent the flushing of unwanted drugs down the toilet and prevent them from ever entering the WWTP,

decreasing the concentration of drugs not eliminated by secondary treatment systems.

Finally and most importantly, the spread of ARB and introduction of PCPPs to the environment can be prevented through consumer education. Physicians prescribe antimicrobial drugs in the absence of a bacterial infection because patients often demand drugs for viruses and colds which will not respond to these drugs in an effort to feel better. This practice is thought to contribute to a rise in ARB in community infections and increasing unmetabolized PCPPs in wastewater. The engagement of patients and doctors working together to treat health holistically rather than looking for quick fixes to illnesses or larger health problems would be a step in the right direction to decreasing ARB gene spread. Consumer education about products that contain antimicrobials would also be beneficial. Antimicrobials used subtheraputically in animal agriculture, products like toothpastes, hand soaps, and deodorants, and antimicrobial hand gels and cleaning products are ubiquitous in the supermarket and in our homes. Using these products can help prevent the spread of infections when used prudently, but the majority of these products are used constantly, defeating the purpose of including drugs in the product. Aside from this, products containing triclosan and other unregulated antimicrobials are not required to be labelled as containing drugs, and make their way into products that consumers may not suspect contain these compounds. Consumer education and more transparent labelling would help ameliorate the overuse of antimicrobials in our homes, slowing community-based ARB infections and decreasing PCPPs entering the environment.

Changing the way we conceptualize medicinal treatment is also an important consideration. Antimicrobials are rapidly becoming obsolete, and drug companies are no longer working on developing new drugs because the immense cost is not worth the development of a drug that will only be effective for a short time (Shnayerson and Plotkin, 2002). Human medicine is in danger of entering a new era where antimicrobials no longer exist, which would drastically change treatment of diseases and infections. While engineering, water resources, policy, and educational advances can be made to slow or prevent this change, the posing of this problem is perhaps indicative of an overall need for a new approach to health care. Instead of treating symptoms, perhaps physical health could be improved through preventative measures of diet, exercise, and mental state. Wellness has long been considered a science by the majority of Americans, who lead the developing world in health problems such as obesity and diabetes and rank 37th overall in international health (International Diabetes Foundation, 2013; WHO, 2013). If holistic health practices and concentration on prevention rather than treatment was adopted by a larger majority of the population, the need for antimicrobials and PCPPs could be decreased, eliminating the problem of these contaminants in the environment altogether. This would require a paradigm shift in the individual's responsibility for and understanding of personal health, a large transition for the majority of the nation, and until then, people will look to medications to ease pain and cease illness, making PCPP and ARB pollution a problem in human, environmental, and animal health.

This thesis incorporates the One Health interdisciplinary concept of interconnectedness of human, animal, and environmental health in an attempt to draw conclusions on inputs of antimicrobial compounds and ARB to the Salish Sea. Although the limited sample size and unrefined geographic scope in this study made relationships between human pollution and environmental and animal health hard to draw, the findings of ARB in scat of the Southern Resident Killer Whales suggest that monitoring the health of this species could help draw conclusions about the health of all the creatures and the waters of the Salish Sea.

This study presents the first findings of antimicrobial resistant bacteria in the scat of *Orcinus orca,* specifically the SRKW of the Salish Sea. While the findings warrant more scientific investigation, this study was unable to pinpoint any environmental or organism traits that cause trends in resistance. Further study to improve upon this research must include the following components to increase understanding of this phenomenon.

Subdivision of study areas: This study only used a coarse-grained spatial analysis to analyze environmental variables as risk factors. Without using extensive GIS mapping to further subdivide the study area, the six sites linked to the environmental variables is only a rough estimate of correlation. Using more advanced GIS, the population and number of septic tanks, as well as other information like watershed outflow volume and number of WWTP outfalls, could be directly spatially related to each sample site rather than related by assigned area, making the spatial assessment of colony growth variability more complete.

Genetic identification of whales for cultured samples: The data from 2013 yielded more information on the diversity and prevalence of resistance. However, without the identification of animal age, sex, and pod, the individual animal risk assessment was not possible for these samples. Reviewing the field log showed that each day a cultured sample was collected, sampling was occurring simultaneously on all 3 pods, so there was no way to narrow down the identity of each sample. With the genetic information on the whales, both environmental and organism risk assessments can be conducted, to gain a better understanding of relative contribution of each of these components to colonization with ARB.

Less time between sample collection and genetic analysis: The results from the DNA concentration testing indicate that the nucleic acids used as a template for PCR and genetic identification degraded. This is supported by the large number of resistant colonies growing on fresh agar and the small number of samples that tested positive for resistant genes. For future studies, immediate culturing followed by colony isolation, identification, and genetic assessment of resistance genes would improve this study's usefulness.

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